# THERAPEUTIC MECHANISMS FOR CANNABINOID-PROMOTED SURVIVAL OF OLIGODENDROCYTES

by

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# DEDICATION

This work is dedicated to my parents and my brother.

As I finish this chapter in my life, I know that this achievement is not just mine, but ours. This work is the culmination of 30 years of many laughs and tears but, most of all, the belief that the little girl who had a less than perfect start could overcome any obstacle and grow up to be whatever she wanted. For all your love and support I will be forever grateful.

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# ABSTRACT

**Therapeutic Mechanisms for Cannabinoid-Promoted Survival of Oligodendrocytes**

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Loss of axonal myelination due to oligodendrocyte cell death is one of the major pathological features in Multiple Sclerosis (MS), and there is currently no effective treatment. Inflammation, a hallmark in the pathology of MS involves activation of microglia and release of cytotoxic factors including peroxynitrite, the most reactive free radical causing toxicity to oligodendrocytes. The cannabinoid system has recently emerged as a promising therapeutic target in MS. We and others have shown that the cannabinoid type 1 and type 2 (CB1 and CB2) receptors are expressed in microglia and oligodendrocytes. Although activation of these receptors is thought to regulate the inflammatory response and cell survival, the mechanisms involved are still not well elucidated. The objective of this dissertation was to study the potential role of cannabinoids in preventing oligodendroglial cell death from two different angles. First, by testing the ability of these compounds in blocking the production of peroxynitrite by microglia and secondly, by determining whether cannabinoids can act directly on oligodendrocytes to provide protection from peroxynitrite induced toxicity. Pharmacological, biochemical and molecular approaches were used to elucidate the mechanisms of peroxynitrite production in reactive microglia and to determine how cannabinoids interfere with the signaling pathways of peroxynitrite formation and its

toxicity to oligodendrocytes *in vitro*. Studies *in vivo* were performed to characterize the effect of a novel synthetic cannabinoid compound in preventing inflammation, demyelination and oligodendrocyte injury in a mouse model of MS.

During the course of this study, we identified that several signaling molecules, such as ERK1/2, $cPLA_2$, NF-kB and iNOS, are involved in the production of peroxynitrite by reactive microglia. Importantly, treatment with cannabinoid agonists was shown to prevent peroxynitrite formation by interfering with these signaling cascades. However, our results also showed that cannabinoid antagonists were unable to block the effect of cannabinoid receptor agonists in this signaling pathway and, surprisingly, the antagonists themselves were also able to affect this signaling pathway and attenuate the generation of peroxynitrite in microglia cells. Moreover, we were able to determine that cannabinoid agonists can protect mature oligodendrocytes against peroxynitrite toxicity through a mechanism involving attenuation of ERK1/2 and $cPLA_2$ phosphorylation. *In vivo*, our results demonstrated that the novel cannabinoid agonist CB-52 can attenuate clinical symptoms in EAE mice when given either before or after disease onset. Mechanistically, CB-52 was shown to decrease peroxynitrite production, microglia/macrophage infiltration, myelin loss, axonal damage and oligodendroglial cell death in EAE mice.

Our results suggest that cannabinoids can promote oligodendrocyte survival by inhibiting the release of cytotoxic factors from reactive microglia, and/or by directly interfering with the cell death pathways in oligodendrocytes. These studies provide further experimental evidence that cannabinoids may serve as therapeutic agents for MS, and other neuroinflammatory and neurodegenerative diseases.

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# CHAPTER 1: Introduction

## MULTIPLE SCLEROSIS

Multiple Sclerosis (MS) was first clinically described in 1868 by Jean Martin Charcot (136), and is believed to be the most common demyelinating disease affecting the central nervous system (CNS) (50; 156). There is an estimated prevalence of about 100 cases per 100,000 people worldwide and 350,000 people in the United States and Canada alone (156). MS usually develops in young adults (39; 171), and results in motor and sensory disabilities (20). Despite extensive research that has been done in the field, there is still no cure for the disease.

Like a well insulated wire, the axons of neurons are partially covered with myelin, a fatty substance which allows for proper conduction of electrical signals along nerve cell axons. Oligodendrocytes are the glial cells responsible for the production of myelin around neuronal axons in the CNS (136; 171). While in a healthy person, neurons effectively communicate with each other, in people with MS this communication is hampered due to myelin loss. MS is an autoimmune disorder, meaning that the myelin loss seen in these patients is the result of their immune system failing to recognize myelin as benign and instead, treating it as a pathogen. As a result, the immune system is activated leading to inflammation and resulting in demyelination, oligodendrocyte cell death and axonal loss (191). Patients experience symptoms that can be classified as primary, secondary or tertiary symptoms, depending on the causes. Primary symptoms are a direct result of demyelination and can include weakness, numbness, and vision impairment among others. Secondary symptoms (e.g. bed sores) can happen as a result of

primary symptoms (paralysis), and tertiary symptoms are social and/or emotional consequences of the disease such as depression.

While the mechanisms underlying this immune response remain unknown (156), it is generally accepted that genetic predisposition and environmental factors play a role in the development of the disease (137; 166). Studies show that descents of northern Europe descent have a higher risk of developing MS compared to Asians and Africans (212), and more women develop MS than men (156). Although MS does not present itself with a pattern of inheritance that we can recognize, people who have other family members with the disease are usually at a higher risk of developing MS themselves (136; 156; 212). However, twins studies show that it is possible for one twin to develop MS while the other is healthy (156; 212). These findings point to an environmental component to the development of the disease. Interestingly, when looking at the incidence of MS worldwide a pattern emerges with the risk increasing as you move away from the Equator, which corresponds to areas of less sunlight. Studies have shown that patients tend to experience more symptoms right after winter and less at the end of summer, suggesting a possible link between vitamin D deficiency and disease activity (31; 82). The phenomenon where a person can decrease his or her chances of developing the disease by moving from an area of high incidence to an area of low incidence and *vice versa* at an early age also supports the importance of environmental risk factors to the development of MS. In addition, studies have also supported the possibility that viral exposure in genetically predisposed people could lead to the activation of the immune system resulting in the development of the disease (212).

## MS Pathology

Different types of cells participate in the development of the disease, such as CD4+ cells, microglia, and oligodendrocytes. Two types of CD4 cells that are important in inflammation are the CD4+ T helper cells and the CD4+ T regulatory cells. These cells are responsible for regulating immune responses against foreign pathogens. CD4+ T helper cells can secrete cytokines which will lead to the recruitment and activation of different immune cells thus leading to inflammation. CD4+ regulatory cells will secrete anti-inflammatory cytokines that will result in suppression of inflammation (156).

One of the cells that can be activated by CD4+ T helper cells are the microglia. Microglia are the smallest of the glia cells and serve a homeostatic surveillance role. Found throughout the CNS, these cells are constantly scanning for foreign pathogens. In case of injury or disease these cells become activated and change their morphology from elongated cells to a round amoeboid shape, and will work to engulf and clear damaged cells and debris. Because of their function, microglia are considered the "macrophages of the CNS" (63).

Oligodendrocytes, as mentioned previously, are the glia cells responsible for the production of myelin in the CNS. Oligodendrocyte cells have many processes resulting in one oligodendrocyte being able to myelinate more than one axon. Thus, the loss of each oligodendrocyte can impact the conduction speed of several neurons. These cells are very sensitive to injury and the absence of mature, myelinating oligodendrocytes renders remyelination impossible (81).

The pathological state starts as an immune-mediated response, where CD4+ T lymphocytes fail to recognize myelin as a benign antigen, instead treating it as a foreign pathogen (64). This type of autoimmunity that is so characteristic of MS, can also be seen

in other conditions such as rheumatoid arthritis and type 1 diabetes (89). The mechanism(s) that trigger this response remain unknown (156), although it is believed to involve some exogenous agent that contribute to disease onset in a predisposed individual (137). It is believed that these genetic and environmental factors can lead to the disruption of the blood brain barrier (BBB), thus allowing autoreactive T cells to access the CNS. The BBB is responsible for restricting access to the CNS, therefore affording protection against toxins and immune cells from the periphery. T cell infiltration is made possible through interactions between adhesion molecules present in these cells and those found in the BBB and it has been reported that the levels of these adhesion molecules are higher in MS patients (72). Upon recognition of the myelin antigen, these infiltrating cells can secrete a number of chemokines and cytokines such as interleukin-2 (IL-2), tumor necrosis factor alpha (TNFα) and interferon gama (IFNγ) leading to the destruction of myelin

Infiltrating T cells are also responsible for the activation of several antigen presenting cells such as microglia. These cells are responsible for engulfing and degrading the myelin. Supporting this concept is the fact that proteins and lipids which result from the breakdown of myelin can be seen inside of these cells (156). Another evidence for the breakdown of myelin comes from examination of the cerebrospinal fluid (CSF) of patients, where myelin protein levels can be elevated (211). This inflammatory process leads to edema and the further break down of the blood brain barrier (156).

An interesting and perhaps important point when trying to understand what triggers this inflammatory process is the realization that T cells that react to myelin are not exclusively found in people that have MS. In fact, they are found in healthy

individuals as well, with the only (although extremely important) difference being the fact that in healthy individuals these T cells are only found in the blood stream and are unable to cross the blood-brain barrier to gain access to the CNS (89). Therefore, when trying to develop new treatment options it becomes crucial to understand this break down of the blood-brain barrier in order to avoid infiltration of the CNS by these T cells.

During this inflammatory process, myelin is not the only part of the CNS that is damaged. There is also a loss of oligodendrocytes and some of the demyelinated axons, which are more sensitive to injury, might be transected. Eventually, through the actions of astroglia, microglia and regulatory/suppressive T cells, the inflammatory process subsides (137; 156). The overall result of these inflammatory episodes or "relapses" will be a slowing of impulse transmission through the pathways involved in the lesion. To some extent, the body does try to regain function by repairing itself. Oligodendrocytes that did survive will start remyelinating axons, however, remyelination is usually incomplete, partially due to the insufficient numbers of mature oligodendrocytes. A major problem in trying to reestablish the lost function is that transected axons are most likely unable to regrow and connect to their target sites, therefore making it impossible for the affected areas to completely recuperate on its own. In addition, the accumulation of scarring due to multiple relapses, in old sites as well as new ones, will eventually lead the affected areas to progressively worsen, and the increase in white matter that is affected will lead to the worsening of the patient's symptoms.

## Microglia Activation

Microglial cells are considered the first line of defense in the CNS and account for 5-20% of the total cells in the adult brain (145). They serve a protective role and are activated during pathogen invasion and injury (119). However, microglia activation is also implicated in a variety of neurological diseases such as Alzheimer's disease, cerebral palsy and MS (119; 218). Once activated, microglia can release a number of factors which can be detrimental to neighboring cells. These cytotoxic factors include cytokines (73; 106), glutamate (56; 103), reactive nitrogen species (RNS) (195), and reactive oxygen species (ROS) (79; 148). The toxic interaction of microglia with neurons (26), and oligodendrocytes (85; 118) has been previously reported. Microglial cells were shown to accumulate at the border of MS lesions (162), suggesting they may contribute to the loss of myelin-forming oligodendrocytes. In fact, selective killing of microglia or inhibition of activation decreased the severity of symptoms and reduced damage to myelin and axons in EAE (71; 84). Understanding the mechanisms by which reactive microglia produce cytotoxic factors will likely reveal a novel target for intervention in the inflammatory process of MS.

## Peroxynitrite Toxicity

Peroxynitrite is the reaction product between superoxide and nitric oxide (197) and believed to be the major toxic species released by activated microglia (119; 218). Nitric oxide is produced from the inducible nitric oxide synthase (iNOS) in reactive microglia. Its production can be increased in pathological conditions such as inflammation (17) and contributes to the pathogenesis in animal models of multiple

sclerosis (47; 55). Although iNOS inhibition has been shown to be protective in EAE, targeted gene deficiency of iNOS was shown to exacerbate the disease severity (99). These results suggest controlling the production of peroxynitrite, but not nitric oxide might be important for the treatment of MS. Recently our group and others have shown that nitric oxide and peroxynitrite may have contrasting effects in the pathogenesis of EAE (98; 120).

Superoxide can be generated from different sources, which include mitochondria, and the actions of lipoxygenases (LOX), cyclooxygenases (COX) and NADPH oxidase (45). In the case of phagocytic cells such as microglia, NADPH oxidase is thought to be the main source for superoxide production (119; 184). This enzymatic complex is composed of five main subunits, namely p47phox, p67phox, gp91phox, p22phox, and rac2 (181). p22phox and gp91phox are integral membrane proteins while the other components of the complex are cytosolic. Upon activation, the cytosolic components translocate to the membrane to form a functional heteromeric complex with the membrane bound subunits (30).

Peroxynitrite is more reactive than nitric oxide or superoxide alone. This molecule can cause protein oxidation and nitration in addition to lipid peroxidation (208). Low concentration of peroxynitrite causes apoptosis, while at high concentrations it can induce necrosis (208). Peroxynitrite has been implicated in different pathological conditions and was found to be increased in the CNS of MS patients as well as in mice with acute EAE, as indicated by an increase in nitrotyrosine, a marker for peroxynitrite (46; 90; 202).

## CANNABINOIDS AND THE ENDOCANNABINOID SYSTEM

There are a number of compounds being studied as a possible treatment for MS and one class of compounds that is showing particular promise are the cannabinoids. Cannabis, or marijuana, as it is mostly known, is one of the illicit drugs that has the longest recorded history of human use. It is also the one most abused illegal substances in Western Societies today (151). Its ability to disrupt sensory perception is widely known as this effect helped to increase its popularity as a recreational drug. This effect was described by French poet Charles Baudelaire who gives a vivid account of the effects of the hemp plant (*Cannabis sativa*) in his 19$^{th}$ century book *Les Paradis Artificiels* (96). However, beside being known as a recreational drug, accounts dating back from the third millennium B.C. already described some medicinal uses for the plant extract, recognizing its usefulness in helping treat some human ailments (140).

Research on the properties of *cannabis sativa* has dramatically increased since 1964 when the chemical structure of $\Delta^9$-tetrahydrocannabinol (THC), which is the major psychoactive ingredient of the plant, was identified by Gaoni and Mechoulam (69). Cannabinoids became the term used to describe the active constituents of cannabis and today we are aware of more than 60 cannabinoids that are present in the plant. In addition, and perhaps one of the most important findings in fueling cannabinoid research, was the discovery of cannabinoid receptors (135; 144) and naturally-occurring cannabinoids (or endogenous cannabinoids) (53; 138) produced by the body . This led to increased studies on the roles of cannabinoids in the body as well as raising the possibility of using cannabinoids as therapeutic agents, with some people comparing today's cannabinoid research to the opiate research of the 1970's (97).

## Discovery and Expression of Cannabinoid Receptors

To date there are two known cannabinoid receptors which are expressed in mammals. The CB1 receptor was cloned in 1990 in Tom Bonner's lab (135) followed by the cloning of the CB2 receptor three years later by Sean Munro and his colleagues (144). After the receptors were identified and cloned, research intensified in search for the endogenous ligands binding to their receptors. This happened in 1992 when the lipid arachidonoyl ethanolamine (also known as anandamide or AEA) was isolated from the porcine brain and was found to not only bind to the receptor, but also to mimic the effects of THC when injected in rodents (53). Subsequently, 2-arachidonoyl glycerol (2-AG), the most abundant endocannabinoid in the CNS, was also identified. The CB1 and CB2 cannabinoid receptors, endogenous ligands AEA and 2-AG and their respective hydrolytic enzymes constitute the endocannabinoid system (Fig.1) (159). Interestingly, the endocannabinoid system is not limited to mammals and it can also be found in birds, amphibians, fish, sea urchins, mollusks and leeches, among others (77). In mammals, CB1 receptors are found mostly in the CNS, although they can also be seen in the peripheral nervous system (PNS), as well as some peripheral organs and tissues. In the brain, CB1 receptors are highly expressed in; the basal ganglia, cerebellum, hippocampus and cerebral cortex, areas important for movement and memory processing (28; 77). The CB2 receptors on the other hand are expressed mostly in cells of the immune and hematopoietic system, although they have also been found in the brain stem and glial cells in the CNS (159). Thus, activation of CB2 receptors is generally thought to modulate the inflammatory response and immune function (151; 159).

## FUNCTION OF THE ENDOCANNABINOID SYSTEM

The role of the endocannabinoid system seems to be of neuromodulation, with endocannabinoids being synthesized "on demand" and released by the cells, and then being quickly inactivated (77). They are thought to be part of a defense system which acts in response to excessive neuronal activity, responding to rises in intracellular calcium or activation of metabotropic receptors. They are synthesized by postsynaptic neurons, released, and then act presynaptically to suppress neurotransmitter release (28; 206).

The endocannabinoid system is thought to play a role in an array of different pathophysiological conditions including, but not limited to diseases of energy metabolism, neuropathic and inflammatory pain, stroke, brain and spinal cord injury, Alzheimer's disease, Parkinson's disease, cardiovascular and respiratory disorders (151).

Both CB receptors are G-protein coupled receptors (GPCRs) and thought to be among the most abundant and widely distributed GPCRs in the brain (77). At the protein level, the CB1 and CB2 receptors are only 44% identical while they show 68% homology in the transmembrane domains. The transmembrane domains are believed to be the location for the ligand binding sites of the receptors (151). It is believed that both receptors act through $G_{i/o}$ proteins leading to lower adenylate cyclase activity (159). For the CB1 receptors, two factors can influence the activation of downstream pathways. First, there is a difference in efficacy for any given agonist depending on whether the receptors are coupled to a $G_i$ or a $G_o$ protein (74). Secondly, it is believed that CB1 receptors have more than one binding site, and that each site is favorably bound by a specific class of ligand (the different classes of ligands will be discussed later) (172). A possible implication for different ligands having a preference to specific G-protein coupled receptors is that it allows for a better therapeutic intervention, which varies

depending on the expression of each G protein in different cell types. Although the specifics about the downstream pathways activated by cannabinoids receptors are not yet known, activation of the phosphatidylinositol-3-kinase/Akt (75), and members of all three families of the multifunctional mitogen-activated protein kinases, such as p44/42 MAP kinase, p38 kinase and JUN-terminal kinase (151) appear be involved.

## CANNABINOID LIGANDS

Cannabinoids can be divided into three main classes depending on where they are obtained (Table 1 and Fig. 2-4). Phytocannabinoids refer to the natural compounds that are derived from the *Cannabis sativa* plant. Endocannabinoids are the endogenous cannabinoids produced by the body. An important difference between these two classes of cannabinoids is that while all endocannabinoids are derived from polyunsaturated fatty acids, phytocannabinoids tend to be alkaloids, thus differing in their chemical structures (77). The third class of cannabinoids relates to the synthetic cannabinoids. These synthetic cannabinoids can be further divided into "classical" and "non-classical", and the classification depends on their degree of kinship with the phytocannabinoids (77). As new synthetic cannabinoid are developed it becomes important to evaluate their actions in different model. One newly synthesized cannabinoid is CB52, which is a novel synthetic cannabinoid compound developed in 2005 as an analog of anandamide (AEA) and $\Delta^9$-THC (Fig. 5). This compound has a high affinity for the CB1 and CB2 receptors, but has not been very well described in the literature (29; 36)

The development of selective CB1 and CB2 agonists and antagonists gave researchers a valuable pharmacological tool to use in order to better understand the

signaling pathways mediated by the cannabinoids. This knowledge will hopefully result in the development of specific agents that can be used for the treatment of various human diseases.

## CANNABINOIDS AND MS

MS patients have been self medicating with cannabinoids for decades and the discovery of the endocannabinoid system led to an increase in research aiming to find a link between cannabinoids and MS.

Most of what we know about cannabinoids and MS originated from studies using animal models of the disease. Among the three most used animal models of MS are the Experimental Autoimmune Encephalomyelitis (EAE), in which an autoimmune disease is induce by injection of myelin antigens; Theiler's murine encephalitis virus-induced demyelinating disease (TMEV-IDD) in which a virus is used to initiate disease development; and the cuprizone model in which demyelination is induced through the ingestion of the copper chelator by the animals (142). In our study, we opted to use the EAE model because it would allow us to evaluate the effect of CB-52 on T cell infiltration, as well as its role on demyelination and axonal damage. This model has been widely used in MS research as it mimics many aspects of the condition.

EAE was first described in 1933 using monkeys (175), and today EAE can be induced in many different species, including rabbits, guinea pigs, mice and rats in addition to monkeys (152). Since its development, EAE animal model has been used to identify many new targets for treatment, in addition to serving as a model system to test potential new drugs (40). Depending on the species used and how the disease is induced,

distinctive pattern and pathology of the disease can be obtained. By immunizing C57BL/6 mice with MOG35-55 and CFA, followed by a "booster" of the MOG peptide a week later, we are able to produce a chronic form of the disease which is thought to resemble the secondary progressive MS. In this type of MS the disease steadily progresses with less distinct episodes of relapses. Animals usually start showing symptoms starting at day 10, after which symptoms will progress quickly and steadily for about 5 days and then remain constant.

To better understand the function of the endocannabinoid system, several laboratories have developed CB1 and CB2 receptor knockout mice (32; 117; 151). When looking at CB1 receptor deficient mice, it was noted that these animals developed more severe EAE, characterized by greater neurodegeneration (168), while in CB2 knockout mice, immunomodulation was found to be absent (32). These studies suggest that the endocannabinoid system may play a role in neurodegenerative/inflammatory conditions such as in EAE and MS and that treatment with cannabinoid compounds may be beneficial not only for symptom management but also for slowing disease progression.

If in fact the endocannabinoid system plays a role in how the disease progresses, increasing the ligand level should be able to attenuate some of the problems generated by the condition. Indeed, when EAE mice were treated with THC researchers found that treatment with this cannabinoid, when compared to the one receiving placebo, not only showed reduced CNS inflammation, but also had their neurological outcome improved as well as increased survival rates (126).

Using the TMEV-IDD model it was reported that treatement with WIN55,212-2, a synthetic non-selective cannabinoid agonist, had a similar effect to what was seen

usung the EAE model, namely a decrease in symptom progression. In addition, a lower expression of proinflammatory cytokines was also reported (48). Another study using the same model reported a reduction in microglial activation and a decrease in the number of CD4+ infiltrating T-cells in the spinal cord (151). These animal studies provide an insight into how the endocannabinoid system behaves during pathological disease states. The possibility that endocannabinoids are somehow involved in the inflammatory process makes this system even more appealing as a therapeutic target for MS research.

Consistent with these preclinical studies, cannabinoids seem to be promising in attenuating MS symptoms and disease progression in humans, but so far, only Sativex, a 1:1 mixture of $^{9}\Delta$-THC and cannabidiol, has been approved and used to reduce pain and spasticity of MS patients in Canada and several European countries (7; 77; 159). The anti-inflammatory, immunosuppressive and neuroprotective properties of various cannabinoids suggest that development of highly selective and more efficacious agents can be clinically useful for the treatment of MS, and other inflammatory and neurodegenerative diseases.

Extracts from *Cannabis Sativa* have been in use for hundreds of years for both medicinal and recreational reasons but not until fairly recently did scientists begin to understand the active principals and how they mediate their actions. When cannabinoid receptors were isolated in the early 1990s, it provided a platform for the elucidation of cannabinoid receptor agonistic ligands. This was the opening the field seemed to have been waiting for because it led to an expansion in the study of the therapeutic effects of cannabinoids. The idea that cannabinoids can be used to treat multiple MS seems promising. As we understand more about MS and how the cannabinoid system interacts

with it, we will be better equipped to offer hope to those patients who suffer from the condition.

Table 1. Cannabinoid agonists and antagonists

| Ligand | CB1 $K_i$ value (nM) | CB2 $K_i$ value (nM) | Reference |
|---|---|---|---|
| **Phytocannabinoids** | | | |
| $\Delta^9$-tetrahydrocannabinol (THC) | 7.3 – 7.4 | 7.1 – 7.5 | (61) |
| **Endogenous Cannabinoids** | | | |
| Anandimide (AEA) | 6.3 – 7.0 | 5.7 – 6.4 | (61; 138) |
| 2-arachidonoylglycerol (2-AG) | 6.3 – 7.2 | 5.8 – 6.8 | (19; 138) |
| **Synthetic General Cannabinoid Agonists** | | | |
| CP55940 | 8.3 – 9.2 | 8.6 – 9.2 | (61) |
| WIN55212-2 | 6.9 – 8.7 | 8.4 – 9.6 | (61; 190) |
| CB-52 | 210 | 30 | (29) |
| **Synthetic CB1-selective Cannabinoid Agonists** | | | |
| arachidonyl cyclopropylamide (ACPA) | 2.2 | 715 | (87) |
| arachidonyl-2-chloroethylamide (ACEA) | 1.4 | > 2000 | (87) |
| Mathanandimine | 17.9 | 868 | (121) |
| **Synthetic CB2-selective Cannabinoid Agonists** | | | |
| AM1241 | 280 | 3.4 | (94) |
| JWH015 | 386 | 13.8 | (190) |
| GW405833 | 4772 | 3.92 | (201) |
| **Synthetic CB1-selective Cannabinoid Antagonists** | | | |
| AM281 | 12 | 4200 | (112) |
| AM251 | 7.49 | 2290 | (113) |
| SR141716A | 12.3 | 702 | (190) |
| **Synthetic CB2-selective Cannabinoid Antagonists** | | | |
| AM630 | 5152 | 31.2 | (179) |
| SR144528 | >10000 | 5.6 | (179) |

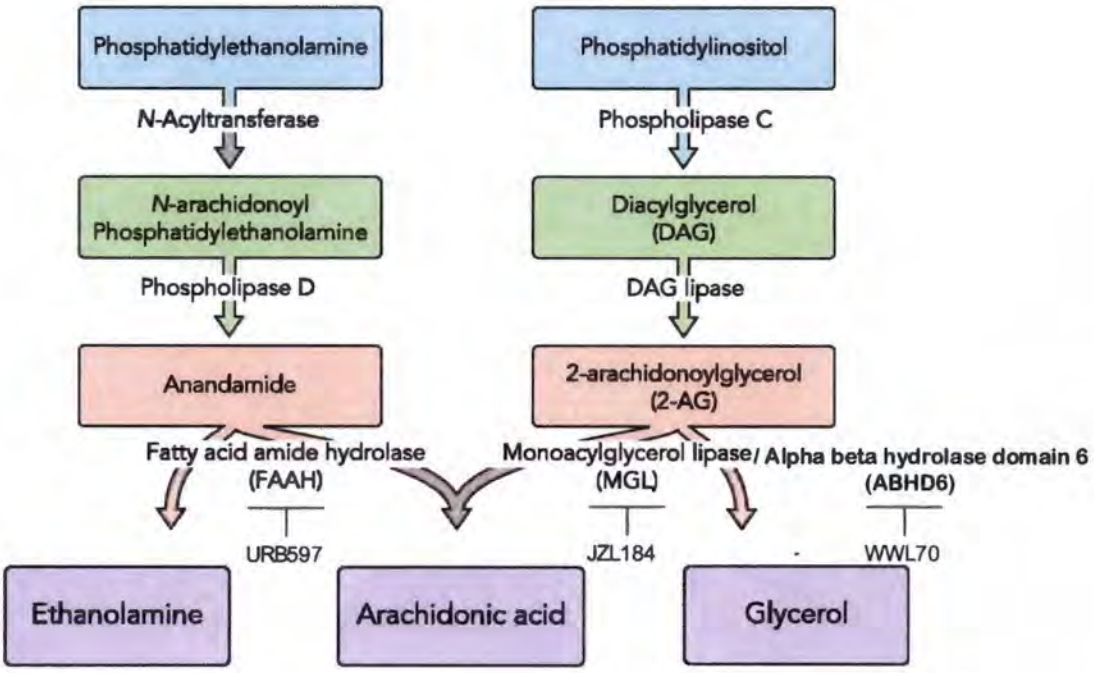



**Figure 1. Main pathways for the synthesis and degradation of AEA and 2-AG**

Diagram shows the main pathways for the endocannabinoids AEA and 2-AG, including enzymes involved in the metabolism and degradation. Inhibitors for the three hydrolytic enzymes are also shown. Image modified from (57)

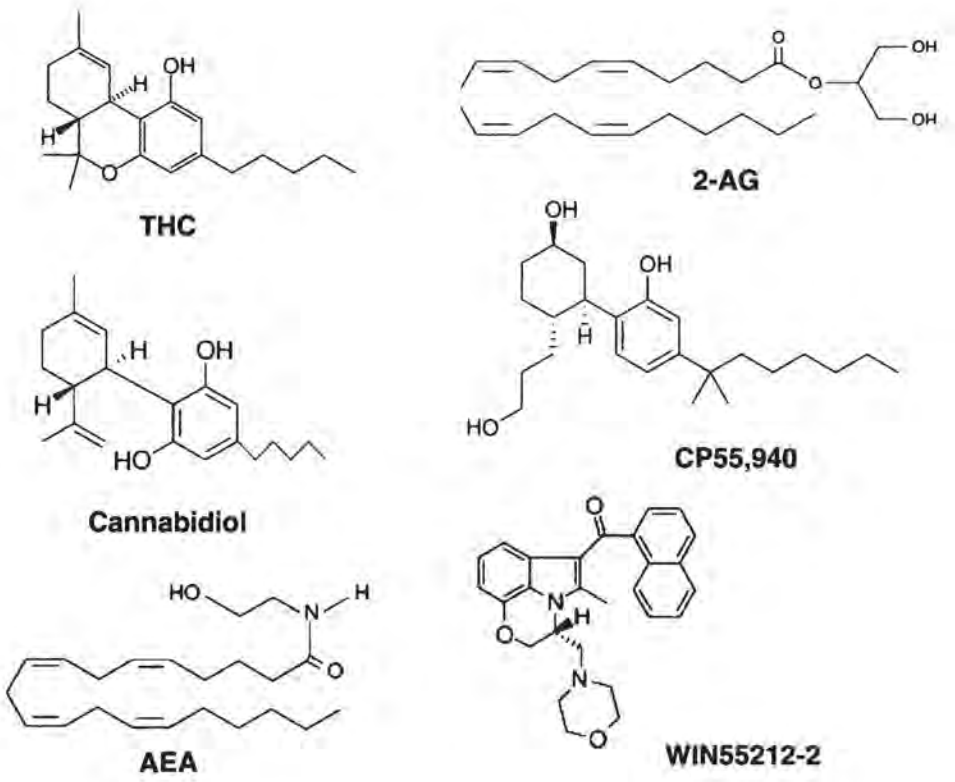



**Figure 2. Structure of general cannabinoid agonists**

Molecular structure of the phytocannabinoids tetrahydrocannabinol (THC) and cannabidiol; endocannabinoids anandamide (AEA) and 2-arachydonoylglycerol (2-AG); and synthetic cannabinoids CP55,940 and WIN55212-2

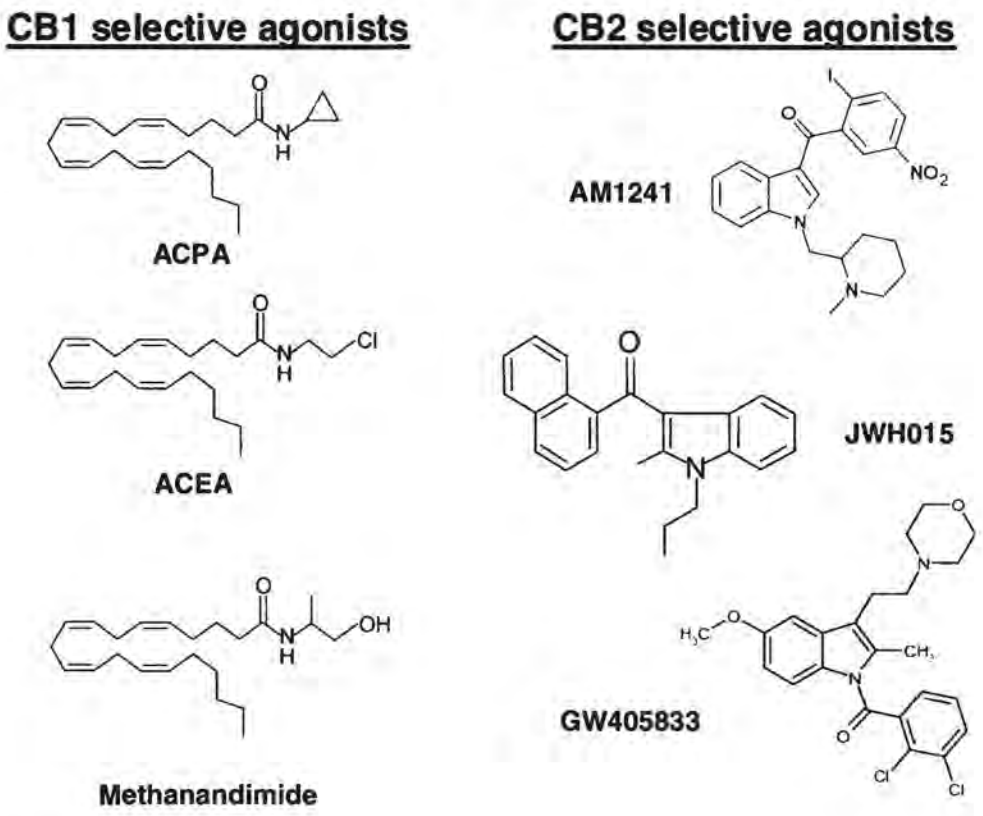



**Figure 3. Structure of selective cannabinoid agonists for the CB1 and CB2 receptors**
Molecular structures for the CB1-selective agonists ACPA, ACEA and methanandimide and the CB2-selective agonists AM1241, JWH015 and GW405833

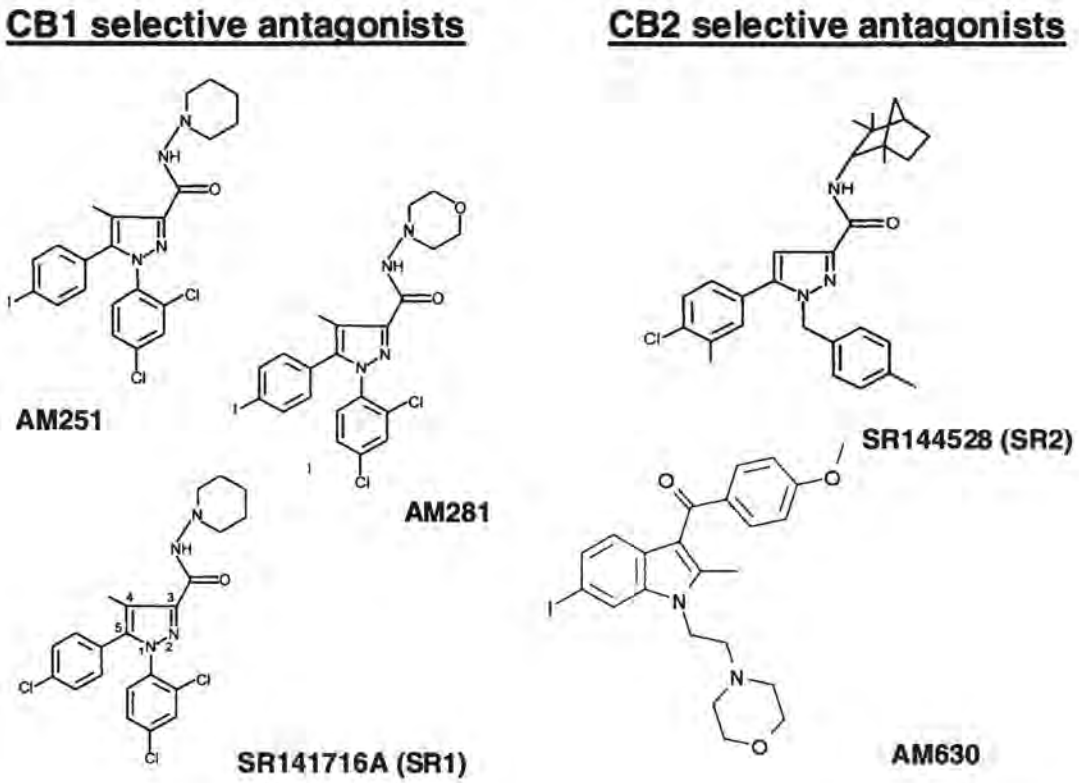



**Figure 4. Structure of selective cannabinoid antagonists for the CB1 and CB2 receptors**

Molecular structures for the CB1-selective antagonists AM251, AM281 and SR141716A (SR1), and the CB2-selective antagonists SR144528 (SR2) and AM630.

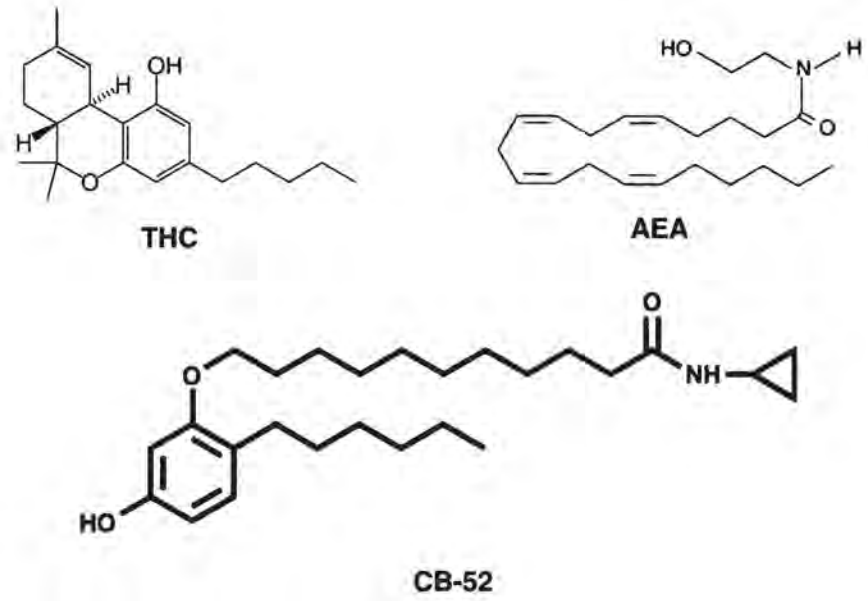



**Figure 5. Structure of the novel cannabinoid agonist CB-52**

Molecular structure of CB52, a cannabinoid agonist created as an analog of THC and AEA. This compound has a high affinity for the central cannabinoid (CB1) and peripheral cannabinoid (CB2) receptors.

# CHAPTER 2: Involvement of ERK1/2, cPLA$_2$ and NF-κB in microglia suppression by cannabinoid receptor agonists and antagonists

Rachel Ribeiro, Jie Wen, Shihe Li, and Yumin Zhang

## Abstract

Cannabinoids have been consistently shown to suppress microglia activation and the release of cytotoxic factors including nitric oxide, superoxide and proinflammatory cytokines. However, the underlying molecular mechanisms and whether the action of cannabinoids is coupled to the activation of cannabinoid type 1 (CB1) and type 2 (CB2) receptors are still poorly defined. In this study we observed that the CB1 and CB2 receptor non-selective or selective agonists dramatically attenuate iNOS induction and ROS generation in LPS-activated microglia. These effects are due to their reduction of phosphorylation of extracellular signal regulated kinase 1/2 (ERK1/2), cytosolic phospholipase $A_2$ (cPLA$_2$) and activation of NF-κB. Surprisingly, instead of reversing the effect of the respective CB1 and CB2 receptor agonists, the antagonists also suppress iNOS induction and ROS generation in activated microglia by similar mechanisms. Taken together, these results indicate that both cannabinoid receptor agonists and antagonists might suppress microglia activation by CB1 and CB2 receptor independent mechanisms, and provide a new insight into the mechanisms of microglia inhibition by cannabinoids.

## INTRODUCTION

Microglia, the resident immune cells in the central nervous system (CNS), are thought to carry out homeostatic surveillance, screening for changes in the microenvironment (35; 63). Upon activation, microglia undergo a dramatic transition from a resting, ramified morphology to a phagocytosis-capable, "macrophage-like" phenotype (165; 213). Although the initial response of microglia to injury is to promote repair by removing debris and dying cells, excessive activation of microglia appears to be toxic to neighboring cells due to their release of inflammatory cytokines (13; 73), glutamate (12; 103), nitric oxide and peroxynitrite (196). Accumulating evidence indicates that cannabinoids can modulate the function of activated microglia and the release of cytotoxic factors (193).

It has been shown that both cannabinoid receptor type 1 (CB1) and type 2 (CB2) are expressed in microglia and astrocytes (194). Unlike the CB1 receptor, which is constitutively expressed in microglia, the expression of CB2 receptor is inducible and modulated in response to the activation stage of microglia (193; 194). Increased expression of CB1 and CB2 receptors has been demonstrated in many inflammatory and neurodegenerative diseases, including multiple sclerosis, traumatic brain and spinal cord injury, Parkinson's disease, Huntington's disease and Alzheimer's disease (1; 22; 33; 139; 151).

Although there is consensus that activation of these receptors can suppress the release of cytotoxic factors from activated microglia and astrocytes, it is still controversial whether and how these receptors mediate the anti-inflammatory action of cannabinoids. It has been reported that the inhibitory effect of WIN55212-2, a non-selective cannabinoid agonist, on TNF-α production from LPS-activated microglia is not

altered by CB1 or CB2 receptor antagonists (60; 170). However, other studies suggested that the release of TNFα and other proinflammatory cytokines, such as IFN-γ, IL-12 and IL-23, is attenuated by signaling pathways coupled to the cannabinoid receptor activation (41; 189). Similarly, WIN55212-2 and other non-selective cannabinoid receptor agonists are also shown to attenuate iNOS induction in activated microglia and glutamate excitotoxicity by cannabinoid receptor dependent and independent mechanisms (58; 146; 155). Although CB2 selective agonists have been shown to be beneficial in several animal models of neurodegenerative diseases, as well as neuropathic and inflammatory pain by inhibiting microglia activation (5; 18; 95; 125; 201; 216; 220), the selective CB1 agonists and cannabidiol (CBD), a non-CB1/CB2 receptor interactive cannabinoid, are also shown to have anti-inflammatory effects by targeting CB1 receptor and the non-CB1/CB2 receptors expressed in microglial cells, respectively (4; 52; 107; 133).

We and others have previously shown that peroxynitrite, the reaction product of nitric oxide and superoxide, is the primary toxic species released from activated microglia causing toxicity to neurons and oligodendrocytes (119; 205; 218; 222; 223). It has been suggested that activation of $cPLA_2$ occurs upstream of iNOS induction in macrophages and astrocytes (163; 215), and phosphorylation of mitogen activated protein kinase (MAPK) is required for the activation of $cPLA_2$ (88; 219). In this study, we examined whether the inhibitory effects of cannabinoids are due to their interference with the signaling pathways leading to iNOS induction. Using microglia cell line BV-2 cells and primary cultures of microglia, we found that the CB1 and CB2 receptor non-selective and selective agonists attenuated iNOS induction and ROS generation in LPS-activated microglia. Surprisingly, the inhibitory effects of these cannabinoids are not reversed by

the cannabinoid receptor antagonists, and, in fact, these antagonists themselves can also attenuate nitric oxide and superoxide production in activated microglia. Furthermore, the inhibitory action of both cannabinoid receptor agonists and antagonists is mediated by a similar signaling pathway, which includes inhibition of ERK1/2 and $cPLA_2$ phosphorylation and suppression of NF-κB activation.

## MATERIALS AND METHODS

### *Materials*

Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). BV-2 cell line was a generous gift from Dr. Feng-Qiao Li, Cognosci. Inc., NC. Bacterial LPS (Escherichia Coli O111:B4) and 3-isobutyl-1-methylxanthine (IBMX) were obtained from Sigma (St. Louis, MO). 2′,7′ dichlorohydrofluorescein diacetate (DCF) was purchased from Molecular Probes, Inc. (Eugene, OR). Antibodies against the phosphorylated and the total extracellular signal regulated kinase 1/2 (ERK1/2), the phosphorylated and the total c-Jun N-terminal kinase (JNK), the phosphorylated and the total $cPLA_2$ were purchased from Cell Signaling Technology (Danvers, MA). Anti-iNOS antibody was purchased from BD Biosciences (San Diego, CA). Antibody against β-actin was obtained from Sigma (St. Louis, MO). U0126 was obtained from Calbiochem (Gibbstown, NJ). Transfection reagents were from Roche (Basel, Switzerland) and the luciferase assay kit was from Promega (Madison, WI). The non-selective cannabinoid receptor agonists CB52, CP55940 and AEA, the CB2 agonists AM1241, JWH015 and GW842166X, the CB1 antagonist SR141716A and the CB2 antagonists AM630 and SR144582 and the adenylyl cyclase activator forskolin

were obtained from Cayman Chemical (Ann Harbor, MI). The CB1 agonists ACPA, ACEA and methanandamide and the CB1 antagonist AM281 were obtained from Tocris (Ellisville, MO). All other reagents were obtained from Sigma (St. Louis, MO).

*Cell culture*

Forebrains of Sprague-Dawley rats at postnatal day 2 were dissected, dissociated and plated in 75 $cm^2$ flasks containing DMEM plus 20% FBS and 1% penicillin/streptomycin. After 10 days, the mixed cultures containing microglia, astrocytes and oligodendrocytes were shaken on an orbital shaker for 1 h at 200 rpm at 37°C. The loosely attached microglial cells were detached into the medium while the astrocytes and oligodendrocytes remain attached to the flask. Microglia in the medium were collected, centrifuged and then plated into 24-well plates at the density of $2 \times 10^5$ cells/well and 96-well plates at a density of $5 \times 10^4$ cells/well.

The murine microglial cell line BV-2 cells were cultured in 100 mm dishes in DMEM containing 10% FBS, 1% penicillin/streptomycin. Cells were maintained at 37°C in an incubator with a humidified atmosphere of 95% air and 5% $CO_2$.

*Exposure of microglia to cannabinoids and LPS*

In each experiment, cannabinoid agonists were added 30 min prior to LPS (1 μg/ml) treatment and remained during the exposure time. When applied, antagonists were added 15 min prior to the agonists. The concentration of the mixed and selective CB1 and CB2 agonists and antagonists were used at 100 nM, unless otherwise stated. All the compounds were dissolved in 0.1% DMSO, which was used as the vehicle control.

*Quantitative real-time PCR*

Total RNA was isolated from cultured BV-2 cells and primary cultures of microglia using RNeasy Mini Kit (Qiagen, Valencia, CA) and cDNA synthesis from total RNA was performed using a ReveriAid First Strand cDNA synthesis kit (Fermentas, Glen Burnie, MD) using 1 μg total RNA and 1 μl oligo (dT)18 following the manufacturer's instructions. Quantitative real time PCR was conducted with cDNA as a template in a 7500 Real time PCR System using SYBR Green PCR master mix (Applied Biosystems, Foster city, CA). The primers used for the CB1 receptor were: forward 5'-ctggttctgatcctggtggt-3' and reverse 5'-tgtctcaggtccttgctcct-3' for BV2 cells; forward 5'-catccagtgtggggagaact-3' and reverse 5'acattggggctgtctttacg-3' for primary microglia. The primers for the CB2 receptor were: forward 5'-tcattgccatcctcttttcc-3' and reverse 5'-gaaccagcatatgagcagca-3' for BV2 cells; forward 5'tgatccccaacgactacctc-3' and reverse 5'tgtcctggtgctcagtcaag-3' for microglia. All samples were run in triplicate for PCR amplification. Relative values for mRNA expression were determined from their optimized threshold cycle (CT) normalized against the CT value of an internal control gene, GAPDH, by using the comparative CT method (User Bulletin 7500, Applied Biosystems).

*cAMP measurement*

Cultures of BV2 cells were washed twice and medium was replaced by Earle's balanced salt solution (EBSS). Different concentrations (10 μM, 1 μM and 100 nM) of CP55940 were added in the absence or presence of the CB1 antagonist AM281 (1 μM)

and/or the CB2 antagonist AM630 (1 μM). Fifteen minutes later, forskolin (100 μM) was added for 10 min. The nonspecific phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 μM) was present in all the treatment conditions. Medium was aspirated and cells were lysed in a 0.1M HCl solution, centrifuged at 1,000 x g for 10 min and the supernatant was then collected. cAMP levels in microglia were measured using a competitive EIA kit from Cayman (Ann Harbor, MI) following the manufacturer's instructions.

*Western blot analysis*

At various times after drug treatment (30 min for $cPLA_2$, ERK and JNK phosphorylation, and 6 h for iNOS measurement), cells were washed with cold phosphate buffered saline (PBS) twice and whole cell lysates were obtained using ice cold protein lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH, 7.4), 1mM EDTA, 1mM EGTA, 1% Triton X-100 with freshly added protease inhibitor cocktail (Complete, Mini tablet, Roche - Mannheim, Germany) and phosphatase inhibitors (glycerophosphate and sodium orthovanadate). The lysates were subject to centrifugation at 10,000 x g for 10 min at 4°C. Aliquots were removed for protein determination. Whole cell lysates were mixed with electrophoresis sample buffer and boiled for 5 min. The proteins were separated on Novex 4-12% Bis-Tris gel, transferred to a PVDF membrane, and then blocked with 5% milk for 1 h at room temperature. Membranes were incubated overnight with primary antibodies against phosphorylated and total ERK1/2, phosphorylated and total JNK, phosphorylated and total $cPLA_2$, iNOS, and monoclonal antibody against β-actin. After incubation, membranes were washed and incubated with horseradish

peroxidase conjugated secondary antibodies. The membranes were incubated in hysignal western blot detection reagent for 1 min and protein was visualized using Image Reader LAS-3000 software. Quantification for Western blot analysis was done by measuring band density using Multi Gauge software.

*siRNA transfection*

To determine the role of $cPLA_2$ phosphorylation in LPS-induced iNOS induction in microglia, the double-stranded siRNA oligonucleotide for $cPLA_2$ (Qiagen, Valencia, CA) was transfected into BV-2 cells with X-treme transfection reagent (60 nmol siRNA/well). The day before transfection, BV-2 cells were plated into a 24-well plate at a density of $2x10^5$ cells/well to assure the cells around 80% confluent at the time of transfection. The transfected cells were continuously incubated at 37°C for 48 h for further experiment. siGLO RISC-free siRNA from Dharmacon was used as a negative control and its fluorescence was also used for evaluating the transfection efficiency.

*Measurement for ROS*

BV2 cells and primary microglia in 96-well plates were pretreated with cannabinoid agonists or antagonists for 15 min, and then LPS (1 μg/ml) was added. Six hours later, wells were washed with EBSS twice and incubated with 20 μM DCF at 37 °C for 30 min. ROS generation was quantified using the Spectra MAX Gemini XS microplate reader with excitation wavelength at 485 nm and emission wavelength at 538 nm at room temperature.

*Nitrite measurement*

Nitrite concentration was measured using a fluorometric assay kit from Cayman Chemical (Ann Arbor, MI). Cannabinoid agonists (100 nM) were added 30 min before the addition of LPS (1 μg/ml). Media was collected at 24 h and the concentration of nitrite was measured following the manufacturer's instructions.

*Plasmid transfection and luciferase assay*

The reporter gene with NF-κB promoter was transfected into BV-2 cells. In brief, cells were trypsinized and plated into a 96-well plate at a density of 5 x$10^4$ cells/well. The transfection was performed with FuGene HD transfection reagent. 1 μg plasmid containing NF-κB promoter or green fluorescent protein (GFP) was mixed with 0.25 μl FuGene HD in a total volume of 5 μl of serum-free DMEM for each reaction. At 24 h after transfection, cells were treated with LPS for 3 h in the presence of cannabinoid receptor agonists and antagonists. The assessment of luciferase activity in the transfected cells was carried out with luciferase reporter assay system from Promega following the manufacturer's instructions.

*Statistical analysis*

Statistical significance was assessed using ANOVA with the Tukey-Kramer post-hoc multiple comparison test. Statistical analysis was performed using the Instat program from GraphPad Software (San Diego, CA). Representative experiments are shown unless noted otherwise. Experiments were performed with triplicate samples, and the data are expressed as mean ± SEM. All experiments were repeated at least 3 times.

## RESULTS

### ERK1/2 and JNK phosphorylation leads to cPLA$_2$ activation and iNOS induction in LPS-activated BV-2 cells

Activation of cPLA$_2$ has been reported to occur downstream MAPK phosphorylation (88) and upstream iNOS induction in macrophages and astrocytes (163; 215). However, it is unclear whether the same signaling pathway is required in activated microglia. To test this possibility, we examined cPLA$_2$ phosphorylation and iNOS induction in murine microglia cell line BV-2 cells following LPS treatment. LPS (1 μg/ml)-induced phosphorylation of cPLA$_2$ was dramatically increased at 30 min and then gradually decreased until 6 h. cPLA$_2$ phosphorylation preceded iNOS induction, which appeared at 3 h, peaked at 6 h and then decreased at 24 h (Fig. 6A). The same phenomenon was also observed when LPS was used at 10 ng/ml and 100 ng/ml, and notably the cell viability was not affected by LPS treatment examined by Alamar blue assay (data not shown). To determine whether cPLA$_2$ phosphorylation is required for the induction of iNOS, BV-2 cells were transfected with siRNA of cPLA$_2$ for 48 h, which almost completely knocked down the expression of cPLA$_2$ (Fig. 6B). Downregulation of cPLA$_2$ or treatment with the cPLA$_2$ inhibitor, AACOCF3 (10 μM), attenuated iNOS induction at 6 h after LPS treatment. In both scenarios, the expression of iNOS was down to 15% of their respective controls (RISC-free siRNA and vehicle controls treated with LPS) (Fig. 6C). Among the inhibitors of various MAPKs, U0126 (5 μM), an inhibitor for the phosphorylation of ERK1/2, and SP600125 (10 μM), a selective inhibitor of JNK, dramatically reduced cPLA$_2$ phosphorylation in BV2 cells treated with LPS, the phosphorylation levels were 47.4 ± 1.2% and 26.9 ± 0.8% of the LPS alone group,

respectively (Fig. 6D). On the other hand, SB203580 (10 μM), an inhibitor of p38 did not have any effect (Fig. 6D). Taken together, the data demonstrate that LPS treatment causes ERK1/2- and JNK- dependent $cPLA_2$ phosphorylation and iNOS induction.

**CB1 and CB2 receptors are expressed in BV2 and primary microglia and their activation attenuates cAMP production triggered by forskolin.**

While the CB1 and CB2 receptors are rarely expressed in microglia in a healthy brain, their expression can be upregulated through the activation of these cells (193). *In vitro*, both receptors have been shown to be expressed in microglia, likely due to the fact that these cells might be in a "primed" state under culture conditions (16; 60; 210). A number of microglia cell lines, because of their high rates of proliferation, have also been shown to express these receptors (34; 210). Consistent with these reports, we also found that both CB1 and CB2 receptors are expressed in BV2 cells and primary microglia detected by quantitative real-time PCR (Fig. 7A-B). We then looked at the ability of these receptors to reduce the production of cAMP activated by forskolin (Fig. 7C-D), which is a common feature for this type of G-protein coupled receptors (Patcher et al, 2006). Treatment with forskolin (100 μM) for 10 min caused a dramatic increase of cAMP production, which was significantly attenuated by the non-selective cannabinoid agonist CP55940 in a concentration dependent manner (Fig. 7C). The inhibitory effect was partially reversed by either the CB1 antagonist AM281 or the CB2 antagonist AM630, and completely blocked when both antagonists were added together (Fig. 7D).

**Cannabinoids attenuate iNOS induction in a dose-dependent manner**

Cannabinoids have been shown to lower iNOS induction in activated microglial cells (58; 209). Figure 3 shows a concentration-dependent inhibition of iNOS by the general cannabinoid receptor agonist AEA (Fig. 8A), the CB1 receptor selective agonist ACPA (Fig. 8B), and the CB2 receptor selective agonist AM1241 (Fig. 8C). At 1 μM and 100 nM, all the cannabinoid compounds significantly reduced the induction of iNOS in LPS-activated BV2 cells. A concentration of 100 nM was chosen in further studies, which is based on the previous report about their binding affinities to CB1 and CB2 receptors (161) and the fact that at concentrations higher than 100 nM, cannabinoids will likely produce off-target effects (194).

**Non-selective cannabinoid receptor agonists attenuate ERK1/2 and $cPLA_2$ phosphorylation, and iNOS induction in activated BV-2 cells**

Although cannabinoids have been shown to reduce iNOS induction in primary cultures of microglia and in BV-2 cells (58; 209), the underlying mechanisms by which cannabinoids attenuate nitric oxide production are poorly understood. Similar to AEA, the other non-selective cannabinoid receptor agonists WIN 55212-2 (100 nM) and CP55940 (100 nM) also dramatically reduced iNOS induction and nitric oxide production in LPS-activated BV-2 cells (Figs. 9A-B). To determine whether the inhibitory action of these cannabinoids is due to their interference with ERK1/2, JNK and $cPLA_2$ phosphorylation, BV2 cells were treated with LPS for 30 min in the absence or presence of AEA, WIN 55212-2 and CP55940, the phosphorylation of ERK1/2, JNK and $cPLA_2$ was assessed by western blot. CP55940, WIN55212-2 and AEA significantly attenuated

ERK1/2 (Fig. 9C) and $cPLA_2$ phosphorylation (Fig. 9D), but had no effect on JNK phosphorylation (data not shown).

**CB1 and CB2 selective agonists attenuate ERK1/2 and $cPLA_2$ phosphorylation and iNOS induction in activated BV2 cells**

We then tested the role of CB1 and CB2 selective agonists on iNOS induction, ERK1/2 and $cPLA_2$ phosphorylation in LPS-activated BV-2 cells. Consistent with the results obtained using the non-selective cannabinoid receptor agonists, CB1 selective agonists ACEA, ACPA and methanandamide (each at 100 nM), significantly attenuated iNOS induction (Fig. 10A), nitric oxide production (data not shown), and the phosphorylation of ERK1/2 and $cPLA_2$ (Figs. 10B and C). The same phenomenon was also observed when the CB2 selective agonists, AM1241, GW842166X and JWH015 were used (Figs. 10D and F). To examine whether the action of cannabinoids can be reversed by the cannabinoid receptor antagonists, the CB1 antagonist AM281 and the CB2 antagonist AM630, were administered 15 min before and during treatment with the cannabinoid receptor agonists. AM281 (100 nM) and AM630 (100 nM) either used alone or in combination did not reverse the inhibitory effect of AEA on iNOS induction (Fig. 11A). The same results were also obtained when these antagonists were used at 1 and 10 μM and the other cannabinoid agonists, WIN55212-2 and CP55940, were used (data not shown). Surprisingly, we found that AM281 or AM630 alone or their combination also completely blocked iNOS induction (Figs. 11A and B). Similarly, the reduced iNOS induction by the CB1 selective agonist ACPA and the CB2 selective agonist AM1241 was not reversed by the respective CB1 and CB2 receptor antagonists (Fig. 11B). These

results suggest that AM281 and AM630 may directly interfere with the signaling pathways leading to nitric oxide production in activated microglia.

**CB1 and CB2 receptor antagonists attenuate ERK1/2 and cPLA$_2$ phosphorylation and iNOS induction in activated BV2 cells**

The above results led us to examine whether the CB1 and/or CB2 receptor antagonists could directly affect the signaling pathway leading to iNOS induction in LPS-activated BV-2 cells. Consistent with the effect of AM281 and AM630, the other commonly used CB1 receptor antagonist SR141716A (100 nM) and the CB2 receptor antagonist SR144582 (100 nM) also reduced iNOS induction (Fig. 12A). Similar to the cannabinoid receptor agonists, these antagonists either used alone or in combination also dramatically attenuated ERK1/2 and cPLA$_2$ phosphorylation (Figs. 12B and C).

**CB1 and CB2 receptor agonists and antagonists attenuate ROS generation in BV-2 cells activated by LPS**

It has been previously shown that peroxynitrite, the reaction product of nitric oxide and superoxide, is the primary toxic species released from activated microglia causing toxicity to its neighboring cells, such as neurons and oligodendrocytes (119; 218). Using DCF as an indicator of ROS generation, we found that treatment with LPS for 6 h caused a 2.5 fold increase in ROS generation (Fig. 13). The non-selective cannabinoid receptor agonist AEA, the CB1 selective agonist ACPA and the CB2 selective agonist AM1241 each at 100 nM, almost completely blocked ROS generation (Fig. 13A). The general cannabinoid agonists CP55940 and WIN55212-2, the CB1

agonists ACEA and methanandamide and the CB2 agonists AM1241, JWH015 and GW842166X also significantly attenuated ROS generation (data not shown). Consistent with their effects on iNOS induction (Fig. 12A), the CB1 receptor antagonists (AM281 and SR141716A) and the CB2 receptor antagonists (AM630 and SR144582) also significantly reduced ROS generation in LPS activated BV-2 cells (Fig. 13B). These results suggest that both CB1 and CB2 receptor agonists and antagonists are able to attenuate peroxynitrite formation in activated microglia.

**Activation of NF-κB in LPS-activated BV-2 cells can be blocked by both cannabinoid receptor agonists and antagonists**

It has been shown that iNOS induction in activated BV-2 cells can be transcriptionally regulated by NF-κB activation (58; 146; 155; 214). Similar to the effect of U0126, a specific inhibitor for ERK1/2 phosphorylation, the $cPLA_2$ inhibitor AACOCF3 also significantly reduced NF-κB activation in LPS-activated BV-2 cells (Fig. 14A). Since cannabinoid receptor agonists and antagonists can attenuate iNOS induction and ERK1/2 and $cPLA_2$ phosphorylation, we anticipated that these cannabinoids could also block NF-κB activation in activated microglia. The non-selective cannabinoid receptor agonist AEA, the CB1 selective agonist ACPA, and the CB2 selective agonist AM1241 completely blocked NF-κB activation (Fig. 14B). Similarly, the general cannabinoid receptor agonists CP55940 and WIN55212-2, the CB1 receptor agonists ACEA and methanandamide and the CB2 receptor agonists AM1241, JWH015 and GW842166X also displayed a complete inhibition of the NF-κB activity (data not shown). In harmony to their inhibitory effects on ERK1/2 phosphorylation and iNOS

induction, the CB1 and CB2 receptor antagonists also significantly attenuated the activation of NF-κB in LPS-activated BV2 cells (Fig. 14C).

**Cannabinoid receptor agonists and antagonists reduce ERK1/2, $cPLA_2$ phosphorylation and iNOS induction in primary cultures of microglia activated by LPS**

Although BV-2 cell line is widely used to study the signaling mechanisms of microglia, many laboratories have reported that the phenotype of BV-2 cells is different from microglia in primary cultures (194). To determine whether the same signaling pathway of iNOS induction is also present and affected by the treatment of cannabinoid receptor agonists and antagonists in primary cultures of microglia, phosphorylation of ERK1/2, $cPLA_2$ and induction of iNOS were also examined in primary microglia following LPS treatment. Similar to the phenomenon found in BV-2 cells, LPS also induced time-dependent ERK1/2 and $cPLA_2$ phosphorylation and iNOS induction (Figs. 15A and B). CB52, WIN55212-2 and AEA significantly attenuated iNOS induction (Fig. 15C), ERK1/2 and $cPLA_2$ phosphorylation activated by LPS (Figs. 15D and E). Consistently, all the cannabinoid receptor agonists and antagonists also attenuated ROS generation in primary cultures of microglia when activated by LPS (Figs. 16A and B).

## DISCUSSION

Cannabinoid receptor antagonists are commonly used to determine the CB1 and CB2 receptor dependent mechanisms of cannabinoids in both *in vitro* and *in vivo* studies. Although numerous studies have demonstrated that CB2 receptor is highly expressed in

activated microglia in many inflammatory and neurodegenerative diseases and likely mediates the anti-inflammatory properties of cannabinoids, the *in vitro* studies using microglia cell lines and primary cultures of microglia continue to produce inconsistent results on the role of CB1 and CB2 receptors in the action of cannabinoids (194). In both BV-2 cells and primary microglia, we found that cannabinoid receptor agonists attenuated iNOS induction and ROS generation following LPS treatment. However, the inhibitory effect was not reversed by the CB1 and CB2 receptor antagonists, and in fact surprisingly, the antagonists themselves also suppressed microglia activation by interfering with ERK1/2 and $cPLA_2$ phosphorylation and NF-κB activation.

**Cannabinoid receptor agonists blocked iNOS induction in activated microglia via inhibition of ERK1/2 and $cPLA_2$ phosphorylation and NF-κB activation.**

Similar to its role in reactive astrocytes and macrophages, activation of $cPLA_2$ also leads to iNOS induction in BV-2 cells and primary microglia when activated by LPS. Among the MAPKs, we found that the phosphorylation of ERK1/2 and JNK, but not p38, causes $cPLA_2$ activation. Although cannabinoids have long been known to possess anti-oxidative and anti-inflammatory properties, the mechanisms of cannabinoid action in microglia are still not well understood. The phosphorylation of ERK1/2 and JNK is increased at 30 min following LPS treatment. The non-selective cannabinoid receptor agonists attenuate the phosphorylation of ERK1/2, but not JNK. It has been shown that AEA can activate MAPK phosphatase-1 (MKP-1) resulting in the dephosphorylation of ERK1/2 and the reduction of iNOS from LPS activated microglia. The induction of MKP-1 is mediated by CB1 and CB2 receptor activation and is

associated with histone H3 phosphorylation of the MKP-1 gene (58). Activation of MKP-3 and the subsequent dephosphorylation of ERK1/2 were also shown to contribute to the inhibitory action of cannabinoids on microglia migration and the release of proinflammatory cytokines (178). The upregulation of MKP-1 and MKP-3 may not be the initial cause for the inhibitory action of cannabinoids on ERK1/2 phosphorylation, since there were no changes in the expression of MKP-1 and MKP-3 at 30 min following LPS treatment in the absence and presence of cannabinoids (data not shown). However, we do not exclude the possibility that the induction of MKP-1 and MKP-3 may contribute to the de-phosphorylation of ERK1/2 in microglia at late time following LPS treatment as reported previously (58; 178).

Because of the low yield and the difficulty for molecular manipulation of targeted molecules in primary cultures of microglia, BV-2 cells are widely used as a surrogate model to study the signaling mechanisms of activated microglia and the action of a variety of anti-inflammatory agents. Although BV-2 cells are thought to be morphologically and functionally similar to the primary cultures of microglia (108), many studies have shown that the phenotype of these cells is different and a recent study demonstrates that the expression of 2-AG hydrolyzing enzyme ABHD6 (serine hydrolase α-β-hydrolase domain 6) is found in BV-2 cells, but not in primary cultures of microglia (131). To determine whether the BV-2 cell line can be used as a model system to study the action of cannabinoids in microglia, we performed parallel studies to elucidate the signaling mechanisms of iNOS induction and ROS generation in BV-2 and primary microglia following LPS treatment. It has been shown that $cPLA_2$ activation occurs upstream of iNOS induction in macrophages and astrocytes (163; 215). Similar to the

$cPLA_2$ inhibitor AACOCF3, downregulation of $cPLA_2$ by siRNA also significantly blocked iNOS induction in LPS-activated BV-2 cells. We revealed that activation of $cPLA_2$ occurs downstream of ERK1/2 phosphorylation, since inhibition of ERK1/2 attenuates $cPLA_2$ phosphorylation, and $cPLA_2$ siRNA has no effect on the phosphorylation of ERK1/2 (data not shown). Inhibitors of ERK1/2 and $cPLA_2$ also attenuate the activation of NF-κB, which is the transcriptional regulator for iNOS expression in LPS-activated BV-2 cells (58; 146; 155; 214). Our results suggest that cannabinoid agonists blocked iNOS induction and ROS generation in BV-2 and primary microglia by attenuating ERK1/2 and $cPLA_2$ phosphorylation and NF-κB activation.

**CB1/CB2 receptor antagonists did not reverse the action of cannabinoid agonists, but actually blocked NO and ROS production themselves.**

To determine whether the inhibition of cannabinoids on iNOS induction and ROS generation is due to their suppression of this signaling cascade and mediated by activation of CB1 and CB2 receptors, microglia were treated with LPS in the presence of cannabinoid agonists and/or antagonists. Although the non-selective, and the selective CB1 and CB2 agonists are shown to block NF-κB activation, iNOS induction and ROS generation, it is still unclear whether the action of these cannabinoid compounds is mediated by activation of CB1 and CB2 receptors (43; 149). Our results have indicated that: 1) both CB1 and CB2 receptors are expressed in BV2 and primary microglia and activation of these receptors can attenuate cAMP production; 2) the inhibitory effects of the non-selective cannabinoid agonists on iNOS induction and ROS generation are not reversed by either the CB1 or CB2 receptor antagonist alone or their combination; 3) the

action of CB1 and CB2 selective agonists can not be antagonized by the respective CB1 and CB2 receptor selective antagonists; and 4) the CB1 and CB2 receptor selective antagonists also block iNOS induction and ROS generation. Interestingly, we found that the CB1 and CB2 receptor antagonists, similar to their agonists, can also attenuate the phosphorylation of ERK1/2 and $cPLA_2$, and activation of NF-κB in LPS-activated microglia.

**The anti-inflammatory effects of cannabinoid receptor agonists and antagonists are likely mediated by CB1/CB2 receptor independent mechanisms.**

Recently, several non-CB1 and non-CB2 receptors have been suggested to mediate the action of several cannabinoid compounds in activated microglia. Stella's group has previously shown that 2-AG can induce migration of microglia through the abnormal cannabidiol (abn-CBD) receptors (66). In hippocampal slice cultures, 2-AG, but not AEA and THC, is shown to protect against NMDA-induced excitotoxicity via activation of the abn-CBD on microglia cells (109; 110). It is known that not all cannabinoid compounds can activate abn-CBD, one of such examples is WIN55212-2, which does not activate abn-CBD (194). In this study, we found that WIN55212-2 as well as other CB1 and CB2 receptor selective and non-selective agonists reduced iNOS induction and ROS generation, suggesting that the action of these cannabinoids is unlikely mediated by activation of abn-CBD. In addition, we found that the abn-CBD antagonist O-1918 did not reverse the inhibitory effects (data not shown). These results are in agreement with a recent report showing that THC and cannabidiol can decrease the production and the release of proinflammatory cytokines, including IL-1β, IL-6 and IFN-

β, from LPS-activated microglia via CB1, CB2 and abn-CBD independent mechanisms (108). The inhibitory effects are attributable to their interference with the NF-κB and IFN-β dependent signaling pathways. The other possible non-CB1/CB2 receptors include transient receptor potential vanilloid 1 (TRPV1) receptor, WIN receptor, and the G-protein coupled receptor GPR55 (161). In addition, the non-receptor dependent mechanisms, such as direct antioxidant effects and induction of peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ), may account for, at least in part, the anti-inflammatory and neuroprotective effects of cannabinoids.

Several studies suggest that the CB1 receptor antagonist/inverse agonist SR141716A (rimonabant) has neuroprotective effects in models of cerebral ischemia, trauma, NMDA induced neuronal damage and Parkinson's disease (65). Although the action of rimonabant in these disease models is unclear, our findings that CB1 and CB2 receptor antagonists can directly suppress iNOS induction and ROS generation in activated microglia may account for part of the protective mechanisms. The common signaling pathway shared by both cannabinoid receptor agonists and antagonists suggests that these well-described CB1/CB2 agonists and antagonists may suppress microglia activation by novel CB1 and CB2 receptor independent mechanisms. In fact, both cannabinoid receptor agonists and antagonists have been shown to activate GPR55, TRPV1 and PPARγ (161), but the molecular targets responsible for the anti-inflammatory action of these agents remain to be determined. Microglia isolated from CB1 and CB2 receptor knockout mice will help to identify potential targets for cannabinoid receptor agonists and antagonists.

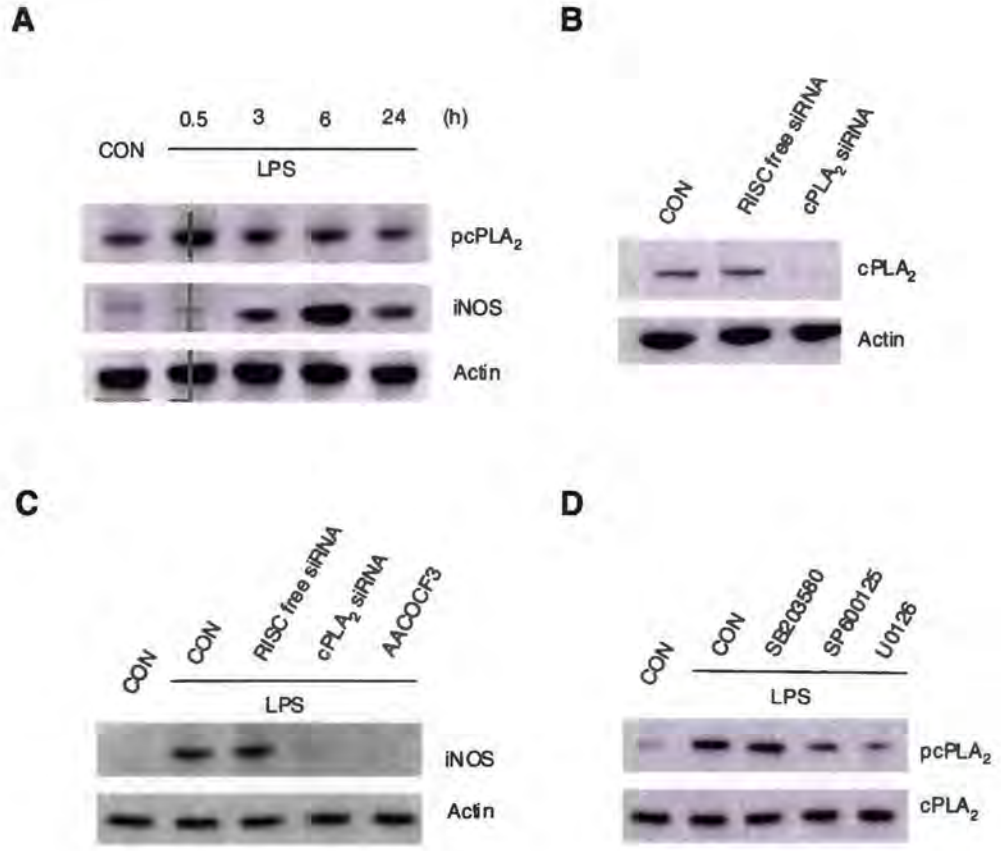



**Figure 6. Phosphorylation of ERK1/2 and JNK led to $cPLA_2$ activation and iNOS induction in LPS-activated BV2 cells**

*A*, BV-2 cells were treated with LPS (1 μg/ml) for various times and the cell lysates were subjected to western blot using antibodies against phosphorylated $cPLA_2$ ($pcPLA_2$), iNOS and beta-actin. A representative experiment of four that were performed is shown.

*B*, Cells were transfected with $cPLA_2$ siRNA and the control RISC-free siRNA for 48 h and then lysed for detection of the expression of $cPLA_2$. A representative experiment of three that were performed is shown.

*C*, Cells were transfected with $cPLA_2$ siRNA and the RISC-free siRNA for 48 h and then treated with LPS for 6 h. Cells without siRNA transfection were also treated with LPS for 6 h in the presence of the $cPLA_2$ inhibitor AACOCF3 (10 μM). A representative experiment of three that were performed is shown.

*D*, Cells were treated with LPS (1 μg/ml) in the presence of SB203580, SP600125 and U0126 for 30 min, and the cell lysates were subjected to western blot using antibodies against the phosphorylated and the non-phosphorylated $cPLA_2$. SP600125 (10 μM) and U0126 (5 μM) attenuated $cPLA_2$ phosphorylation, but SB203580 (10 μM) had no effect. A representative experiment of three that were performed is shown (Fig.6B-C generated by Wen, J)

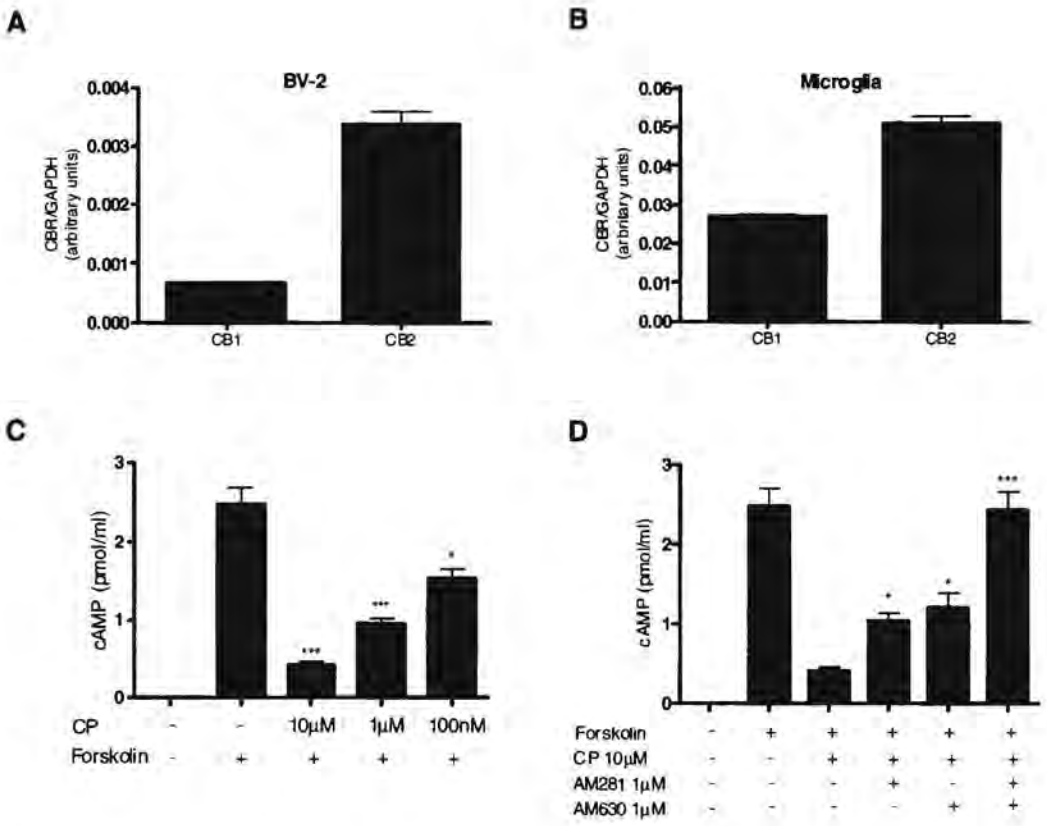



**Figure 7. The non-selective cannabinoid agonist CP55940 reduced forskolin-induced cAMP production in BV2 cells in a CB1/CB2 receptor dependent manner**

*A-B,* RNA samples isolated from BV2 and primary microglia were analyzed by quantitative real-time PCR using primers specific for mouse and rat CB1 and CB2 receptors. GAPDH mRNA was used as an internal control. The mRNA levels of the CB1 and CB2 receptors in BV2 cells (A) and primary microglia (B) were indicated by the ratio of the expression of cannabinoid receptors (CBR) and GAPDH. The data shown are pooled from three different experiments.

*C,* BV2 cells were pre-incubated with various concentrations of the non-selective cannabinoid agonist CP55940 and then treated with forskolin (100 μM) for 10 min. CP55940 dose-dependently reduced cAMP production activated by forskolin. *$p < 0.05$ and ***$p < 0.001$ compared to the forskolin alone group. The data shown are pooled from three different experiments.

*D,* The reduction of forskolin-induced cAMP production by CP55940 (CP; 10 μM) was partially reversed by either the CB1 receptor antagonist AM281 (1 μM) or the CB2 receptor antagonist AM630 (1 μM), and completely blocked when AM281 and AM630 were co-applied. *$p < 0.05$ and ***$p < 0.001$ compared to the forskolin group treated with CP alone. The data are pooled from three different experiments (Fig. 7A-B generated by Wen, J).

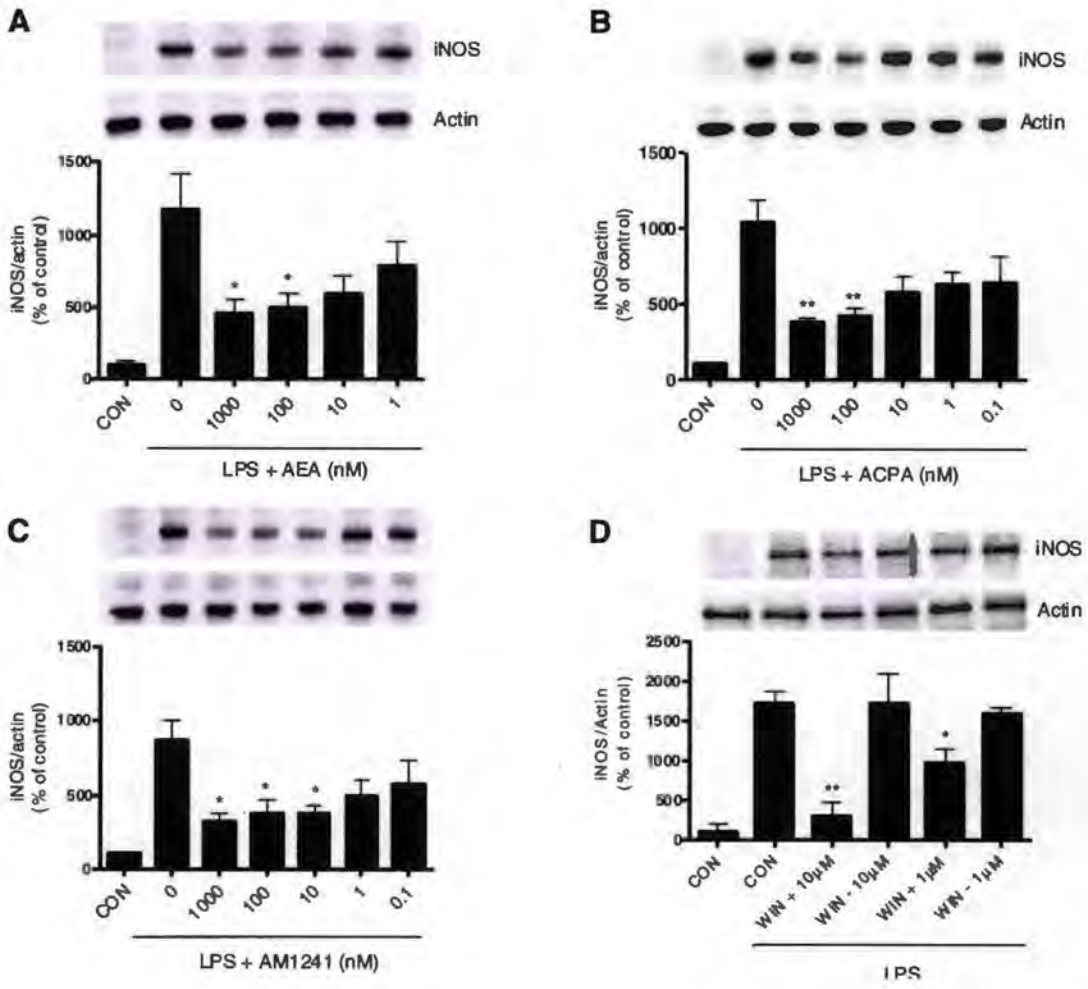



**Figure 8. Cannabinoids attenuated iNOS induction in a dose-dependent manner**

*A,* BV-2 cells were treated with LPS (1 μg/ml) in the presence of AEA at various concentrations for 6 h and iNOS induction was detected by western blot. AEA at 1 μM and 100 nM significantly attenuated iNOS induction (*$p < 0.05$ compared to LPS alone group). The data shown are pooled from four different experiments. *B-C,* A selective CB1 agonist ACPA attenuated iNOS induction at 1 μM and 100 nM (**$p<0.01$ compared to LPS alone group. B), whereas the CB2 selective agonist AM1241 showed a significant decrease in iNOS induction at 1 μM, 100 nM and 10 nM (*$p < 0.05$ compared to LPS alone group. C).

*D,* WIN55212-2 (WIN+), but not its inactive enantiomer WIN55212-3 (WIN-), was found to significantly decrease iNOS induction in BV2 cells (*$p<0.05$ and **$p < 0.01$ compared to LPS alone group).

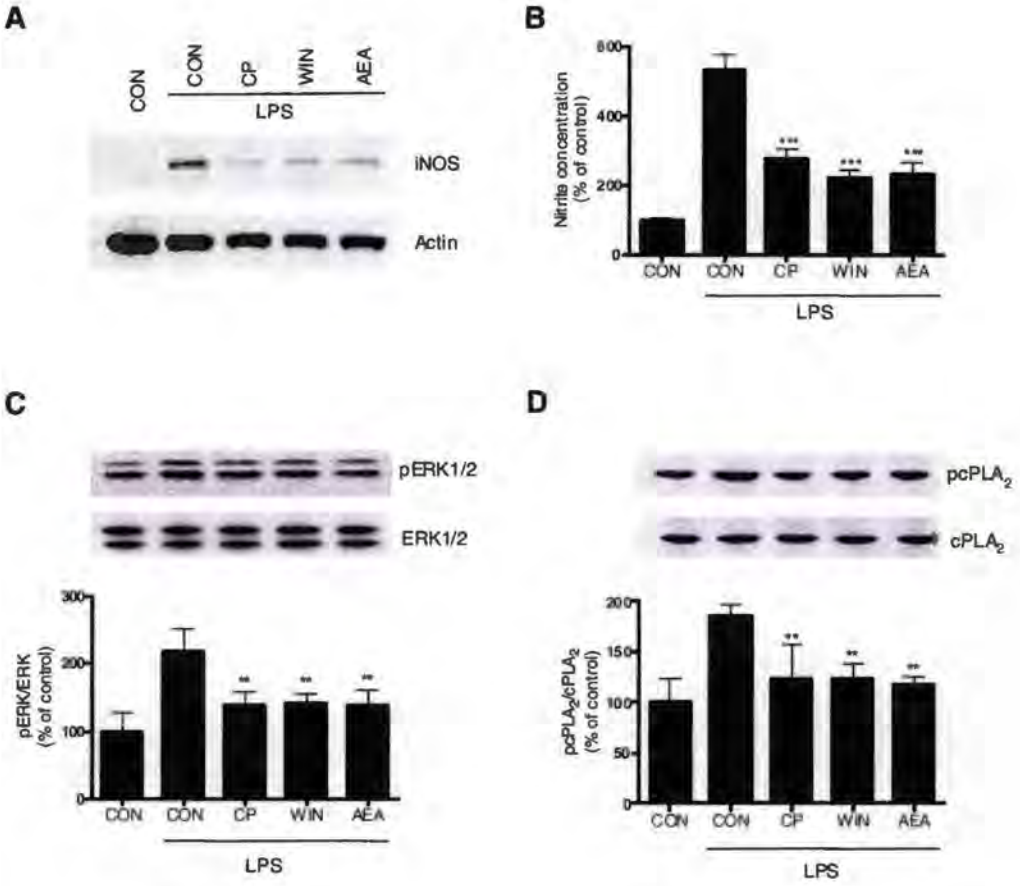



**Figure 9. Cannabinoid receptor non-selective agonists attenuated iNOS induction, nitric oxide production, ERK1/2 and cPLA$_2$ phosphorylation in BV-2 cells treated with LPS**

*A*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of the cannabinoid receptor non-selective agonists CP55940 (CP), WIN55212-2 (WIN) and AEA for 6 h and then lysed for western blot analysis. All the cannabinoid compounds at 100 nM dramatically blocked iNOS induction. A representative experiment of six that were performed is shown.

*B*, At 24 h following LPS (1 μg/ml) treatment with/without CP, WIN and AEA, culture media from 24-well plates were collected for measurement of nitrite production by activated microglia. ***$p < 0.001$ was obtained when the cannabinoid treated groups were compared to the LPS alone group. The data shown are pooled from three different experiments.

*C-D*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of CP, WIN and AEA for 30 min and then lysed for western blot analysis. These cannabinoid compounds at 100 nM significantly attenuated the phosphorylation of ERK1/2 (C) and cPLA$_2$ (D) in LPS-activated BV2 cells. **$p < 0.01$ was obtained when the drug treated groups were compared to the LPS alone group. The data shown are pooled from four different experiments.

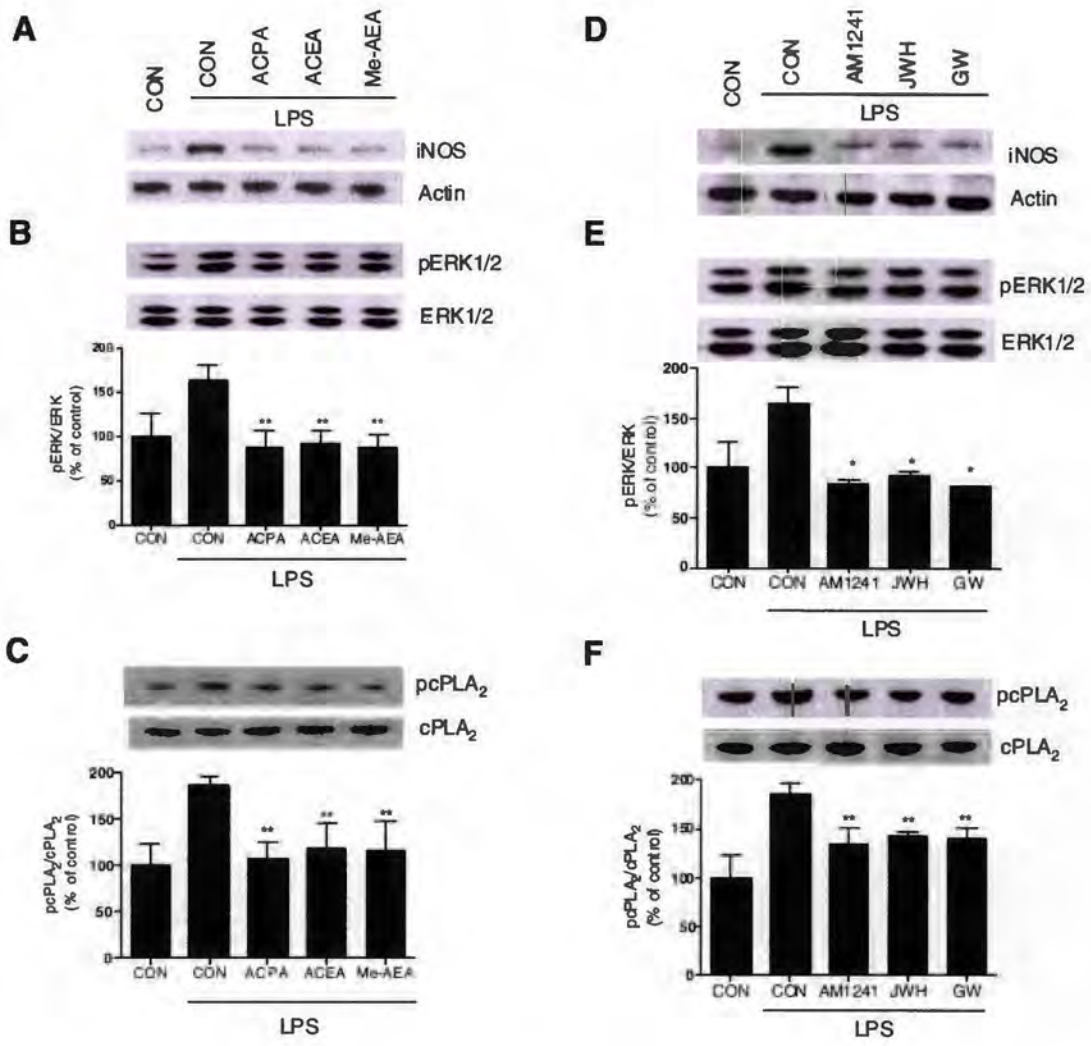
**Figure 10. CB1 and CB2 receptor selective agonists attenuated iNOS induction, ERK1/2 and cPLA$_2$ phosphorylation in BV-2 cells treated with LPS**

*A*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of the CB1 selective agonists ACPA, ACEA and methanandamide (Me-AEA) for 6 h and then lysed for western blot analysis. The cannabinoid compounds at 100 nM almost completely blocked iNOS induction. A representative experiment of three that were performed is shown.

*B-C*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of ACPA, ACEA and Me-AEA (100 nM) for 30 min and then lysed for western blot analysis. The phosphorylation of ERK1/2 and cPLA$_2$ was significantly attenuated by the CB1 receptor selective agonists. $^{**}p < 0.01$ was obtained when the drug treated groups were compared to the LPS alone group. The data shown are pooled from three different experiments.

*D*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of the CB2 receptor selective agonists AM1241, GW842166X (GW) and JWH015 (JWH) for 6 h and then lysed for western blot analysis. All the cannabinoid compounds at 100 nM almost completely blocked iNOS induction. A representative experiment of three that were performed is shown.

*E-F*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of AM1241, GW842166X (GW) and JWH015 (JW) for 30 min and then lysed for western blot analysis. The increased ERK1/2 and cPLA$_2$ phosphorylation was significantly attenuated by the CB1 receptor selective agonists. $^{*}p < 0.05$ and $^{**}p < 0.01$ were obtained when the drug treated groups were compared to the LPS alone group. The data shown are pooled from three different experiments.


A
CON
CON
ACPA
ACEA
Me-AEA
LPS
iNOS
Actin
B
pERK1/2
ERK1/2
pERK/ERK (% of control)
CON
CON
ACPA
ACEA
Me-AEA
LPS
C
pcPLA2
cPLA2
pcPLA2/cPLA2 (% of control)
CON
CON
ACPA
ACEA
Me-AEA
LPS
D
CON
CON
AM1241
JWH
GW
LPS
iNOS
Actin
E
pERK1/2
ERK1/2
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CON
CON
AM1241
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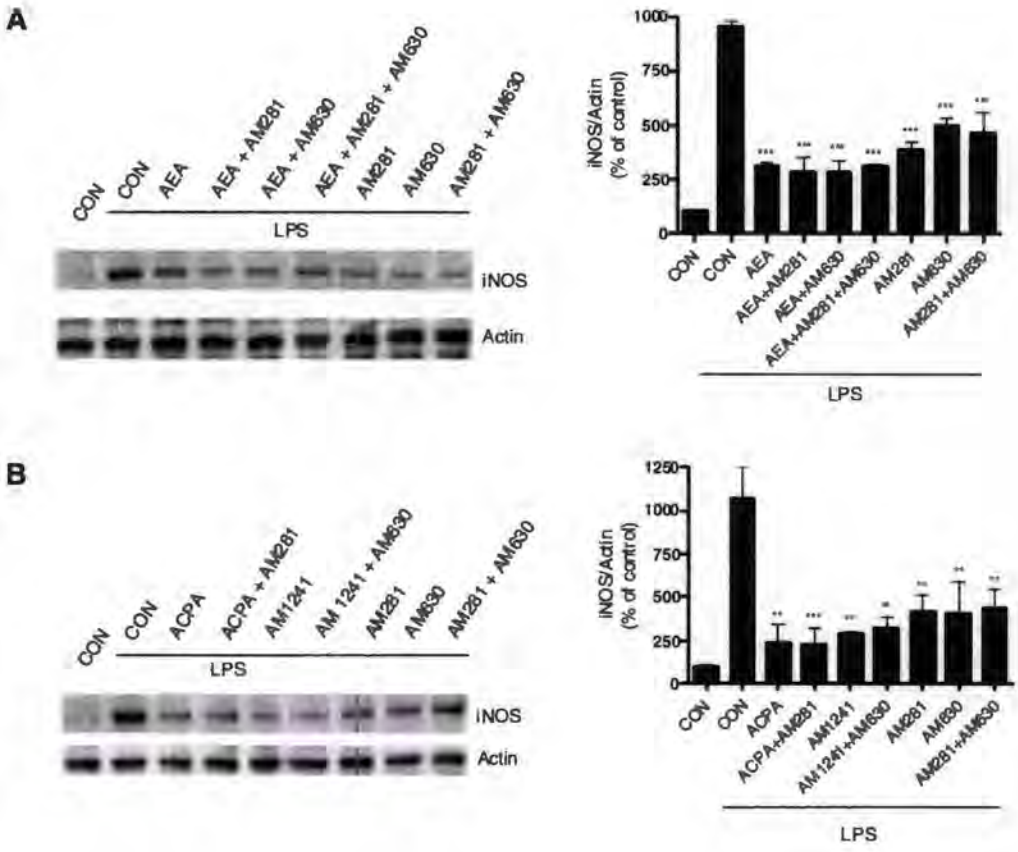



**Figure 11. The inhibitory effects of CB1 and CB2 receptor agonists on LPS-induced iNOS induction was not reversed by their respective antagonists**

*A*, BV-2 cells were treated with LPS (1 μg/ml) in the presence AEA, AEA together with AM281 and/or AM630 (all at 100 nM) for 6 h and then lysed for western blot analysis. The induction of iNOS was completely blocked in all the treatment conditions. ***$p < 0.001$ was obtained when the cannabinoid treated groups were compared to the LPS alone group (n = 3).

*B*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of CB1 selective agonist ACPA, together with the CB1 antagonist AM281, the CB2 selective agonist AM1241 with and without the CB2 antagonist AM630, the antagonists alone or their combination for 6 h and then lysed for western blot analysis. The induction of iNOS was attenuated by the selective agonist and this effect was not blocked by the presence of the antagonist. Interestingly, the antagonists alone or in combination were also able to attenuate iNOS induction. All drugs were used at 100 nM. **$p < 0.01$ and **$p < 0.001$ were obtained when the cannabinoid treated groups were compared to the LPS alone group (n = 3).

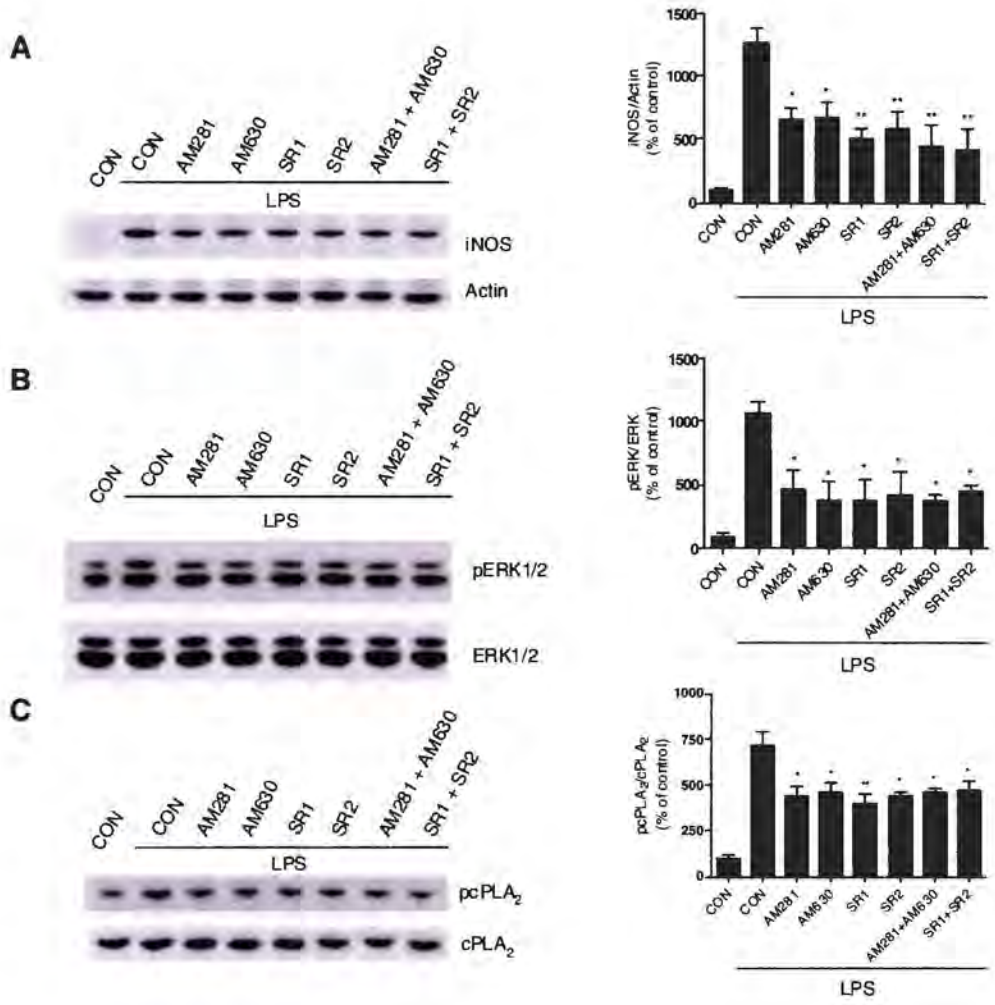



**Figure 12. CB1 and CB2 receptor antagonists, either used alone or in combination, attenuated iNOS induction, ERK1/2 and cPLA$_2$ phosphorylation in LPS-activated BV-2 cells**

*A*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of the CB1 antagonists AM281 and SR141716A (SR1) and the CB2 antagonists AM630 and SR144582 (SR2) for 6 h, and then lysed for western blot analysis. These antagonists alone or in combination attenuated iNOS induction. *$p < 0.05$ and **$p < 0.01$ were obtained when the cannabinoid treated groups were compared to the LPS alone group (n = 3).

*B-C*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of the CB1 antagonists AM281 and SR1 and the CB2 antagonists AM630 and SR2 for 30 min, and then lysed for western blot analysis. These antagonists alone or in combination attenuated ERK1/2 and cPLA$_2$ phosphorylation. All drugs were used at a concentration of 100 nM. *$p < 0.05$ and **$p < 0.01$ were obtained when the cannabinoid treated groups were compared to the LPS alone group (n = 3).

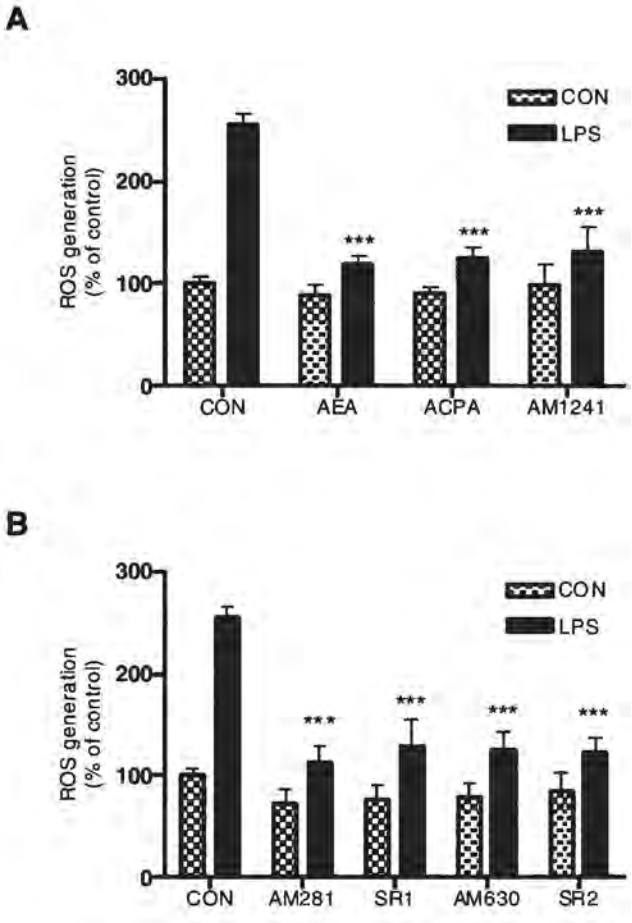



**Figure 13. CB1 and CB2 receptor agonists and antagonists reduced ROS generation in LPS activated BV-2 cells**

*A*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of the non-selective cannabinoid receptor agonists AEA, the CB1 selective agonist ACPA and the CB2 selective agonist AM1241, all at 100 nM for 6 h, and then washed and incubated with DCF for 30 min. The DCF fluorescence intensity, indicative of ROS generation, was measured using a fluorescent plate reader. ***$p < 0.001$ was obtained when the drug treated groups were compared to the LPS alone group. The data shown are pooled from 4 different experiments.

*B*, BV-2 cells were treated with LPS (1 μg/ml) in the presence of the CB1 selective antagonists AM281 and SR1, and the CB2 selective antagonists AM630 and SR2 for 6 h, then washed and incubated with DCF for 30 min. The CB1 and CB2 selective antagonists significantly attenuated ROS generation in LPS-activated BV2 cells. ***$p < 0.001$ was obtained when the drug treated groups were compared to the LPS alone group. The data shown are pooled from 4 different experiments.

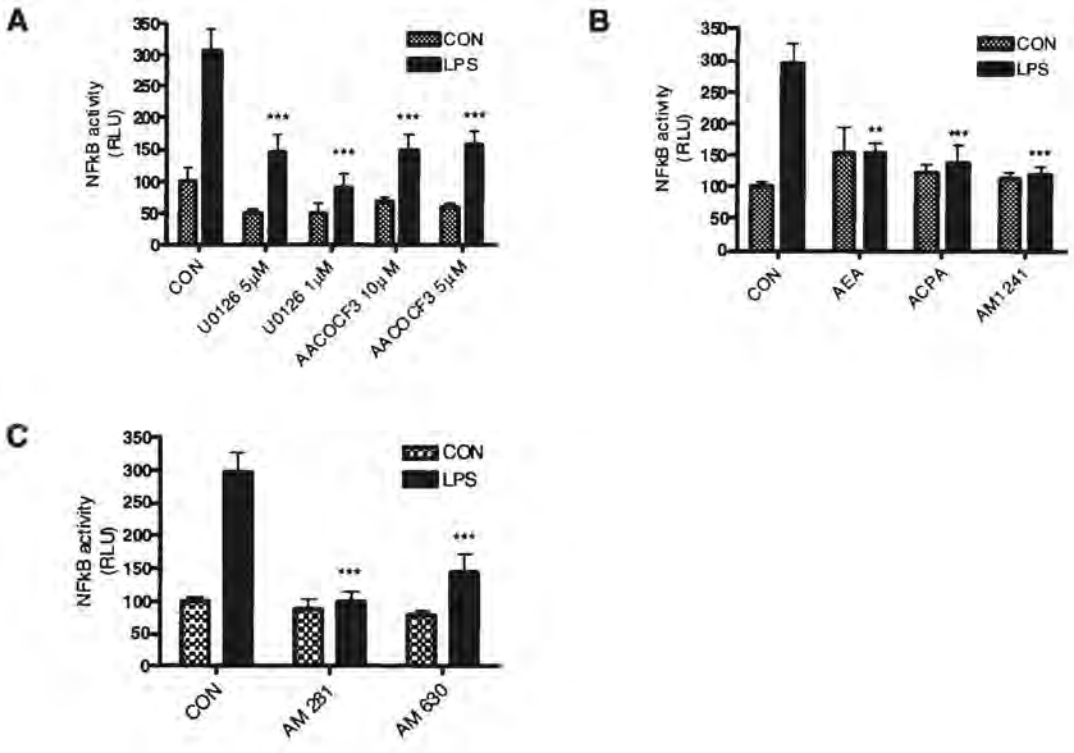



**Figure 14. LPS induced NF-κB activation in BV-2 cells was reduced by the inhibitors of ERK1/2 phosphorylation and cPLA$_2$ activation and the cannabinoid receptor agonists and antagonists**

*A*, BV-2 cells in 96-well plates were transiently transfected with Luc-reporter gene with NF-κB promoter for 24 h and then treated with LPS (1 μg/ml) for 6 h in the presence of ERK1/2 and cPLA$_2$ inhibitors. The luminescence from the cells was evaluated by luciferase reporter assay. RLU (the relative luminescence unit) reflects the activity of NF-κB. ***$p < 0.001$ was obtained when the drug treated groups were compared to the LPS alone group. The data shown are pooled from three different experiments.

*B*, The CB1 and CB2 receptor non-selective or selective agonists used at 100 nM completely reduced the increased NF-κB activity by LPS. **$p < 0.01$ and ***$p < 0.001$ were obtained when the drug treated LPS groups were compared to the LPS alone group. The data shown are pooled from three different experiments.

*C*, The CB1 receptor selective antagonist AM281 (100 nM) and the CB2 receptor selective antagonist AM630 (100 nM) significantly attenuated NF-κB activity. ***$p < 0.001$ was obtained when the drug treated groups were compared to the LPS alone group. The data shown are pooled from three different experiments.

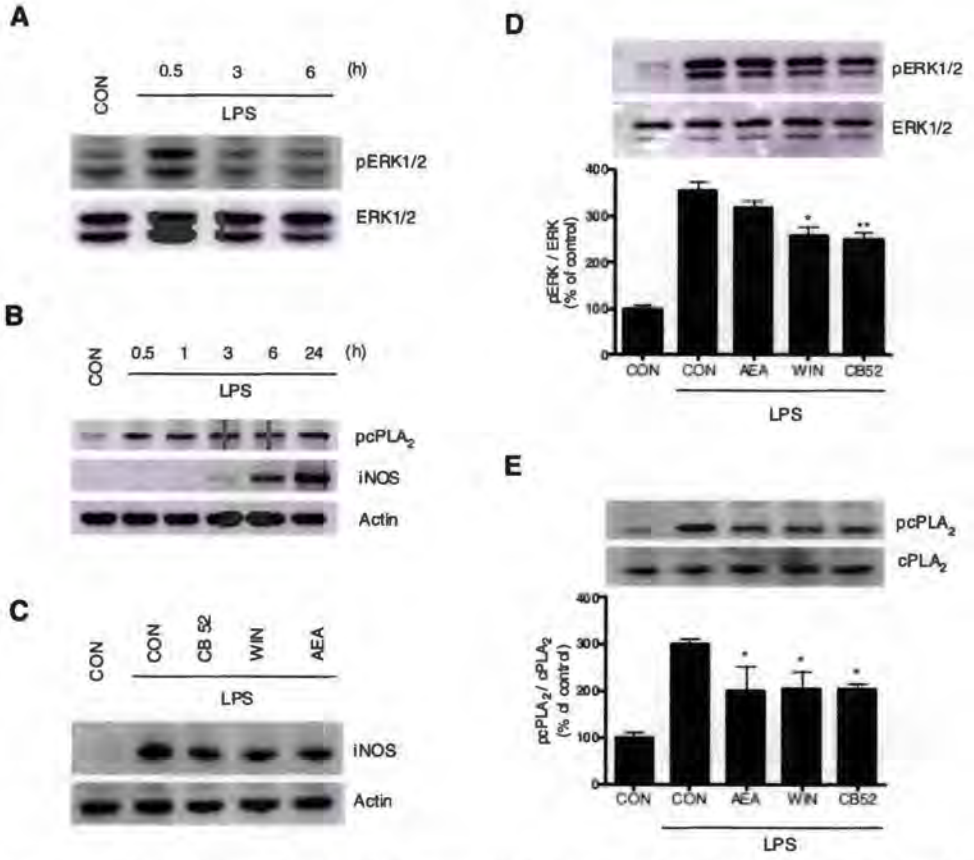



**Figure 15. Cannabinoid receptor agonists attenuated iNOS induction, ERK1/2 and $cPLA_2$ phosphorylation in primary microglia treated with LPS**

*A*, Primary microglia were treated with LPS (1 μg/ml) for various times and cell lysates were subjected to western blot using antibodies against phosphorylated and total ERK1/2. The representative experiment of three that were performed is shown.

*B*, Microglia were treated with LPS (1 μg/ml) for various times and the cell lysates were subjected to western blot using antibodies against phosphorylated $cPLA_2$, iNOS and actin. A representative experiment of four that were performed is shown.

*C*, Microglia were treated with LPS (1 μg/ml) in the presence of the cannabinoid receptor non-selective agonists CB52, WIN55212-2 (WIN) and AEA for 6 h and then lysed for western blot analysis. These cannabinoids attenuated iNOS induction in activated microglia. A representative experiment of three that were performed is shown.

*D-E*, Microglia were treated with LPS (1 μg/ml) in the presence of AEA, WIN and CB52 for 30 min and then lysed for western blot analysis. ERK1/2 phosphorylation was significantly attenuated by CB52 and WIN, but not by AEA. The increased phosphorylation of $cPLA_2$ was significantly reduced by all three cannabinoid agonists. $*p < 0.05$ and $**p < 0.01$ were obtained when the drug treated groups were compared to the LPS alone group. The data are pooled from 3 different experiments (Fig. 15B generated by Li, S).

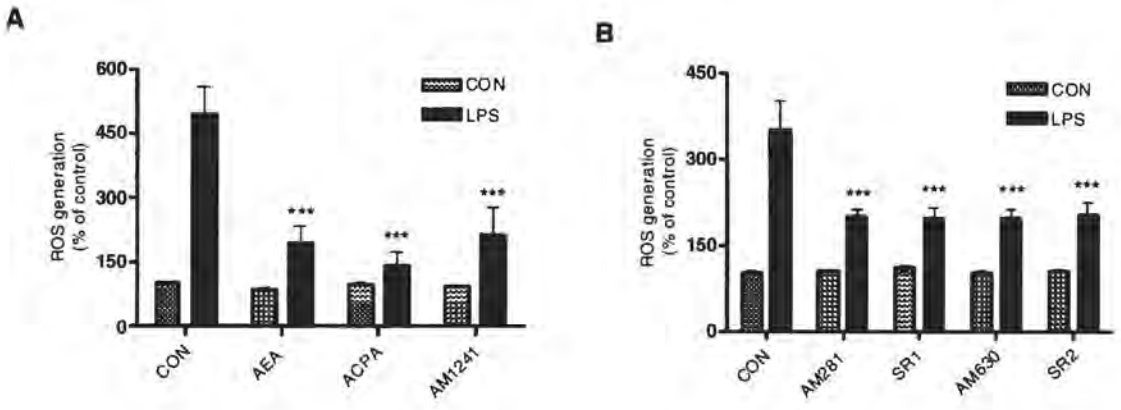



**Figure 16. CB1 and CB2 receptor agonists and antagonists attenuated ROS generation in primary microglia following LPS treatment**

*A*, Microglia were treated with LPS (1 μg/ml) in the presence of the non-selective cannabinoid receptor agonist AEA, the CB1 selective agonist ACPA and the CB2 selective agonist AM1241 for 6 h, and then washed and incubated with DCF for 30 min. The DCF fluorescence intensity, indicative of ROS generation, was measured using a fluorescent plate reader. ***$p < 0.001$ was obtained when the drug treated groups were compared to the LPS alone group. All drugs were used at 100 nM. The data shown are pooled from 4 different experiments.

*B*, Microglia cells were treated with LPS (1 μg/ml) in the presence of the CB1 receptor selective antagonists AM281 and SR1, and the CB2 receptor selective agonists AM630 and SR2 for 6 h, then washed and incubated with DCF for 30 min. The CB1 and CB2 receptor selective antagonists significantly attenuated ROS generation in primary microglia following LPS treatment. ***$p < 0.001$ was obtained when the drug treated groups were compared to the LPS alone group. All drugs were used at 100 nM. The data shown are pooled from 4 different experiments.

# CHAPTER 3: Therapeutic Potential of a Novel Cannabinoid Agent CB52 in the Mouse Model of Experimental Autoimmune Encephalomyelitis

Rachel Ribeiro, Adam Vana, Fengshan Yu, and Yumin Zhang

**ABSTRACT**

Multiple Sclerosis (MS) is a demyelinating disease which causes inflammation, demyelination, and axonal injury. Currently, there is no cure for the condition. The endocannabinoid system has recently emerged as a promising therapeutic target for MS. The protective mechanisms of cannabinoids are thought to be mediated by activation of cannabinoid receptor 1 (CB1) and 2 (CB2) expressed primarily in neurons and immune cells respectively. However, the molecular mechanisms and the contribution of each receptor in ameliorating disease progression are still debatable. Although CB1 and CB2 are expressed in oligodendrocytes, the myelin producing cells in the central nervous system, the role of cannabinoids in oligodendrocyte survival has not been well investigated. Using primary cultures of mature oligodendrocytes, we tested the effect of a novel synthetic cannabinoid CB-52 on oligodendrocyte toxicity induced by peroxynitrite, the primary toxic species released by microglia. Interestingly, we found that CB-52 is more potent than a number of broad and selective CB1 and CB2 agonists in protecting oligodendrocytes against peroxynitrite-induced toxicity. The protection provided by CB-52 is likely due to its reduction of ERK1/2 phosphorylation and ROS generation. Using experimental autoimmune encephalomyelitis (EAE), an animal model of MS, we found that CB-52 reduces microglia activation, nitrotyrosine formation, T cell infiltration, oligodendrocyte toxicity, myelin loss and axonal damage in the spinal cord white matter

and alleviates the clinical scores when given either before or after disease onset. These effects are reversed by the CB1 receptor antagonist, but not by the CB2 receptor antagonist, suggesting that activation of CB1 receptors contributes significantly to the anti-inflammatory and neuroprotective effects of cannabinoids on MS.

## INTRODUCTION

Multiple Sclerosis (MS) is an autoimmune disease affecting the central nervous system (CNS). It is the most common demyelinating disease in young adults and results in motor and sensory deficits (164). MS is characterized by chronic inflammation resulting from the infiltration of T cells and activation of microglia/macrophages culminating in the loss of oligodendrocytes, demyelination and axonal damage (168). Although no cure exists, advances in research have led us to a better understanding of the pathogenic mechanisms, which aid the development of novel and effective agents for the treatment of MS.

Cannabinoids are a class of compounds being studied for the treatment for MS and other neurological diseases (6; 10; 59; 115; 182; 203; 207). Preclinical and clinical studies have demonstrated that cannabinoids can alleviate MS symptoms due to their anti-inflammatory (6), antioxidant (91), and anti-excitotoxic properties (78; 168). Cannabinoids have been shown to be protective to oligodendrocytes, the myelinating cells of the CNS, by promoting the survival and differentiation of progenitor oligodendrocytes *in vitro* (143) and *in vivo* (192). Therefore, cannabinoids can be potentially used as effective agents in limiting demyelination and promoting remyelination.

Experimental autoimmune encephalomyelitis (EAE) is a commonly used animal model that mimics the symptoms of MS in humans (8; 217). The first study using cannabinoids in this model system showed that $\Delta^9$-tetrahydrocannabinol (THC), the main active component of the cannabis plant, has immunomodulatory function, as indicated by

the reduced numbers of infiltrating cells in the spinal cord (126). Following this study many groups have further demonstrated the beneficial effects of various cannabinoids in different animal models of MS (111). The beneficial effects of cannabinoids are mostly due to their activation of cannabinoid type 1 (CB1) and CB2 receptors (135; 144), although increasing evidence points to the contribution by novel receptors and receptor-independent mechanisms to the action of cannabinoids (100; 101; 108; 170; 210). Studies using knockout animals showed that a deficiency in either CB1 (168) or CB2 (153) receptors resulted in more severe EAE.. The regulatory action of cannabinoids in EAE-induced neurodegeneration is largely due to CB2 receptor-mediated modulation of the immune and inflammatory response and CB1 receptor mediated neuroprotection (128), although the relative contribution of each receptor might be dependent upon the specific cannabinoid compounds applied. Because activation of CB1 receptors can produce psychotropic effects, current efforts are focused on searching for agents that increase the therapeutic efficacy and meanwhile reduce the unwanted side effects.

CB-52 is a novel cannabinoid compound synthesized on the basis of the structural modification of the endocannabinoid ligands anandamide (AEA) and $\Delta^9$- THC. It is shown to exhibit high affinity for the central CB1 and peripheral CB2 receptors (29). We have recently reported that like other non-selective, as well as selective CB1 and CB2 agonists, CB-52 can significantly reduce the induction of inducible nitric oxide synthase (iNOS) and the generation of reactive oxygen species (ROS) in lipopolysaccharide-activated microglia. However, the anti-inflammatory effects seem not to be mediated by activation of CB1 and CB2 receptors (173). In the present study we tested whether CB-52 was protective against oligodendrocyte toxicity induced by peroxynitrite, a reaction

product of nitric oxide and superoxide, and a major toxic factor released by activated microglia (119; 218). Interestingly, our results demonstrated that CB-52 is more potent and less toxic than a number of cannabinoid receptor agonists in protecting oligodendrocytes from peroxynitrite-induced toxicity. Moreover, we also found that CB-52 reduced neuroinflammation, oligodendrocyte injury, myelin loss and the clinical scores in mice with EAE. Surprisingly, different from the results found *in vitro*, the therapeutic effect of CB-52 *in vivo* seems to be mediated by activation of CB1 receptors.

## MATERIAL AND METHODS

### *Materials*

Non-selective CB1 and CB2 receptor agonists CB-52, CP55940 and AEA were from Cayman Chemical (Ann Arbor, MI), WIN55212-2 was obtained from Sigma Aldrich (St. Louis, MO). CB1 selective agonists ACPA, ACEA, and methanandamide were purchased from Tocris (Ellisville, MO). CB2 selective agonists AM1241 and JWH015 were from Cayman Chemical. CB1 antagonist AM281 was from Tocris and CB2 antagonist AM630 was from Cayman Chemical. The peroxynitrite generator SIN-1 was from Cayman Chemical. $ZnCl_2$ and lipopolysaccharide (LPS) were purchased from Sigma.

### *Oligodendrocyte cell culture*

Primary cultures of oligodendrocytes were prepared from 2-3 day-old Sprague-Dawley rat brain. Cultures were maintained in DMEM containing 20% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin in 75 $cm^2$ flasks incubated in 95%

air/5% $CO_2$ at 37 °C. At 10 days, the flasks were shaken for 1 h on an orbital shaker (200 rpm) at 37 °C to remove microglia. They were then changed to new media and shaken overnight. Oligodendrocytes were harvested and seeded onto poly-ornithine coated plates [96-well (2.3 x $10^6$ cells/plate), 24-well (3 x $10^6$ cells/plate)] in a basal chemically-defined medium (BDM) [DMEM with 1 mg/ml bovine serum albumin (BSA), 50 µg/ml apo-transferrin, 5 µg/ml insulin, 30 nM sodium selenite, 10 nM biotin, 10 nM hydrocortisone] plus 10 ng/ml of both PDGF and bFGF. For culturing mature oligodendrocytes, cells at day 7 were changed to BDM plus T3 (15 nM) and CNTF (10 ng/ml) for another 2 weeks.

*Exposure of oligodendrocytes to cannabinoids and SIN-1*

In each experiment, cannabinoid agonists were added 30 min prior to SIN-1 exposure and maintained during the treatment. When used, antagonists were added 15 min prior to agonists. All compounds were dissolved in 0.1% DMSO, which was used as the vehicle control.

*Toxicity Assay*

Oligodendrocytes were treated with SIN-1 in the presence or absence of cannabinoids for 2 h, washed twice with Hank's balanced salt solution (HBSS) containing 0.1% BSA, and then placed in BDM with T3 and CNTF. After the cells were incubated for 20-24 h, the culture medium was replaced with Earle's balanced salt solution (EBSS) plus a 1:100 dilution of Alamar Blue (Trek Diagnostic Systems, Inc., Westlake, OH). After 2 h exposure, the cell viability was assessed using a fluorescent

plate reader (SpectraMax Gemini XS, GMI Inc., MN) with excitation wavelength at 530 nm and emission wavelength at 590 nm.

*Western blot analysis*

At 2 h after SIN-1 treatment, oligodendrocytes were lysed and the protein concentration in lysates was measured. After the SDS-PAGE, the membrane was blocked with 5% nonfat milk for 1 h and then incubated overnight at 4 °C with the primary antibody for phosphorylated ERK42/44 or total ERK42/44 (Cell Signaling, Beverly, MA) diluted at 1:2000 in TBST containing 5% BSA. After washing, the membrane was incubated for 1 h at RT with an HRP-conjugated anti-rabbit secondary antibody diluted at 1:2000. The proteins were visualized by enhanced chemiluminescence (ECL). Quantification for Western blot analysis was done by measuring band density using Multi Gauge software.

*Measurement for ROS*

Intracellular free radical generation was evaluated with 2', 7'-dichlorohydrofluorescein diacetate (DCF; Molecular Probes, Eugene, OR). Briefly, after the cells in 96-well plates were treated with SIN-1 with/without cannabinoids for 2 h, they were loaded with DCF (10 μM) for 30 min. The fluorescence of the cells in each well was measured using a fluorescence plate-reader with excitation wavelength at 480 nm and emission wavelength at 530 nm.

*Induction of experimental autoimmune encephalomyelitis (EAE)*

EAE was induced in 7-wk-old female C57BL/6 mice (Jackson Labs) as we previously described (120; 204). EAE was induced by subcutaneous injection of 200 μg myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (H-MEVGWYRSPFSRVVHLYRNGK-OH) in Complete Freunds Adjuvant (CFA, DIFCO) with 500 μg mycobacterium tuberculosis (DIFCO). Immediately following MOG peptide injection and 24 h later mice were intraperitoneally (i.p.) administered 200 μg pertusis toxin (List Biological Labs). At one week after induction EAE mice received a booster of 200 μg of MOG in incomplete Freunds Adjuvant without mycobacterium tuberculosis. Mice were assessed daily to evaluate the extent of dysfunction following EAE induction. Neurological signs were assessed as follows: 0, normal; 1, dysfunction of tail or one limb; 2, dysfunction of tail and one limb or two limbs; 3, limp tail and dysfunction of two limbs; 4, disturbed function in tail and three limbs or dysfunction of all limbs; 5, moribund; 6, death.

*Immunohistochemistry (IHC)*

Animals were euthanized using 90 mg ketamine/10 mg xylazine per kg, i.p.), then intracardially perfused with ice cold 1M phosphate buffer. Spinal cords were dissected prior to post-fixation in 4% paraformaldehyde at 4 °C overnight. Tissue was then cryoprotected in 30% sucrose (Sigma) in 1M phosphate buffer at 4 °C overnight. After being cryoprotected the tissue was embedded in Tissue Tek OCT (Sakura, Torrance, CA) and stored at -80 °C until utilization. Transverse sections of lumbar spinal cords were cut at 14 μm with cryostat (Leica model CM1900, Bannockburn, IL) and mounted onto Superfrost Plus slides (Fisher, Pittsburgh, PA) for immunohistological analysis. Slides

were washed three times with PBS, and blocked with 1% Triton X-100 in PBS containing 5% donkey serum for 20 min at room temperature. The slides were then incubated with the appropriate primary antibody diluted in PBS containing 2% donkey serum. Primary antibodies used were for: Microglia/Macrophages marker F4/80 (1:100, eBioscience); Nitrotyrosine, a marker for peroxynitrite formation (1:100, Chemicon), CC1, a marker for mature oligodendrocyte (1:100, Calbiochem), and CD4 T-lymphocytes (1:100, BD Pharmingen). Primary antibodies were incubated overnight at 4 °C. After three washes with PBS at 5 min each, the appropriate secondary antibody conjugated with either Alexia Fluor 488 or Alexia Fluor 594 (Molecular Probes) was added and then incubated for 1 h at room temperature. After three washes with PBS, the slides were mounted with Fluoro Shield together with DAPI (Sigma) and kept dark at 4 °C. Images were captured with a fluorescence microscope (Nikon Eclipse E800) equipped with a Nikon digital camera.

*Oil Red Staining*

To perform Oil Red O staining, frozen tissue were sectioned at 14 μm thick and mounted on slides. The slides were air dried for 60 min at room temperature and then placed in absolute propylene glycol for 5 min. The sections were further incubated in pre-warmed Oil Red O solution for 10 min in 60 °C water bath and followed by differentiation in 85% propylene glycol solution for 5 min. Finally the section were mounted with VectaMount (Vector, CA) aqueous mounting medium and visualized with a Zeiss (Oberkochen, Germany) Axioplan 2ie microscope.

*Statistical analysis*

Statistical significance was assessed using ANOVA with the Tukey-Kramer post-hoc multiple comparison test. Statistical analysis was performed using the Instat program from GraphPad Software (San Diego, CA). Representative experiments are shown unless noted otherwise. Experiments were performed with triplicate samples, and the data are expressed as mean ± SEM. All experiments were repeated at least 3 times.

## RESULTS

### Cannabinoids protect mature oligodendrocytes against peroxynitrite- and zinc-induced toxicity.

Although both CB1 and CB2 receptors are expressed in various stages of oligodendrocytes, it is unclear whether activation of these receptors can prevent oxidative stress toxicity to mature, myelin producing, oligodendrocytes. Using a novel cannabinoid agent CB-52, and three commonly used non-selective CB1/CB2 receptor agonists CP55940, WIN55212-2 and AEA, we tested the role of these cannabinoid compounds on oligodendrocyte toxicity induced by SIN-1, a peroxynitrite generator. As shown in Figure 12, all four cannabinoids dose-dependently protected mature oligodendrocytes against SIN-1 induced toxicity. However, both CP55940 and AEA were themselves toxic at 10 μM (Fig. 17B and D), whereas CB-52 (10 μM) and WIN55212-2 (10 μM) did not have any toxicity. Notably, a complete protection was achieved when CB-52 was used at 10 μM (Fig. 17A).

We have previously found that peroxynitrite toxicity to mature oligodendrocytes is mediated by intracellular zinc release, ERK1/2 phosphorylation and ROS generation (221; 222). Thus, we also tested whether these cannabinoids could prevent mature oligodendrocytes from zinc-induced toxicity. AEA (3 μM), WIN 55212-2 (10 μM) and CB-52 (10 μM) significantly attenuated oligodendrocyte toxicity induced by zinc chloride ($ZnCl_2$) at 150 μM. When 200 μM $ZnCl_2$ was used, only CB-52, but not WIN 55212-2 and AEA, was shown to have protective effects (Fig. 18A). These results suggest that CB-52 is more potent than AEA and WIN 55212-2 to prevent oligodendrocytes from peroxynitrite- or zinc-induced toxicity.

**Cannabinoid agonists attenuate ERK1/2 phosphorylation and ROS generation in oligodendrocytes treated with SIN-1 or zinc.**

To further characterize the protective mechanisms of cannabinoids on oligodendrocytes, the phosphorylation of ERK1/2 induced by SIN-1 or zinc was assessed by western blot analysis. We found that after a 2 h exposure to either SIN-1 (Fig. 19A) or zinc (Fig. 18B), only CB-52 was able to significantly attenuate ERK1/2 phosphorylation. All four cannabinoids tested significantly attenuated ROS generation at 6 h after SIN-1 treatment. A complete reduction of ROS was observed in the presence of CB-52 and CP55940, whereas the reduction of ROS by WIN55212-2 and AEA was 30% to 40%, respectively (Fig. 19B). CB-52 was also more effective than WIN55212-2 and AEA in attenuating ROS generation caused by zinc (data not shown).

**Cannabinoids protect mature oligodendrocytes against peroxynitrite and zinc toxicity through a mechanism that is partially mediated by the CB2 receptor.**

Considering its high potency, we decided to focus our studies on the protective effects of CB52 on mature oligodendrocytes. To determine if the effect seen with CB-52 was mediated by activation of CB1 and/or CB2 receptors, the CB1 antagonist AM281 (10 μM) and the CB2 antagonist AM630 (10 μM) either alone or in combination were added 15 min prior to CB-52 treatment. As shown in Figure 20A, the protective effect of CB-52 was partially, but significantly attenuated by AM630, but not by AM281. Addition of AM281 did not further enhance the blocking effects exerted by AM630 suggesting activation of CB2, but not CB1 receptor is responsible for part of the protective effects of this compound. Notably, the antagonists themselves did not have any protective effect. To further test the role of CB2 receptor in the survival of oligodendrocytes, oligodendrocytes were treated with SIN-1 in the presence of CB1 or CB2 receptor selective agonists. The CB2 selective agonists AM1241 and JWH015 significantly attenuated SIN-1 induced toxicity, but interestingly the CB1 selective agonists ACPA, ACEA and methanandamide did not have any effect (Fig. 20B). Similarly, the protective effect of CB-52 on zinc-induced toxicity was also partially mediated by activation of CB2 receptor, and the selective CB2 agonists, but not CB1 agonists also exerted a protective effect (Fig. 21). Taken together, these results suggest that the protective effect of CB-52 to mature oligodendrocytes on peroxynitrite- and zinc-induced toxicity is most likely mediated by the CB2 receptor activation.

**CB-52 is protective in the EAE mouse model when given before or after symptom development and its effects are mediated by CB1 receptor activation.**

Given the findings from the in vitro studies that CB-52 seems to be more effective than other commonly used cannabinoids in attenuating oligodendrocyte cell death and the evidence that this effect is mediated by the CB2 receptor, we decided to test the role and the cannabinoid receptor dependency of CB-52 in EAE, a mouse model of MS. In the first set of experiments (Fig. 22A), mice were treated with CB-52 (2 mg/kg body weight, i.p.) in the absence or presence of the CB1 antagonist AM281 (2 mg/kg) or the CB2 antagonist AM630 (2 mg/kg) starting at day 3 after MOG injection and then once a day until day 30. Without treatment, the clinical symptoms developed on day 10, and rapidly reached clinical scores 3.0-3.5 from day 16 to day 30. After CB-52 treatment, there was a slight delay in the disease onset, but the clinical scores were significantly reduced from day 13 until the end of the experimental period. In this group the maximal clinical score was 1.5, which is dramatically reduced when compared to the untreated EAE group (clinical score of 3.5). Surprisingly, the reduction of clinical scores by CB-52 was eliminated by co-administration of AM281, but not AM630, suggesting activation of CB1 receptor contributes to the therapeutic effect of CB-52, which is distinct from our observations in oligodendrocytes in culture.

To be more clinical relevant, we also performed the CB-52 treatment immediately after the disease onset (Fig. 22B). Mice were administered CB-52 (2 mg/kg, i.p.) with/without AM281 or AM630 starting from day 12 and then once daily afterwards. Again, the EAE mice reached and maintained a high clinical score of 3.0 from day 16 to day 30, post-symptom treatment with CB-52 significantly reduced clinical scores starting

from day 17, and further reduction was seen until the end of experiment. The reduction of CB-52 on clinical scores was completely eliminated by co-treatment with AM281, but not by AM630, although co-administration of AM630 seemed to partially reverse the clinical scores. These results clearly indicate that CB1 receptor activation plays a dominant role for the action of CB-52 in the EAE mouse model.

In the third set of experiment (Fig. 22C) we were interested in determining whether continuous activation of the cannabinoid receptor is necessary for the therapeutic action of CB-52. To test this idea, animals started receiving CB-52 after symptom development at day 12, followed by the co-administration of the CB1 or CB2 antagonist beginning at day 20. Our hypothesis was that if transient receptor activation or short-term treatment with CB-52 is sufficient to trigger the signaling pathway leading to protection, delayed treatment with antagonists should not affect the action of CB-52; On the contrary, if continuous activation of cannabinoid receptor is necessary, delayed treatment with antagonists may still reverse the protective effect of this cannabinoid compound. This study found that delayed administration of CB1 antagonist, but not CB2 antagonist seemed to reverse the protective effect of CB-52, suggesting chronic treatment might be important for sustaining the CB1 receptor signaling and maintaining the protective effect of this cannabinoid compound.

**CB-52 attenuates accumulation of microglia/macrophages and peroxynitrite production in the mouse EAE spinal cord.**

On day 30 after EAE induction, animals were perfused with 4% paraformaldehyde and spinal cords were dissected and sectioned for

immunohistochemistry. Figure 18 shows representative images of microglia/macrophages by F4/80 and the staining of nitrotyrosine, a marker for peroxynitrite formation, in the spinal cord dorsal columns in our first set of experimental groups (i.e. animals that received CB52 with/without antagonists starting from day 3 after induction). Our results show that there was a dramatic increase in the staining of F4/80 and nitrotyrosine in EAE animals (Fig. 23B) compared to the control animals without EAE induction (Fig. 23A). Treatment with CB-52 completely blocked F4/80 and nitrotyrosine staining (Fig. 23C), indicating CB-52 is effective in blocking the accumulation of microglia/macrophages and the release of peroxynitrite. Consistent with the effects on the clinical scores, co-treatment with the CB1 antagonist (Fig. 23D) but not the CB2 antagonist (Fig. 23E) reversed the effects of CB52. Similar results were also observed with post-symptom treatment by CB-52 at day 12 together with the CB1 and CB2 antagonists and when antagonist treatment was delayed until day 20 (Figures 24 and 25, respectively).

**CB-52 attenuates T-cell infiltration in the spinal cords of EAE mice.**

EAE is primarily mediated by the infiltration of $CD4^+$ T lymphocytes from the periphery to the CNS, and once in the CNS, these T cells can be activated by local antigen presenting cells and release the inflammatory mediators (111). At 30 days after EAE induction, the infiltration of $CD4^+$ T cells was clearly observed in the spinal cord white matter, which was almost completely blocked by treatment with CB-52 either before (Fig. 26A and B) or after (Fig. 26C and D) symptom development. Interestingly, the blocking effect of T cell infiltration by CB-52 was also reversed by the co- or delayed- administration of CB1 antagonist AM281, but not the CB2 antagonist AM630

(Fig. 26A-D). These results suggest that CB-52 blocks T cell infiltration through activation of CB1 receptors.

**CB-52 prevents oligodendrocyte cell loss and demyelination in the spinal cords of EAE mice.**

Oligodendrocyte cell death and demyelination are two major components in MS and EAE (23; 67; 114). Using CC1, a marker for mature oligodendrocytes, we examined whether CB-52 treatment can affect the survival of oligodendrocytes (Fig.27A). As demonstrated previously, the number of CC1 positive cells in the spinal cord white matter is significantly reduced in EAE animals when compared to controls (120; 204). As anticipated, CB-52 is effective in preventing the loss of these cells in the spinal cord white matter when given before (Fig.27A) or after (Fig. 27B and C) symptom development. Contrary to our in vitro studies, however, we found that blocking the CB1, but not the CB2 receptor reversed the protective effect of CB-52 no matter when the antagonists were given at day 3 (Fig.27A) or day 12 (Fig. 27B). Delayed administration of CB1 antagonist AM281 seemed also reduced the protective effect of CB-52, although this reduction did not reach significant difference when compared to the CB-52 alone treated group (Fig.27C).

We then looked at demyelination by staining the spinal cord sections with Oil Red. In these sections myelin staining appears to be red, and the white or bleached areas in the white matter are indicative of demyelination (Fig. 28A). Consistent with the loss of mature oligodendrocytes, demyelination was greater in EAE animals when compared to

control animals, and CB-52 was very effective in preventing demyelination when given 3 days (Fig. 28A and B) or 12 days (Fig. 28C and D) after induction. The reduction of demyelination was also blocked by CB1 antagonist, but not by the CB2 antagonist regardless the antagonists were given starting from day 3 (Fig. 28A and B), day 12 (Fig. 28C) or day 20 (Fig. 28D).

**CB-52 prevents axonal injury in the EAE spinal cord white matter.**

We also studied the effect of CB-52 on axonal injury by staining spinal cord sections with an axonal marker NF200. In EAE animals, there was a dramatic decrease in the levels of $NF200^+$ staining (Fig. 29A), indicating axonal damage occurred in these animals. Treatment with CB-52 either before (Fig. 29A and B) or after (Fig. 29C and D) symptom development significantly reduced the loss of $NF200^+$ axons. Co-administration of AM281 appeared to attenuate the protective effect of CB-52 on axonal loss (Fig. 29A-D), although the significant difference was only observed in the delayed AM281 treated group (Fig. 29D). Once again, the contribution of CB2 receptor in the action of CB-52 is quite limited, if there is any.

## DISCUSSION

Accumulating evidence points to the therapeutic potential of cannabinoids in inflammatory and neurodegenerative diseases such as MS (169). In addition to ameliorating the clinical symptoms in animal models (9) and MS patients (176), cannabinoids are also being recognized for their ability to control disease progression (51). Although much progress has been achieved in the last several decades, questions

still remain regarding the protective mechanisms of various cannabinoids and the involvement of CB1 and CB2 receptors. In this study we examined the role of various cannabinoids on mature oligodendrocyte toxicity induced by peroxynitrite, and found that CB2 agonists, but not CB1 agonists, have significant protective effects. Furthermore, we found that CB-52, a newly developed AEA and $\Delta^9$-THC hybrid, is more effective than other commonly used cannabinoids and its protection of oligodendrocytes is partially mediated by activation of the CB2 receptor. In the EAE mouse model, our results showed that CB-52 can lower clinical scores when given either before or after symptom development. Administration of CB-52 also reduced the number of inflammatory cells, prevented the loss of oligodendrocytes, demyelination, and axonal injury in the spinal cord white matter. Surprisingly, we found that contrary to our *in vitro* studies, the CB1 and not the CB2 receptors seem to be critical for the protective effects of CB-52 observed *in vivo*.

**Cannabinoids protect mature oligodendrocytes against peroxynitrite-induced toxicity by attenuating ERK phosphorylation and ROS generation, and this effect is partially mediated through CB2 receptor activation.**

Oligodendrocyte cell death is one of the major characteristics of MS and EAE. Preventing mature oligodendrocytes from injury and/or allowing oligodendrocyte progenitors to develop into mature, myelin producing oligodendrocytes is of critical importance. Several studies have shown that cannabinoids can promote the survival of oligodendrocytes by reducing depolarization-evoked calcium influx (134), and preventing apoptosis in progenitor cells (143). However, it has not been reported whether

cannabinoids can prevent oxidative stress induced toxicity to mature oligodendrocytes. We have previously shown that peroxynitrite, a reaction product of nitric oxide and superoxide, is a major toxic factor released from activated microglia and injures oligodendrocytes via a signaling pathway involving intracellular zinc release, ERK phosphorylation and ROS generation (221; 222) . Here, we were able to show that cannabinoids can directly affect the survival of primary mature oligodendrocytes when exposed to peroxynitrite or extracellular zinc, and that the protection is likely attributable to their inhibition of ERK phosphorylation and ROS generation. In agreement to the reports by others (21; 143), we also found that the CB1 and CB2 receptors are expressed in oligodendrocytes by real-time PCR and western blot (data not shown). However, their role in these cells remains unclear. Our results suggest that activation of CB2 receptor, but not CB1 receptor, might be responsible for the protective effect of CB-52 and other cannabinoid agents on oxidative stress induced toxicity. These results inspired us to test the therapeutic efficacy of CB-52 *in vivo*, considering the notion that CB2 receptor activation does not cause psychotropic side effects.

**CB-52 is effective in lowering clinical scores in EAE animals when given either before or after symptom development.**

EAE is a well-described and commonly used animal model for MS. Its pathological and clinical features are similar to those seen in patients, including the inflammatory components leading to demyelination and neurological dysfunction (15; 116). Using this model, we found that CB-52 can reduce clinical symptoms when given either before (3 days after MOG immunization) or after disease onset (12 days after

MOG immunization when the symptoms became apparent). When EAE animals were treated together with the CB1 and CB2 antagonists, we found that blocking the CB1 but not the CB2 receptor reversed the reduction of clinical scores, suggesting the action of CB-52 is mediated through the activation of CB1 receptors. Although this result is consistent with a recent study indicating that the protective effect of a non-selective CB1/CB2 agonist WIN55212-2 is mediated by CB1, but not CB2 activation in the EAE mouse model, we were surprised by our findings since the protective effect of CB-52 against peroxynitrite- or zinc-induced toxicity is partially mediated by CB2 receptor in cultured oligodendrocytes and the notion that modulation of CB2 receptor may be more relevant for controlling the immune-aspects of the disease (128; 153; 180).

**CB-52 treatment reduces inflammation, oligodendrocyte toxicity, demyelination and axonal loss via CB1 receptor mediated mechanisms.**

To further determine the role of CB-52 in the pathological changes after EAE induction and the involvement of CB1 and CB2 receptors, we assessed microglia activation, T cell infiltration, oligodendrocyte toxicity, demyelination and axonal injury. In agreement with the effect on clinical scores, CB-52 treatment attenuated T-cell infiltration, accumulation of microglia/macrophages, and the loss of mature oligodendrocytes, myelin and axons. Again, all these effects are mediated by the activation of CB1, rather than the CB2 receptors. These results are different from the previous reports showing CB2-mediated inhibition of microglia activation (167; 193) and T cells (128), but are in agreement with those showing that the CB1 receptor can affect the inflammatory response (51; 168). Although the results obtained by CB-52 *in vitro* and

*in vivo* seem to be paradoxical, several possibilities might be taken into consideration. First, the expression of CB1/CB2 receptors in virtually all the immune cells, neurons and glia cells makes it difficult to sort out the specific role of these receptors in each cell type and the overall contribution of these receptors *in vivo*; Second, although our results have demonstrated that the protective effect of CB-52 on peroxynitrite-induced toxicity to oligodendrocytes is partially mediated by CB2 receptor, it is unclear whether activation of CB1 receptor can be involved in other toxic conditions, for instance, the toxicity triggered by cytokines, chemokines or glutamate; Third, the CB2 receptors expressed in T cells are highly regulated by endogenous cannabinoid ligands in the CNS during EAE, and activation of these receptors does not seem to require exogenous agonist for the suppressive effects (111; 128). This idea is supported by a recent finding that HU-308, a CB2 receptor selective agonist, did not exact any protective effect in EAE (52).

Although there is a continuous argument on the relative contribution of CB1 and CB2 receptors in the animal models of MS, the fact that mice with either CB1 or CB2 receptor deletion developed more severe EAE (153; 168), points to the importance of both receptors in the progression of disease. In addition, it becomes increasingly appreciated that novel receptors and receptor-independent mechanisms might also contribute to the protective action of cannabinoids (14; 54; 70). Development of novel cannabinoid compounds with increased therapeutic efficacy and the reduced side effects will like benefit the patients with MS and other neurological disorders.

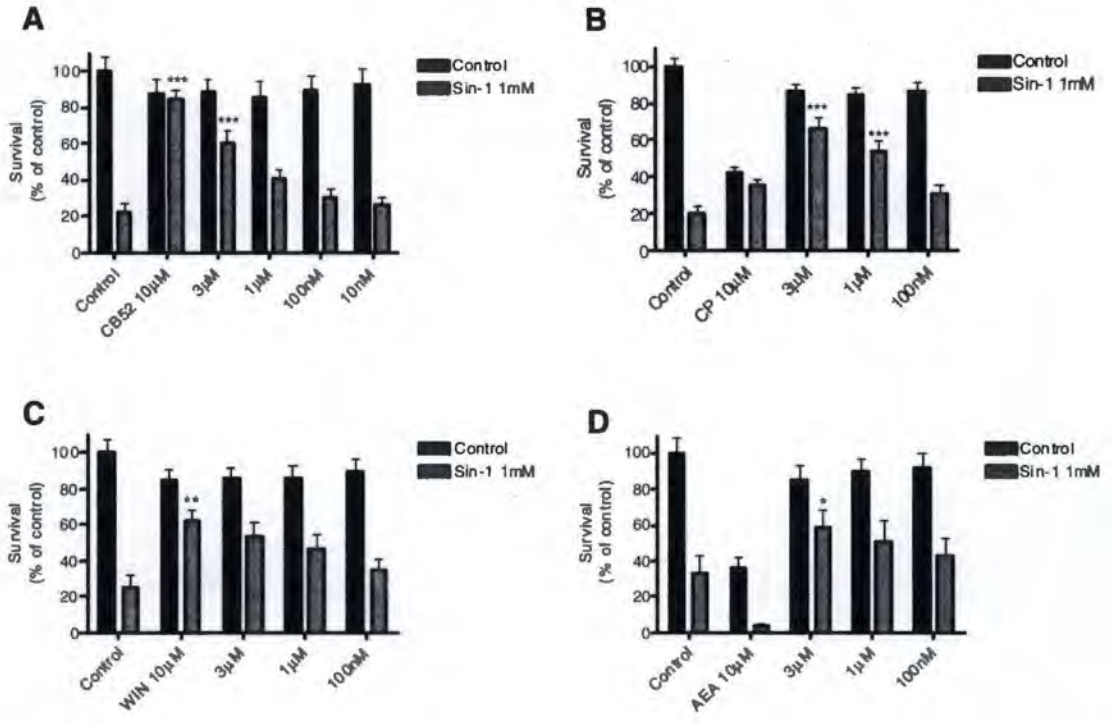



**Figure 17. Dose-dependent protection of the non-selective cannabinoid agonists CB-52, CP55, 940, WIN55-212, and AEA on SIN-1 induced toxicity to mature oligodendrocytes.**

Primary oligodendrocytes were exposed to SIN-1 (1mM) for 2 h in the presence of different concentrations (10 µM-10 nM) of the non-selective cannabinoid agonists CB-52 (A), CP55940 (B), WIN55212 (C), and AEA (D). While all 4 compounds can attenuate SIN-1 toxicity in mature oligodendrocytes, CB-52 (A) showed a better effect when compared with the other three drugs (n=5-9; *, $p < 0.05$, **, $p < 0.01$, and ***, $p < 0.001$ were obtained when the drug treated groups were compared to the SIN-1 alone treated group).

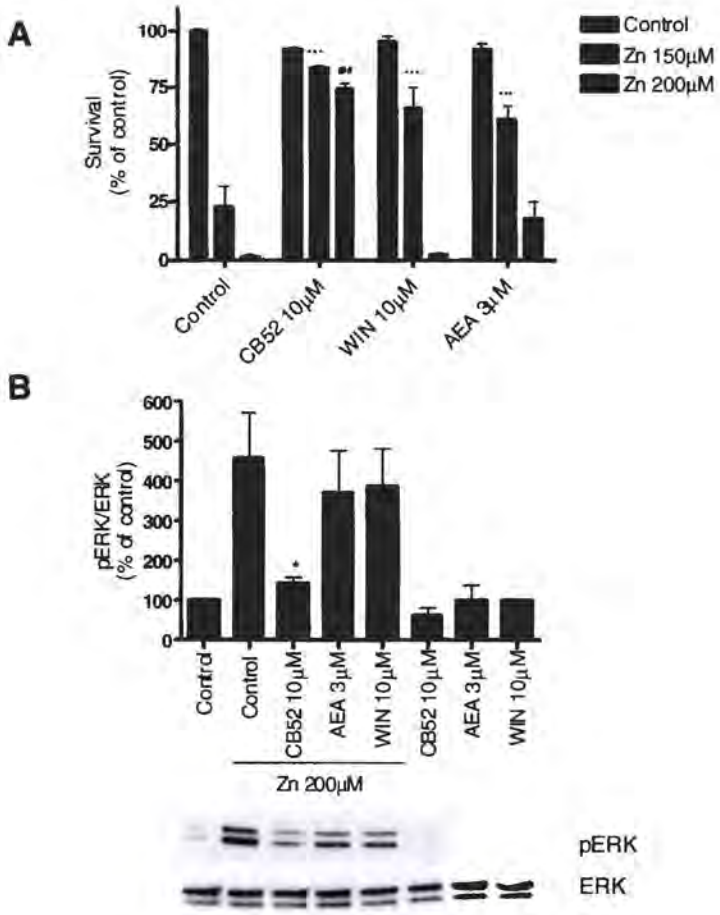



**Figure 18. CB-52 is more effective than other non-selective cannabinoids in preventing oligodendrocyte toxicity cell death induced by zinc**

A, Mature oligodendrocytes were exposed to zinc (150 μM and 200 μM) for 2 h in the presence of CB-52, WIN and AEA. While all 3 drugs can attenuate zinc toxicity in oligodendrocytes, CB-52 showed a better effect when compared with the other three drugs (n=5; ***, $p < 0.001$ and ###, $p < 0.001$ compared to the respective zinc alone group).

B, Mature oligodendrocytes were treated with zinc (200 μM) for 2 h in the presence of various non-selective cannabinoid agonists and lysed for western blot analysis. While all three cannabinoids tested were able to decrease ERK1/2 phosphorylation in these cells, CB-52 was showed to have a greater effect when compared to WIN and AEA.

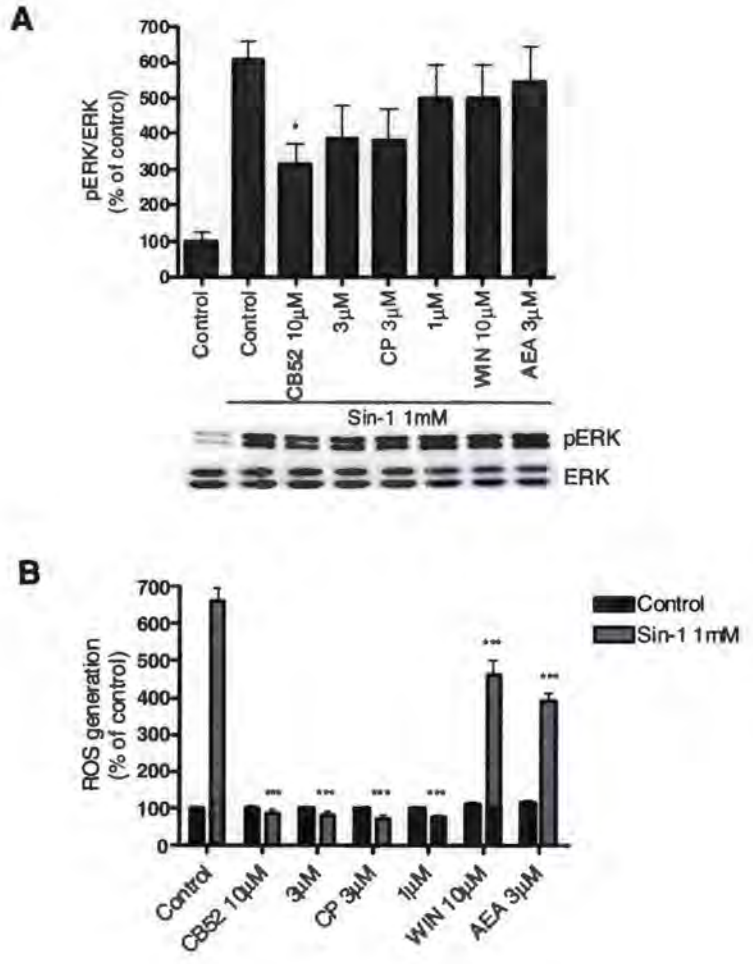



**Figure 19. Cannabinoid agonists decrease ERK1/2 phosphorylation and ROS generation in oligodendrocytes treated with SIN-1**

A, Mature oligodendrocytes were treated with SIN-1 (1mM) for 2 h in the presence of different concentrations of non-selective cannabinoid agonists and lysed for western blot analysis. Although most cannabinoids seem to reduce the ERK1/2 phosphorylation, the significance was only achieved by the CB-52 treatment. (n=3; *, $p < 0.05$ was obtained when the CB52 treated group was compared to the SIN-1 alone group).

B, Mature oligodendrocytes were treated with SIN-1 (1 mM) for 6 h in the presence of different concentrations of non-selective cannabinoid agonists and then incubated with DCF for 30 min. The increased fluorescent intensity, indicative of ROS generation, was then measured. While all drugs tested were able to attenuate ROS production, a complete inhibition of ROS generation was shown with CB-52 and CP55940 (n= 5; ***, $p < 0.001$ was obtained when the drug treated groups were compared to the SIN-1 alone group).

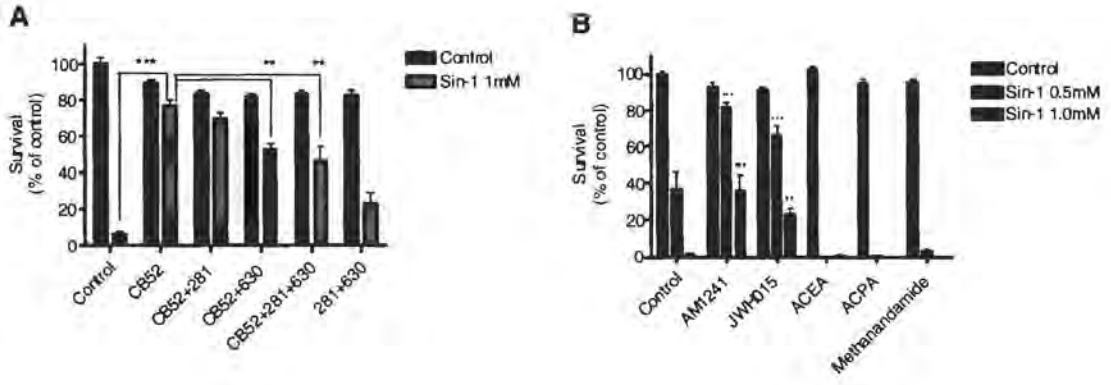



**Figure 20. The protective effect of cannabinoids on mature oligodendrocytes is partially mediated by activation of the CB2 receptor**

A, Mature oligodendrocytes were treated with SIN-1 (1 mM) for 2 h in the presence of CB-52 and the CB1 antagonist AM281 and CB2 antagonist AM630. Toxicity was then evaluated using Alamar Blue. CB-52 is able to attenuate SIN-1 toxicity in mature oligodendrocytes and this effect was partially blocked in the presence of the CB2 antagonist but not the CB1 antagonist (n=5; **, $p < 0.01$, and ***, $p < 0.001$).

B, Mature oligodendrocytes were treated with SIN-1 (1 mM) for 2 h in the presence of the CB1 selective agonists ACPA, ACEA and methanandamide and the CB2 selective agonists AM1241 and JWH015. Toxicity was then evaluated using Alamar Blue. The CB2 agonists but not the CB1 agonists were able to significantly attenuate SIN-1 toxicity in mature oligodendrocytes. (n=5; **, $p < 0.01$, ***, $p < 0.001$, ##, $p < 0.01$ and, ###, $p < 0.001$ compared to the respective SIN-1 alone group).

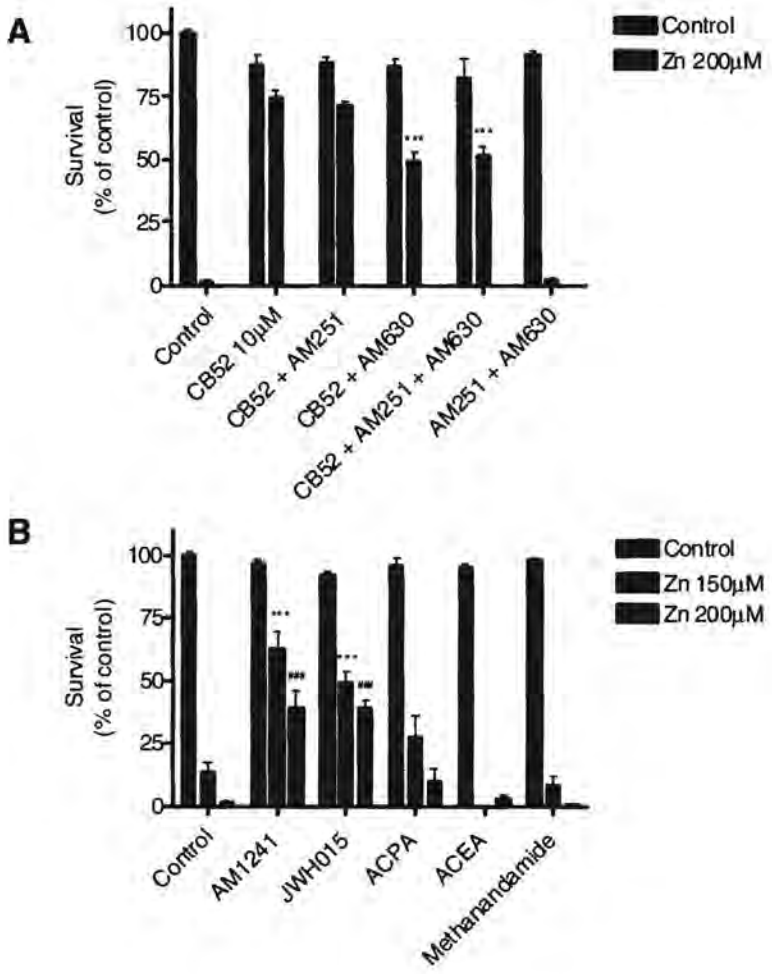



**Figure 21. Cannabinoids protect mature oligodendrocytes from zinc toxicity by a mechanism that is partially mediated by the CB2 receptor**

A, Mature oligodendrocytes were treated with zinc (200 μM) for 2 h in the presence of CB-52 and the CB1 antagonist AM251 and CB2 antagonist AM630. Toxicity was then evaluated using Alamar Blue. CB-52 was able to attenuate zinc toxicity in mature oligodendrocytes and this effect was partially blocked by the CB2 antagonist but not the CB1 antagonist (n=5; ***, $p < 0.001$).

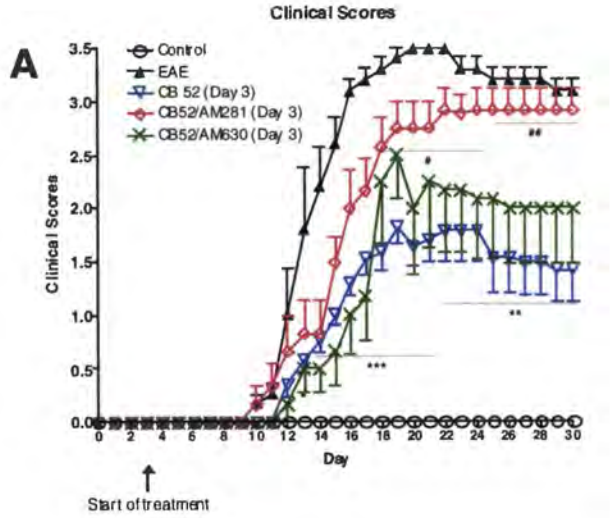
B, Mature oligodendrocytes were treated with zinc (150 μM and 200 μM) for 2 h in the presence of the CB1 selective agonists ACPA, ACEA and methanandamide and the CB2 selective agonists AM1241 and JWH015. Toxicity was then evaluated using Alamar Blue. The CB2 agonists but not the CB1 agonists were able to partially attenuate zinc toxicity in mature oligodendrocytes. (n=5; ***, $p < 0.001$ and ###, $p < 0.001$).

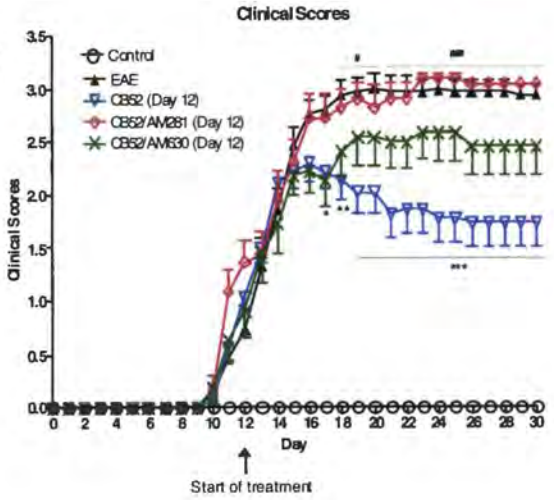
**Figure 22. CB-52 alleviates clinical score in EAE animals and this effect can be blocked by the CB1 antagonist**

A, EAE mice were given CB-52 or a combination of CB-52 with AM281 or AM630 starting from day 3 and then once a day until the end of the experiment. Clinical scores were significantly decreased in the CB-52 treated group (n=13) when compared to the EAE group (n=13; ** $p < 0.01$, *** $p < 0.001$). The effect of CB-52 was reversed when animals were co-administered with CB1 antagonist AM281 (n=13; # $p < 0.05$, ## $p < 0.01$), but not the CB2 antagonist AM630.

B, EAE mice were given CB-52 or a combination of CB-52 with AM281 or AM630 starting from day 12 and then once a day until the end of the experiment. Clinical scores were significantly decreased in the CB-52 treated group (n=12) when compared to the EAE group (n=16; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) and this effect was reversed when animals were co-administered with the CB1 antagonist AM281 (n=12; # $p < 0.05$, and ### $p < 0.001$).

C, EAE mice were treated with CB-52 at day 12 post immunization and the CB1 and CB2 antagonists were given at day 21. Clinical scores were significantly decreased in the CB-52 treated group (n=8) when compared to the EAE group (n=8; *$p < 0.05$, and **$p < 0.01$). Although treatment with CB1 antagonist did not significantly blocked the effect of CB-52 (n=8), there were increased clinical scores in these animals.


A
Clinical Scores
Control
EAE
CB 52 (Day 3)
CB52/AM281 (Day 3)
CB52/AM630 (Day 3)
Clinical Scores
Day
Start of treatment


B
Clinical Scores
Control
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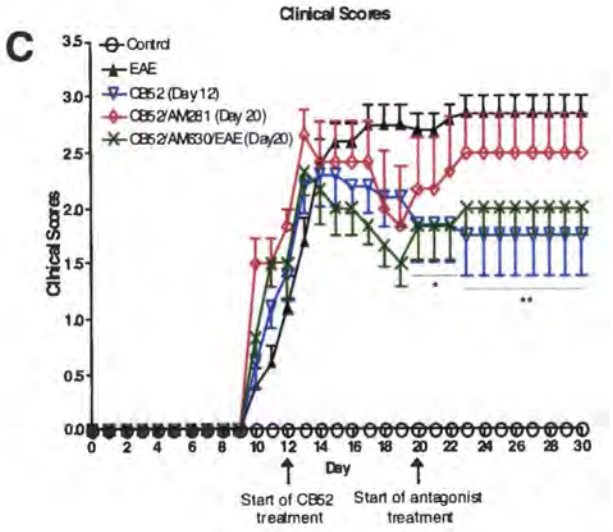
CB52 (Day 12)
CB52/AM281 (Day 12)
CB52/AM630 (Day 12)
Clinical Scores
Day
Start of treatment


C
Clinical Scores
Control
EAE
CB52 (Day 12)
CB52/AM281 (Day 20)
CB52/AM630/EAE (Day20)
Clinical Scores
Day
Start of CB52 treatment
Start of antagonist treatment

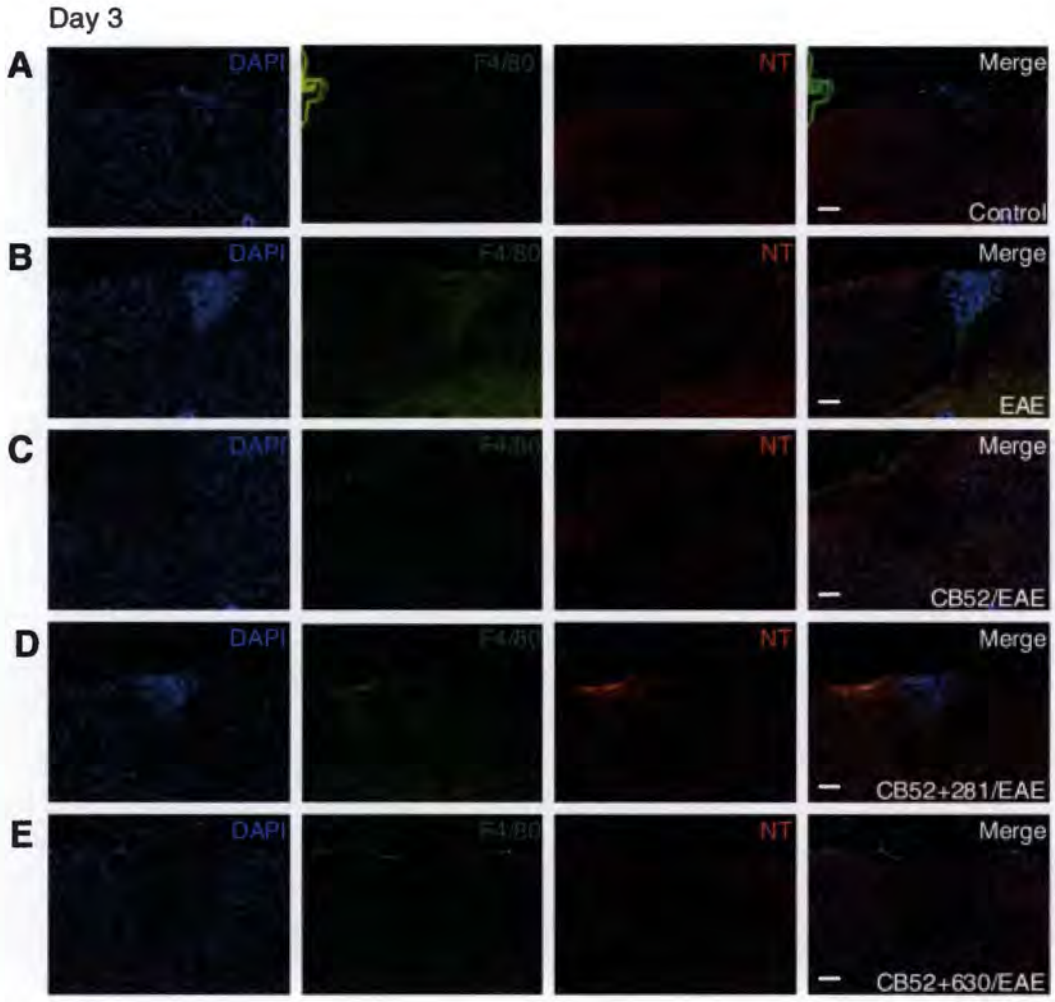



**Figure 23. A significant increase of microglia/macrophages and peroxynitrite formation is found in the spinal cord white matter of EAE mice, which is attenuated in the CB-52 treated groups**

Lumbar sections of EAE animals treated with CB-52 and the CB1/CB2 antagonists starting at day 3 post induction were stained for microglia/macrophages and nitrotyrosine immunoreactivity, which is indicative of peroxynitrite formation. EAE animals showed increased staining for microglia/macrophages and peroxynitrite formation (B) when compared to control animals (A). Treatment with CB-52 significantly attenuated the accumulation of microglia/macrophages and reduced the nitrotyrosine formation in these animals (C). This effect was blocked when animals were treated together with the CB1 antagonist AM281 (D), but not the CB2 antagonist AM630 (E).

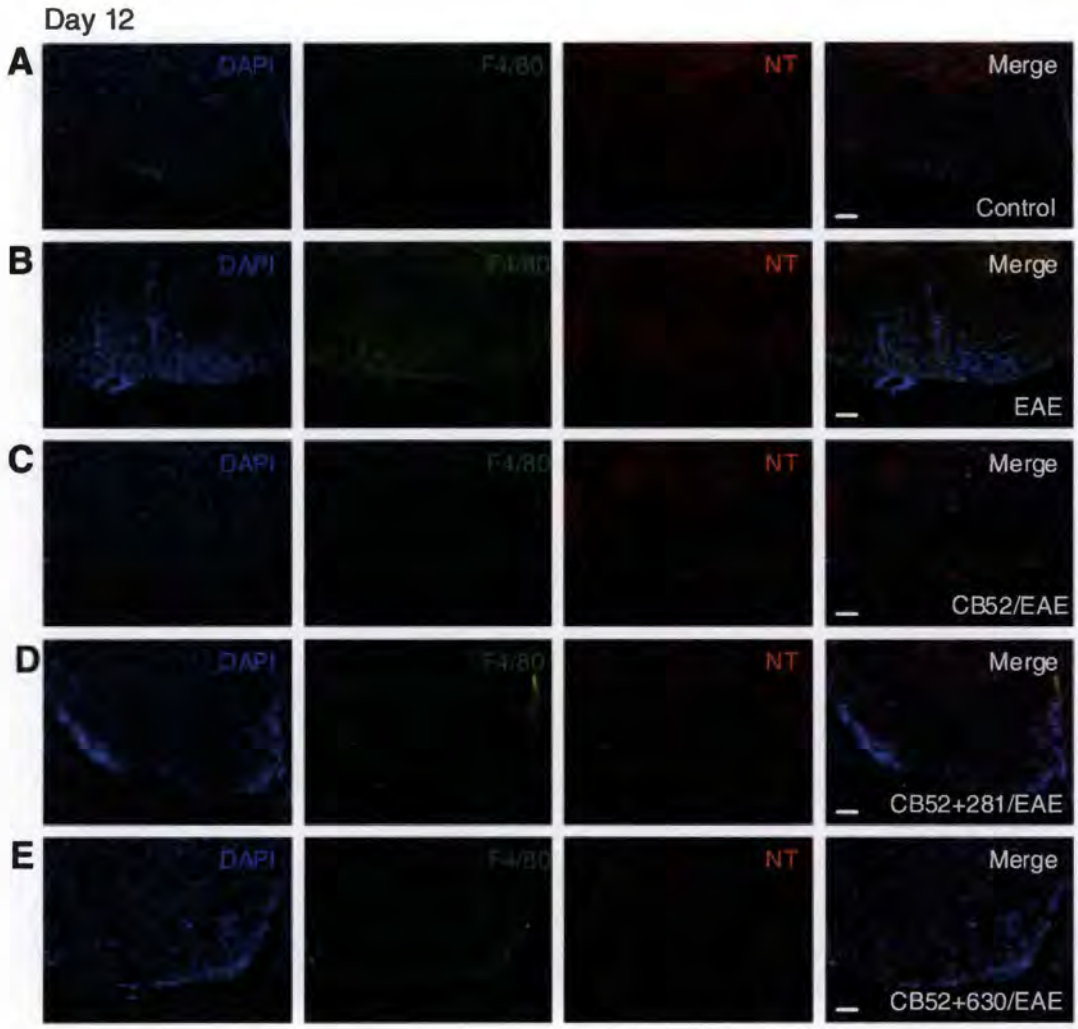



**Figure 24. Post-symptom treatment with CB-52 reduced the number of microglia/macrophages and nitrotyrosine immunoreactivity in EAE spinal cord white matter via cannabinoid receptor activation**

Lumbar sections of EAE animals treated with CB-52 and the CB1/CB2 antagonists starting at day 12 post induction were stained for microglia/macrophages and nitrotyrosine immunoreactivity, which is indicative of peroxynitrite formation. The images show a portion of the ventral white matter. EAE animals demonstrated an increase of microglia/macrophages and nitrotyrosine staining (B) when compared to control animals (A). Treatment with CB-52 significantly attenuated the accumulation of microglia/macrophages and reduced the nitrotyrosine formation in these animals (C). Treatment with the CB1 antagonist AM281 (D) resulted in a greater blocking effect, than treatment with the CB2 antagonist AM630 (E).

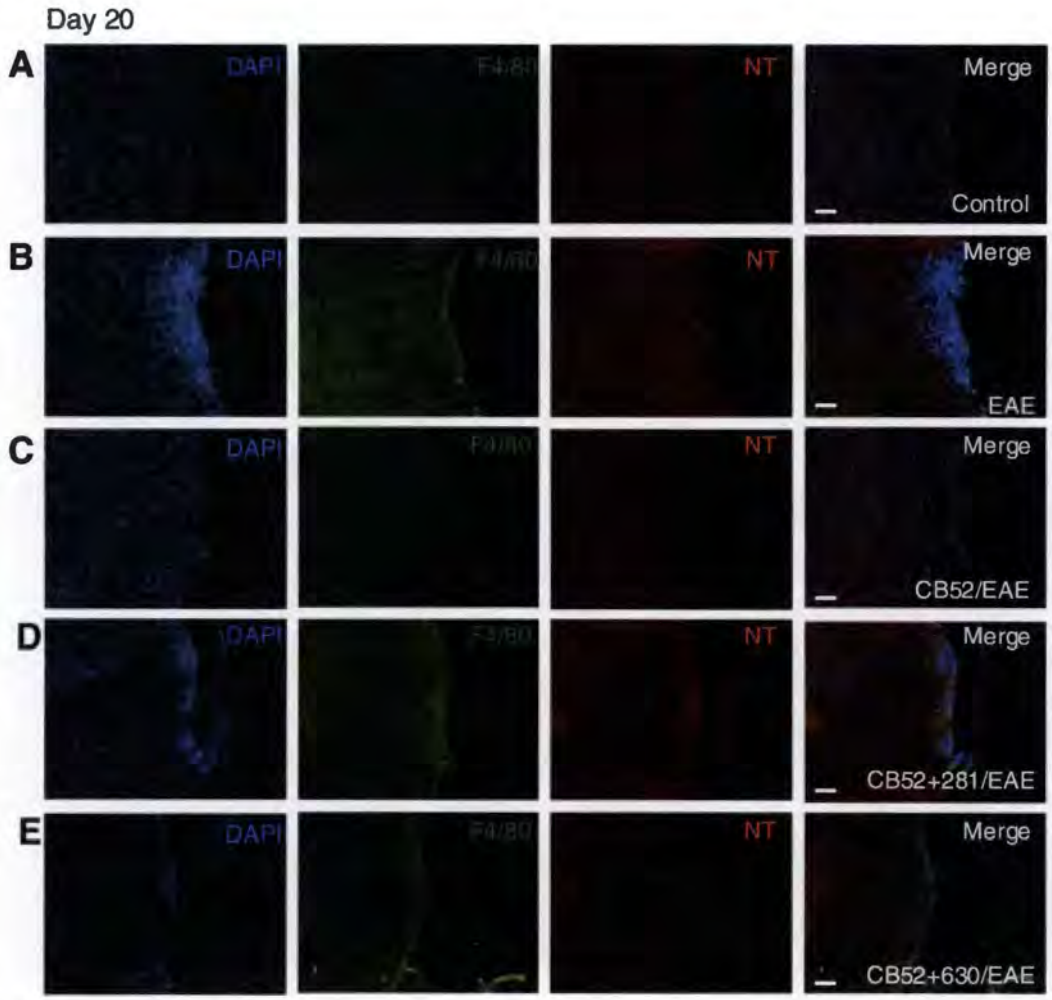



**Figure 25. Delayed treatment with CB1 antagonist partially reversed the inhibitory effect of CB-52 post-treatment on microglia/macrophage accumulation and nitrotyrosine immunoreactivity in EAE spinal cord white matter**
Images show a portion of the lateral white matter stained for microglia/macrophages and nitrotyrosine in animals treated with CB52 at 12 days after induction followed by treatment with the CB1 and CB2 antagonists at 20 days post induction. EAE animals showed increased staining for microglia/macrophages and peroxynitrite formation (B) when compared to control animals (A). Treatment with CB-52 significantly attenuated the accumulation of microglia/macrophages and reduced the nitrotyrosine formation in these animals (C). Treatment with the CB1 antagonist AM281 at 20 days post induction (D) resulted in a greater blocking effect whereas treatment with the CB2 antagonist AM630 only had a slightly blocking effect (E).

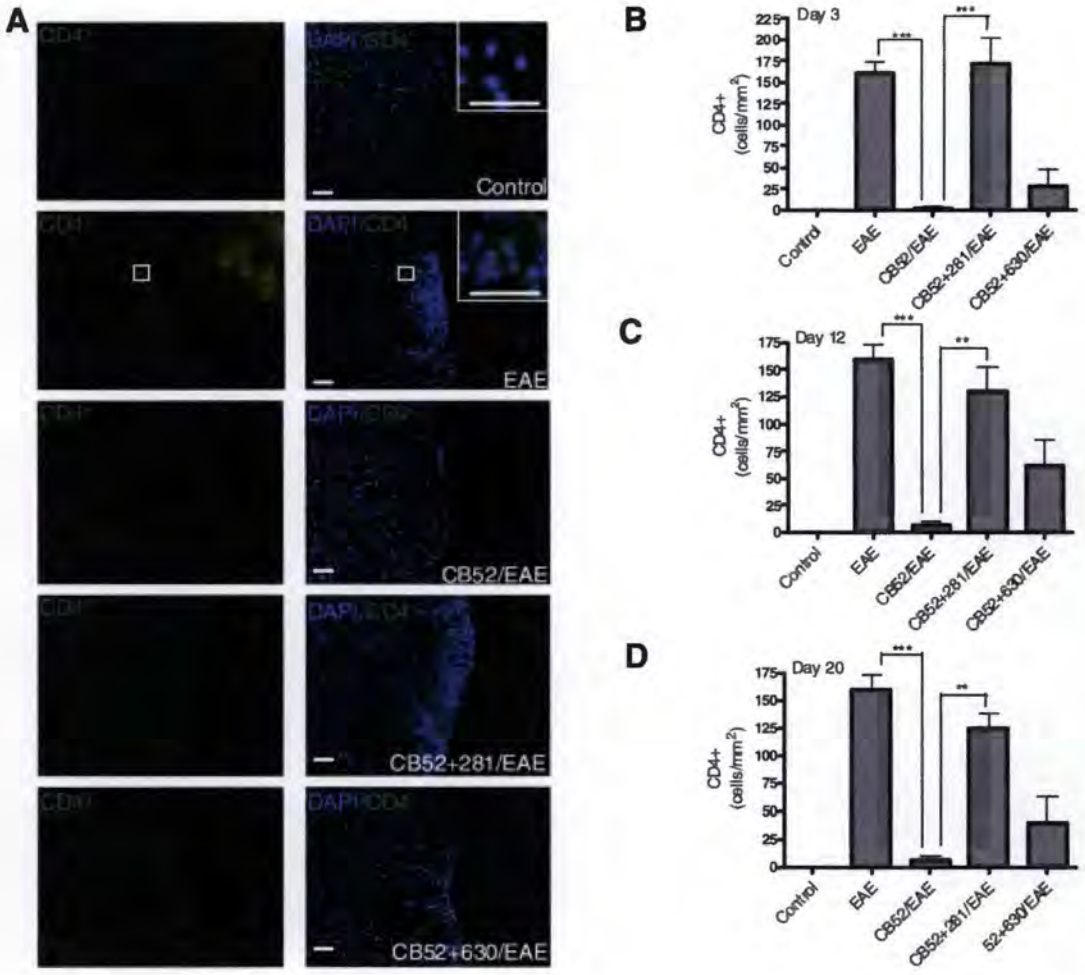



**Figure 26. Infiltration of T cells in EAE animals is decreased with CB-52 treatment**
A, Lumbar spinal sections of EAE animals were stained for T cells (CD4+). An increase in T cells can be seen throughout the white matter of the spinal cord in EAE control animals. Images represent day 3 treatment group.
B, CB-52 blocked the T cell infiltration in the spinal cord of EAE animals when given 3 days post induction. This effect was blocked by the CB2 antagonist (AM630), but not the CB1 antagonist (CB281). (n=4-6; ***, $p < 0.001$).
C-D, Post-symptom treatment with CB-52 decreased T cell infiltration. This effect was block by the CB2 antagonist (AM630) when given at 12 (C) or 21 days (D) post induction. (n=4-6; **, $p < 0.01$, and ***, $p < 0.001$).

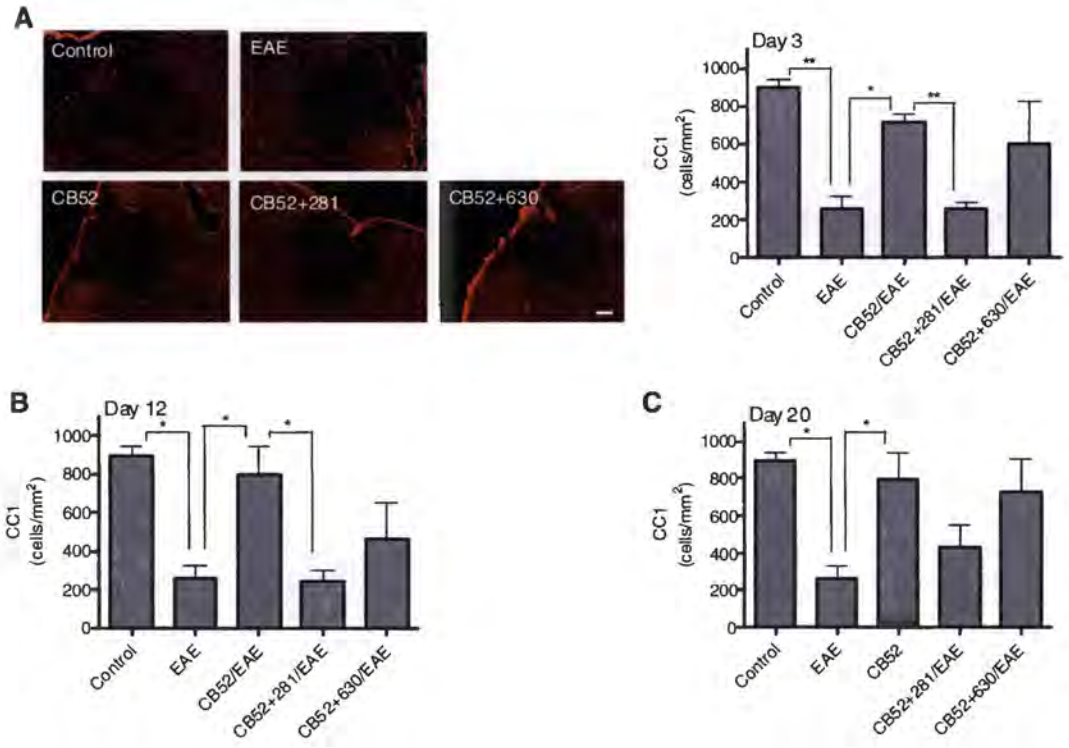



**Figure 27. CB-52 prevents the loss of mature oligodendrocytes in the spinal cords of EAE animals**

A, At 28 days post immunization, the density of mature oligodendrocytes in the spinal cord white matter of EAE mice was significantly less than that found in control animals. The loss of oligodendrocytes was prevented by treatment with CB-52 at day 3 post- induction. This effect was blocked by the CB1 antagonist (AM281), but not the CB2 antagonist (AM630). (n=4; *, $p < 0.05$, and **, $p < 0.01$).

B-C, CB-52 was also effective in preventing the loss of mature oligodendrocytes when given after symptom development (B, C), and this effect was blocked when the CB1 antagonist was given at 12 days post induction (B), but not at 21 days post induction, although a similar trend was also observed (C). (n=5; *, $p < 0.05$).

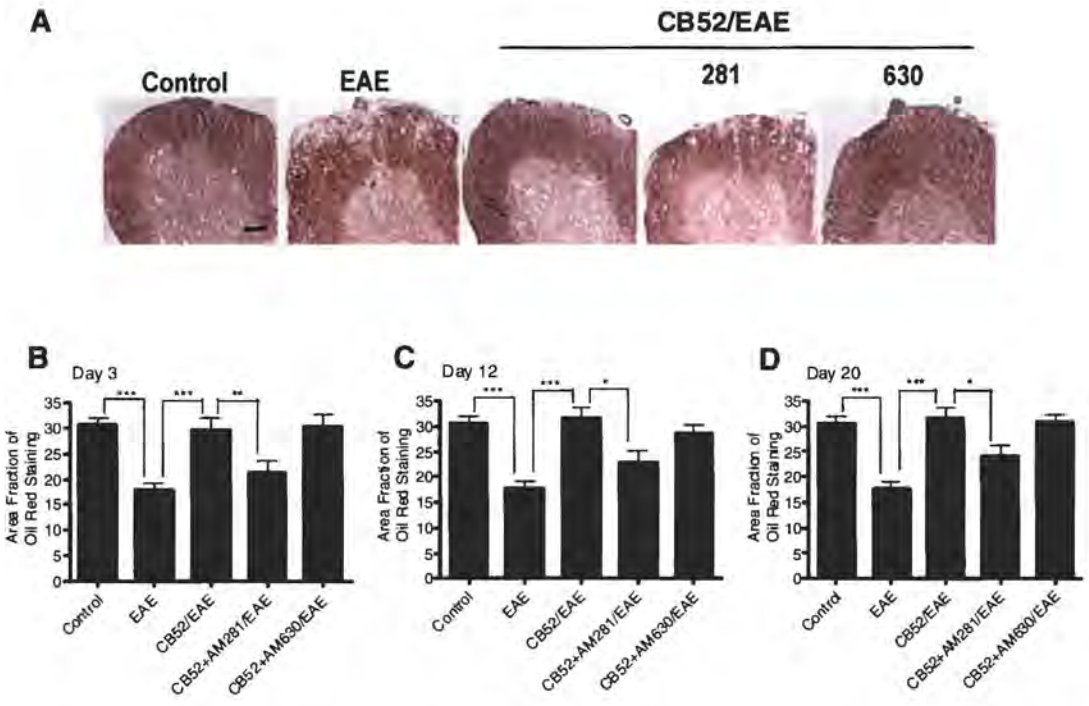



**Figure 28. CB-52 reduces myelin loss in the spinal cord of EAE animals**

A, Myelin loss was measured by staining sections of lumbar spinal cords with Oil Red where intact myelin can be seen as a red staining and demyelinated areas appear white.

B, CB-52 treatment starting at day 3 post induction attenuated myelin loss when compared to the EAE control animals. This effect was blocked by the CB1 antagonist (AM281), but not the CB2 antagonist (AM630). (n=5; **, $p < 0.01$, and ***, $p < 0.001$).

C-D, CB-52 was also effective in preventing myelin loss when given after symptom development on day 12 (C, D). This protective effect was blocked by the CB1 antagonist (AM281) when given at 12 (C) or 21 days (D) post induction. (n=5; *, $p < 0.05$, and ***, $p < 0.001$) (Figure generated by Yu, F).

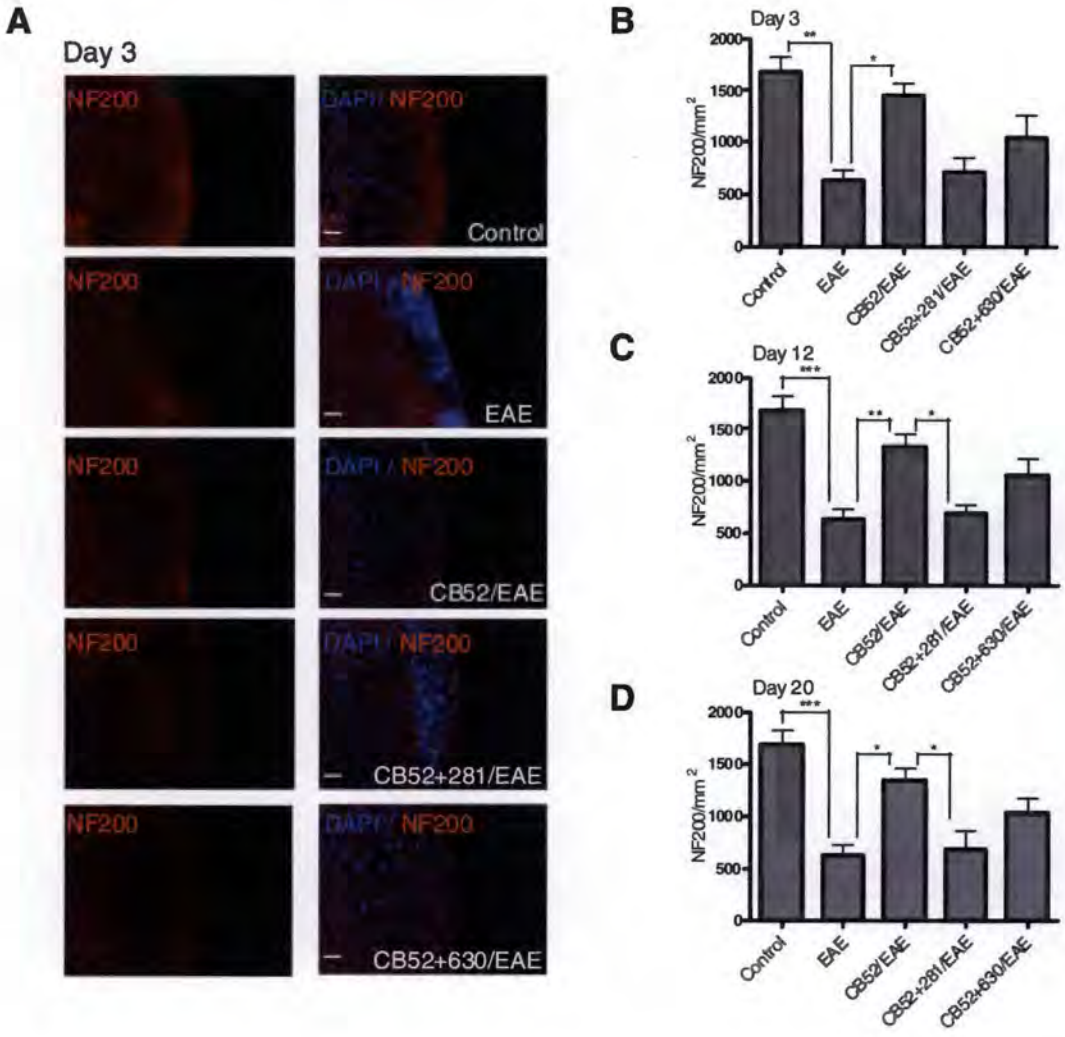



**Figure 29. Treatment with CB-52 decreases axonal loss in EAE animals**

A, Axonal loss was measured by the density of NF200+ axons in lumbar spinal cord sections. EAE animals show decreased NF200+ staining, which was rescued after CB-52 treatment.

B, CB-52 given before symptom development (day 3) significantly decreased axonal loss caused from EAE. While no significant effect can be seen when either antagonist was used, the CB1 antagonist seemed to be more effective in reversing the effects of CB-52 (n=4; *, $p < 0.05$, **, and $p < 0.01$).

C-D, CB-52 was also effective in preventing axonal loss when given after symptom development on day 12 (C, D). This protective effect was blocked by the CB1 antagonist (AM281) when given at 12 (C) or 21 days (D) post induction (n=4; *, $p < 0.05$, **, $p < 0.01$ and ***, $p < 0.001$).

# CHAPTER 4: Future Directions

Today, our understanding of how regulation of the endocannabinoid system can help ameliorate the development and progression of MS is much greater than it was 20 years ago. Although still not definitive, we have gained increased knowledge of where the cannabinoid receptors are expressed and how endocannabinoids are synthesized and hydrolyzed. This knowledge led to cannabinoids being tested in a number of different conditions, including MS (6; 10; 59; 182; 188; 203; 207), where they have been shown to be helpful in ameliorating symptoms and slowing inflammation and degeneration. However, as we learn more about cannabinoids a new question emerges. Is it possible to increase the therapeutic efficacy and meanwhile decrease the potential side effects of any given cannabinoid compound? Since the psychotropic effects of cannabinoids are mediated by the activation of the CB1 receptor, many laboratories have focused on studying the beneficial effects of CB2 receptor activation by using selective CB2 agonists. Although a valid approach, studies show that CB1 receptor activation plays an important role in the therapeutic effects of cannabinoids and therefore should not be ignored (128).

A different approach involves manipulating the levels of endocannabinoids instead of the use of exogenous cannabinoids. This takes advantage of the "on demand" nature of endocannabinoid synthesis, where cannabinoids are produced only where and when needed. This would then limit cannabinoid receptor activation, specially CB1 receptors, where not necessary and therefore attenuate possible side affects (93; 147). AEA and 2-AG are the two most studied endocannabinoids in the CNS (3; 44) and their levels are tightly regulated by the enzymes responsible for their synthesis and

degradation. 2-AG is believed to be the most abundant endocannabinoid in the CNS and three major enzymes believed to be responsible for its hydrolysis: monoacylglycerol lipase (MAGL), α/β-hydrolase domain 6 (ABHD6) and α/β-hydrolase domain 12 (ABHD12) (93). MAGL is believed to be the major hydrolytic enzyme for 2-AG, accounting for 85% of 2-AG hydrolysis, while the other 15% is attributed to the activity of ABHD6 and ABHD12 (25; 199). Recently, a new compound, JZL184 that works as a specific MAGL inhibitor became available. This compound is able to decrease 2-AG hydrolysis by 85% and result in an 8-fold increase in 2-AG levels in mouse brain (122). Chronic MAGL inhibition, however, was shown to produce symptoms similar to those seen with CB1 receptor activation, including analgesia, hypomotility, and hypothermia (104). The same phenomenon was also seen in MAGL -/- animals (38). An alternative approach is to inhibit ABHD6 which was shown to result in a moderate increase in 2-AG levels (130; 188), thus possibly avoiding CB1-like effects seen with MAGL inhibition.

Using a selective MAGL inhibitor JZL184 and a selective ABHD6 inhibitor WWL70, we examined the effect of inhibiting each of these enzymes in the disease progression of EAE animals (Fig. 30). In the first set of experiments mice were treated with JZL184 (10 mg/kg body weight, i.p.) or WWL70 (10 mg/kg body weight, i.p.) starting at day 3 after MOG injection and then once daily until day 30. Animals in the EAE vehicle group showed symptom development starting at day 10 which progressed rapidly to 3.5 on day 19 where it remained constant until the end of experimental period (30 days after EAE induction). Inhibition of MAGL with JZL184 caused a significant reduction in clinical scores when compared to the EAE vehicle group. Significant lower scores were also achieved when animals were treated with the ABHD6 inhibitor

WWL70. We next performed another set of experiments where animals were not given the inhibitors until after at least 50% of the animals exhibited clinical symptoms (day12). Similar to the previous observation in EAE animals, clinical symptoms started to develop on day 10, rapidly progressed to day 14 and then stabilized until day 30 with an average score of 3.5. In this set of experiments WWL70 treatment resulted in a significant decrease in clinical scores when compared to the EAE vehicle group. Interestingly, post-symptom treatment with JZL184 did not significantly reduce the clinical scores, even though MAGL is the enzyme responsible for the majority of 2-AG hydrolysis (25; 199). These preliminary results indicate that inhibition of ABHD6, but not MAGL, may provide a greater therapeutic effect in this model system. It has been reported that chronic inhibition or targeted gene deletion of MAGL can cause CB1 receptor desensitization (185; 187), and result in hypomotility and hyperflexia (122; 123), both of which could affect clinical scores in these animals. Although these studies can help explain our findings it is still not clear why treatment with JZL184 starting at day 3 and not at day 12 resulted in significant lower clinical scores. One possible explanation is that pre-symptom treatment with JZL184 can suppress the function of CD4 T-lymphocytes in periphery and their infiltration to the CNS, and therefore reduce the severity of the disease.

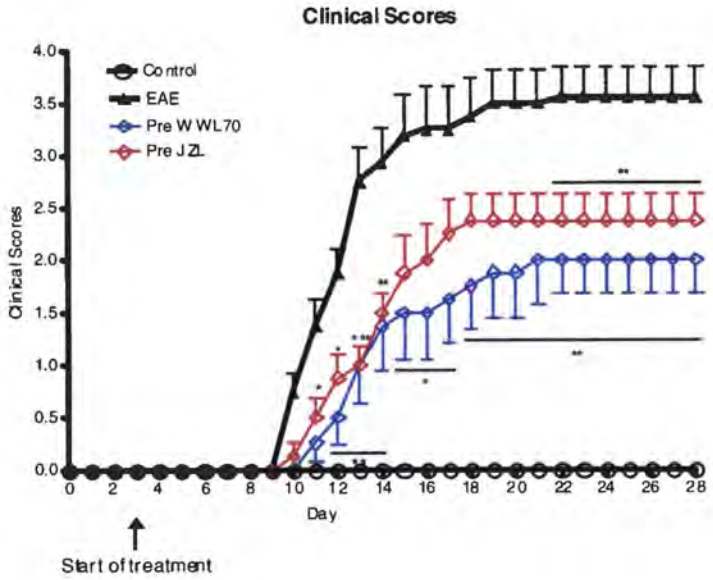
Our results suggest that inhibition of ABHD6 may present a better therapeutic option. Unlike inhibition of MAGL, selective inhibition of ABHD6 only results in a modest increase in 2-AG levels, and therefore can avoid desensitization of CB1 receptors (129; 130; 187). In fact, our group has recently demonstrated that chronic inhibition of ABHD6 results in the upregulation of CB1 and CB2 receptors in a mouse model of TBI (199). Furthermore, it has been shown that MAGL is found in presynaptic neurons, and

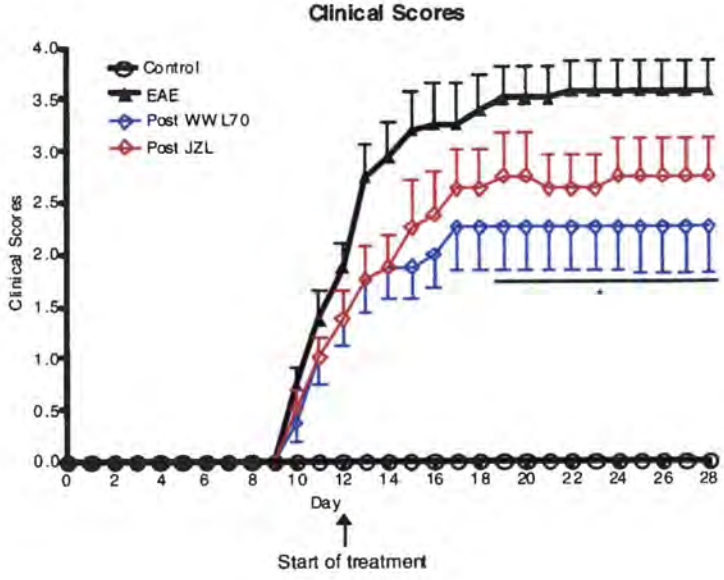
ABHD6 is a membrane protein localized in postsynaptic neurons. This difference in localization suggests that these two enzymes may have distinct roles in 2-AG hydrolysis, allowing ABHD6 to degrade 2-AG close to the site of its production and prior to its being able to reach cannabinoid receptors (129; 185; 199). Taken together, our preliminary studies suggest that a moderate increase of 2-AG by inhibition of ABHD6 may be more beneficial at therapeutics.

**Figure 30. Inhibition of MAGL and ABHD6 reduces clinical scores in EAE animals**

A, At day 3 post immunization, mice were given the MAGL inhibitor JZL184 (10 mg/kg, i.p.) or the ABHD6 inhibitor WWL70 (10 mg/kg, i.p.) once a day until the end of the experiment. Clinical scores were significantly decreased in both the JZL184 treated group (n=8) and the WWL70 treated group (n=8) when compared to EAE group (n=8; *p< 0.05, ** p< 0.01, *** p<0.001).

B, At day 12 post immunization, mice were given MAGL inhibitor JZL184 (10 mg/kg, i.p.) or the ABHD6 inhibitor WWL70 (10 mg/kg, i.p.) once a day until the end of the experiment. Clinical scores were significantly decreased in the WWL70 treated group (n=8) when compared to EAE group (n=8, *p<0.05). Although the clinical scores in the JZL184 treated group seemed to be reduced, but no significance was reached compared to the EAE-vehicle group.


Clinical Scores
Control
EAE
Pre W WL70
Pre JZL
Clinical Scores
Day
Start of treatment


Clinical Scores
Control
EAE
Post WW L70
Post JZL
Clinical Scores
Day
Start of treatment

# CHAPTER 5: Discussion

MS can be a devastating condition for which there is still no cure. Symptoms are a consequence of myelin loss and the transection of neuronal axons, leading to the inability of neurons to effectively conduct signals. Inflammation and oligodendrocyte loss are two of the major characteristics in MS. The specific aims presented in this study were focused on better understanding the effect of cannabinoids on two specific cell types; microglia and oligodendrocytes. In addition, we aimed to characterize a new synthetic cannabinoid in an animal model of MS.

The overall purpose of this thesis was to increase scientific knowledge pertaining to disease development and to further our understanding of cannabinoids as a possible treatment for this disease. Cannabinoids are a class of compounds that have been self-administered by MS patients for decades to relieve pain and spasticity. The discovery of the endocannabinoid system in the human body led to increased interest in these compounds as scientists try to understand how this system works and whether its manipulation can serve as a therapeutic strategy in various pathological conditions, including MS. Cannabinoids are thought to be ideal compounds for the treatment of MS since they possess both immunomodulatory and neuroprotective properties. The use of cannabinoids may not only help symptoms relief, but might also help to delay or even to halt the disease progression.

**Cannabinoids interfere with the signaling pathway leading to peroxynitrite formation in reactive microglia**

The first part of this work dealt with understanding the mechanisms of peroxynitrite production by activated microglia and the role of cannabinoids on its production. Microglia activation is considered to be an important contributor for the progression of a number of inflammatory diseases, including MS. Thus, understanding the process leading to the production and release of toxic factors by reactive microglia can be beneficial in the treatment of inflammatory diseases. Peroxynitrite is the major toxic factor released by microglia (119; 218; 222; 223), but the mechanisms leading to its production are still not clear. Elucidation of the mechanisms leading to peroxynitrite production and release allows us to effectively target this pathway, and therefore reduce the injuries caused by the release of this toxic factor. Limiting the levels of toxic factors released in the CNS could alleviate damage and create an environment that is more conducive to self repair. Increased lipid peroxidation has been demonstrated in the cerebral spinal fluid (CSF) and serum of MS patients indicating the oxidative stress during the MS development (92; 177). Using an *in vitro* model we were able to show that peroxynitrite formation in activated microglia occurs via a pathway involving the phosphorylation of the ERK1/2 and JNK MAPKs, $cPLA_2$, and activation of NF-κB and iNOS. This is in agreement with studies showing that release of IL-12 and IL-23 occurs via a mechanism involving ERK1/2 and JNK (42) and that $cPLA_2$ phosphorylation leads to the activation of iNOS in macrophages and astrocytes (163; 215). Therefore, agents

blocking this pathway should be able to attenuate the release of cytotoxic factors. including peroxynitrite.

Although it is generally accepted that cannabinoids can suppress cytotoxic factors released from microglia, the mechanisms for this suppression are not fully understood. We were interested in identifying whether the suppressive effect of cannabinoids on peroxynitrite production occurred via the pathway we identified. We found that, in fact, cannabinoids attenuated ERK1/2 and $cPLA_2$ phosphorylation, and reduced NF-κB activity, iNOS induction and ROS generation.

**Involvement of the CB1 and CB2 receptors in attenuating peroxynitrite release by microglia**

Another important question that remains unclear is how cannabinoid receptors are involved in suppressing the production of cytotoxic factors by activated microglia. It is still quite debatable whether and how cannabinoid receptors are involved with the inflammatory response. Puffenbarger and colleagues (2000) found that cannabinoids were able to decrease the release of cytokines but that this effect was not blocked by CB1 or CB2 antagonists. Interestingly, when pairs of cannabinoid enantiomers were used both drugs showed blocking effect, leading them to conclude that the effect of cannabinoids was not receptor-mediated. In another study, it was shown that the suppressive effect of cannabinoids on TNFα release in activated microglia was not reversed by the cannabinoid receptor antagonists. However, when WIN55,212-2 enantiomers were used, only the one which has a higher affinity to CB1 and CB2 receptors had a blocking effect. This result suggested that the effect of cannabinoids may be due to CB1/CB2 receptor

independent mechanisms (60). On the other hand, several studies showed that the attenuation of cytokine release in activated microglia by cannabinoids can be blocked by CB1/2 receptor antagonists and that this effect is therefore, receptor mediated (42; 58). Our study found that while the effect of cannabinoids seems to be stereoselective, the use of antagonists was unsuccessful in blocking the effect of cannabinoids that is in agreement with reports pointing to a receptor mediated mechanism other than CB1/2 receptors. In fact, there is a consensus that the CB1 and CB2 receptors are not the only receptors activated by cannabinoids. The human GPR55 receptor first isolated in 1999 (186) has been shown to be activated by several cannabinoids, and postulated to be a third cannabinoid receptor. However, not only was this receptor shown to have a very low homology when compared to CB1/2 receptors (10-15%), their pharmacological properties varies tremendously (11; 83; 160). Nevertheless, this receptor indeed responds to a broad -range of cannabinoids, including phyto- , endo- and synthetic cannabinoids and its activation has been suggested to have neuromodulatory effects (160; 194). In addition to GPR55, cannabinoids have also been found to interact with a number of other receptors and ion channels. Receptors thought to be able to interact with cannabinoid agonists and antagonists include: transient receptor potential vanilloid subfamily, member 1 (TRPV1), peroxisome proliferator-activated receptor (PPAR), muscarinic acetylcholine, opioid, adenosine, 5-HT, angiotensin, prostanoid, dopamine, melatonin, and tachykinin receptors. Among ion channels calcium, sodium and potassium channels are know to respond to cannabinoids (160). The discovery of the CB1/2 receptors' ability to form homo- and heterodimers adds another layer of complexity to the study of these receptors. CB1 receptors have been found to form dimers with dopamine (127), opioid

(174) and orexin-1 (86) receptors and these dimers, once formed, may have different biochemical properties in response to their ligands compared to the individual receptors (62). All these interactions, which can happen at concentrations as low as the nanomolar range makes it more challenging to study the effect of cannabinoids on the CB1 and CB2 receptors. The complex role played by the endocannabinoid system in the body suggests that the current research is still only touching the surface on the topic.

One strategy which can help add to pharmacological studies such as the one presented here is through the use of molecular techniques such as knockout animals. Such strategy helps to isolate each receptor so that its function can be more clearly studied. Animals with global inactivation of CB1 (117; 224) and CB2 (32) receptors have been generated and these animals were shown to be more susceptible to disease in several animal models of MS (153; 168). In addition, tissue specific CB1 knockout for neurons (132), spinal cord nociceptors (2) and interneurons (157) and hepatocytes (150) also exist. Although a microglia specific knockout for the CB1 and CB2 receptors is not yet available, cells isolated from global knockout animals could help to clarify the specific roles played by these receptors.

## CANNABINOIDS AND OLIGODENDROCYTES

### Cannabinoids can directly protect oligodendrocytes against peroxynitrite toxicity

The second specific aim of this study dealt with investigating whether cannabinoids have a direct protective effect on mature oligodendrocytes when exposed to peroxynitrite. While in the CNS most of the research on cannabinoids has been focused

on their effect on neurons and microglia, less is known about how this class of compounds can affect oligodendrocytes.

Oligodendrocyte and myelin loss are two key characteristics in MS development. Oligodendrocytes are responsible not only for producing myelin around neuronal axons and therefore affecting conduction of nerve impulses, but they are also important for the overall health of the axon by maintaining their nodal region (134). Finding agents that can directly affect the survival of these cells may prove beneficial in preventing and/or treating symptoms resulting from their loss.

There are two major approaches when dealing with oligodendrocyte/myelin loss. One is to promote progenitors developing into mature oligodendrocytes so that demyelinated axons can be remyelinated, and the other is to prevent existing mature oligodendrocytes from injury. Cannabinoids have been shown to be effective in both strategies by protecting oligodendrocyte progenitors from apoptosis (143) and preventing mature oligodendrocytes from injury induced by calcium-influx (134). Here, we focused our attention on protecting existing oligodendrocytes from the likely toxic environment that develops with MS. More specifically, our study focused on peroxynitrite toxicity to oligodendrocytes. We have been previously able to show that peroxynitrite leads to mature oligodendrocyte death through the activation of a pathway that involves intracellular zinc release, ERK phosphorylation and ROS generation (221; 222). We were able to show for the first time that exposing mature oligodendrocytes to a number of general cannabinoid agonists resulted in decreased cell loss when these cells were exposed to peroxynitrite. Because intracellular zinc release seems to be one of the first steps in the signaling pathway leading to cell death, we also induced toxicity through

extracellular zinc exposure and found the cannabinoid agents have the similar effects. These results are in agreement with the antioxidative properties attributed to cannabinoids (91). We also found that not all cannabinoid agonists protected oligodendrocytes to the same extent. In our study CB-52, an analog of THC and AEA appears to be a better compound than several other general agonists in protecting oligodendrocytes from injury, in addition to our findings that no toxicity was shown by itself, unlike several other cannabinoid agents, e.g., AEA and CP55,940.

**Activation of CB2 receptors is implicated in the protective action of cannabinoids on oligodendrocytes**

Oligodendrocytes have been shown to express both CB1 and CB2 receptors in culture and *in vivo* (21; 134; 143), but little is know regarding the role of these receptors in progenitor and mature oligodendrocytes. A previous study from 2002 found that cannabinoids can prevent progenitor cell death induced by tropic factor withdrawal and that this effect was mediated by both CB1 and CB2 receptors (143). More recently, in 2009 another study presented evidence to show that cannabinoids can inhibit calcium influx due to transient membrane depolarization in oligodendrocytes. While both CB1 selective and general cannabinoid agonists had a positive effect, CB2 selective agonists were found to be ineffective. Since the use of CB1 antagonist resulted in a partial blocking effect, the authors concluded that the effect seen was partially mediated by the CB1 receptor (134). More recently in 2011, Gomez and colleagues showed that activation of both CB1 and CB2 receptors resulted in enhanced progenitor differentiation (76). Here, we are able to add to this increasing body of knowledge by showing that the

protective effect of cannabinoids on oligodendrocyte appears to be partially mediated by the CB2 receptor. The findings that the cannabinoid receptor antagonists only partially block the effect of cannabinoid receptor agonists, suggests that receptors other than the CB1 and CB2 might contribute to the protective action of cannabinoids.

## CANNABINOIDS IN THE TREATMENT OF EAE AND MS

Cannabinoids have been shown to be useful in different animal models of MS and cannabis-based treatments are also available for human patients in some countries. Studies show that cannabinoids seem to confer both anti-inflammatory and neuroprotective effects, which are thought to be mediated by the CB2 and CB1 receptors, respectively (183). Although our understanding of the therapeutic potential of cannabinoids has grown tremendously in the last two decades, our knowledge concerning how this class of compounds works and how we can manipulate them to take full advantage of their benefits in treating MS remains limited. There is still much to be understood regarding to how the endocannabinoid system works and how to avoid potential side effects, including psychotropic effects and neurotoxicity (37; 65). For this reason, every new synthetic cannabinoid that is developed should be screened and tested using *in vitro* and *in vivo* models for efficacy and potential side effects. Just like the development of any new drug, the overall goal is to develop an agent which possesses a high level of efficiency, while presenting no or minimum side effects. Since the discovery of the endocannabinoid system, and as scientists continue to search for the ideal cannabinoid-based medicine, a number of synthetic cannabinoid agonists and

antagonists have been, and continue to be, created and tested. In this study we wanted to test the effect of CB-52 in the EAE model.

CB-52 was synthesized in 2005 (29), but with the exception of a few pharmacological tests (36), this drug has not been very well described in the literature. At first, CB-52 was added to the group of cannabinoids to be screened in our *in vitro* experiments using oligodendrocytes as described previously. Our results show that CB-52 is more potent than other more commonly used cannabinoids in protecting oligodendrocytes against peroxynitrite and zinc toxicity. In addition, the protective effect of CB-52 in the EAE model seems to be partially mediated by activation of CB2 receptor. This effect seems to be contradictory to the first set of pharmacological studies in which CB-52 is shown to behave as a partial CB1 agonist and a neutral CB2 antagonist *in vitro* (36). It is possible that oligodendrocytes may have a different CB1/CB2 receptor profile as compared to the N18TG2 cells used in the previous study.

The ideal cannabinoid-based drug should be devoid of psychoactive side effects, which are believed to be mediated by activation of the CB1 receptor, while still displaying a highly therapeutic efficacy. Thus, since our *in vitro* results showed that CB-52 may act through the CB2 receptors we decided to further investigate this novel drug in an *in vivo* study, by using the EAE model. We analyzed the drug effect and pathological changes by employing immunohistochemistry techniques. We found CB-52 to be comparable to other cannabinoids by being very effective in ameliorating many aspects of the disease, including decreased T cell infiltration, microglia activation, oligodendrocyte and myelin loss, and axonal damage.

Inflammation is a central component in EAE and MS and although cannabinoids have been shown to be effective in treating this aspect of the disease, the immunomodulatory and anti-inflammatory effects of cannabinoids have not been fully elucidated. Cannabinoids are known to affect a number of immune cells such as macrophages, microglia and T cells. They can limit the amount of cytokines and chemokines released by immune cells in addition to lowering their rate of proliferation and migration (49; 105; 198). Because of the high levels of CB2 receptors expressed in immune cells when compared to CB1 receptors, it has been assumed that the effect of cannabinoids on these cells is due dominantly to the action of these compounds on the CB2 receptors. Further research on the topic has shown, however, that cannabinoids can affect immune response through activation of the CB1 receptor, as well as activation of receptors other than CB1/CB2 (107). In this *in vivo* study, we showed that CB-52 can attenuate the number of activated microglia cells and lower T cell infiltration in the spinal cord of EAE mice. We found this effect to be blocked when animals were co-administered with the CB1 antagonist AM281, but not with the CB2 antagonist AM630. It is possible that the action of CB-52 *in vivo* may be preferentially through the CB1 receptor. Also, it has been demonstrated that CB1 receptors can be upregulated in T cells by treatment with cannabinoids (27). A higher CB1 receptor expression may therefore help explain our findings and other studies that show the importance of CB1 receptor activation in attenuating the inflammatory response.

Inflammation is one of the major characteristics of MS and much research has been focused on trying to suppress the activity of the immune system in hopes to stop disease progression. However, although this proved to be a helpful approach,

immunosuppressive treatments alone are not able to prevent disease progression, resulting in increased disabilities to patients. Researchers now recognize that preventing or treating the neurodegeneration associated with the disease is vital if we want to prevent the chronic disability that is associated with the disease. One reason why cannabinoids are thought to be a good option in the treatment of MS is due to their ability to act not only as immunosuppressants but also act as neuroprotectants to inhibit neurodegeneration in animal models of the disease (168). Although there is no consensus in the literature on whether axonal damage results as a consequence of myelin loss or as a result of the inflammatory insult before demyelination (23; 168; 200), it remains clear that both demyelination and axonal injury are of major importance in disease progression.

Oligodendrocytes are extremely vulnerable to the cytotoxic environment that is characteristic of MS and EAE and the loss of these cells results is myelin loss around neuronal axons. Without myelin, axons become more vulnerable to excitotoxic, inflammatory and oxidative insults (102; 168). In this thesis work we show that cannabinoids are protective to oligodendrocytes *in vitro* and *in vivo*, and can also prevent axonal damage in EAE. While our *in vitro* studies showed that activation of the CB2 receptor was partially responsible for the protective effect of cannabinoids, when CB-52 was used in the EAE model, blockade of the CB1 and not the CB2 receptors resulted in increased oligodendrocyte survival and prevented myelin loss and axonal damage.

It is important to highlight that the overall goal of our animal studies was to characterize the effect of CB-52 in the EAE model, and while our animal studies showed an overall CB1-dependent effect with CB-52 treatment, we cannot define the specific cell types involved based on these results. It is still possible that the effect of CB-52 on

oligodendrocytes *in vivo* is due mostly to the activation of the CB2 receptor, but that when looking at the system as a whole the overall effect of CB1 overshadows that of the CB2. The CB1 receptor is the most abundant G-protein coupled receptor in the CNS and it is found mostly in neurons where it is responsible for regulating neurotransmission (198). Excitotoxicity is also a major contributor to the neurotoxicity seen in MS and EAE and we hypothesize that CB-52 might be effective in attenuating glutamate levels due to the protection attributed to cannabinoid in models of excitotoxicity and our results showing a CB1 mediated effect for this compound *in vivo*. In addition, in this work we did not address the effect of CB-52 on the breakdown of the blood-brain barrier (BBB). One of the early events during MS and EAE is the impairment of the integrity of the BBB, allowing peripheral immune cells access to the CNS. Although most reports point to cannabinoids protecting the BBB via activation of the CB2 receptor, the participation of CB1 receptors has also been demonstrated (141).

The results described in this thesis work illustrate how reliance in just *in vitro* or *in vivo* models fails to convey a complete picture relating to how a drug actually acts. While *in vitro* models are helpful and important in allowing researchers to narrow their field to study specific mechanisms in specific cell types, the findings should be better validated *in vivo* in order to draw more significance. Cells are known to behave differently when grown *in vitro* as they lack the complex interactions to which they are exposed in a system. Only after a drug can be studied in a more complete system can we start to fully evaluate its potential. Cell systems are a convenient way to evaluate how drugs act in specific cell types, which is more complex to be examined *in vivo*. In the case of cannabinoids where researchers are trying to tease out the role of two (or most

certainly several) receptors in a system where they are expressed in various cell types a molecular approach through the use of conditional knockouts may help clarify the pharmacological data available today. As mentioned previously, the overall goal in the cannabinoid field is to produce a cannabis-based drug that possesses highly therapeutic effect with low to no side effects. In order to accomplish this goal, it is imperative for researchers to understand how each drug in this class of compounds acts in the different cell types, as well as its effect in the disease state. This understanding will then allow for development of more effective drugs.

**Modulation of the endocannabinoid system as an alternative approach for treating MS**

While the search for a treatment which is based mostly on the activation of the CB2 receptors continues to grow, an increased number of studies point to the importance of CB1 receptor activation for the protective effect of cannabinoids. Knockout studies showed that knockout of either the CB1 or CB2 receptors resulted in more severe EAE (153; 168), and the CB1 receptor is thought to be responsible for the neuroprotective effect of cannabinoids (128). In addition, this and several other studies have shown that CB1 receptors can also play a role in modulating immune cells and inflammation (51; 168; 173). Thus, targeting both CB1 and CB2 receptors might achieve the maximum therapeutic effects. An alternative approach which is gaining popularity in the field is to modulate the endocannabinoid system by increasing endocannabinoid levels, instead of the use of exogenous cannabinoids, as described previously. By increasing the levels of endocannabinoids only when and where they are synthesized as a compensative

mechanism the occurrence and magnitude of possible side effects can be lowered or eliminated.

AEA and 2-AG are the most studied endocannabinoids and their levels are closely regulated by enzymes responsible for their synthesis and catabolism. AEA is believed to be synthesized through the transfer of arachidonic acid from membrane phospholipids to phophatidylethanolamine (PE) to form N-acyl phosphatidylethanolamine (NAPE) followed by hydrolysis by phospholipase D (PLD) to generate AEA. After release, AEA is taken up by cells and hydrolyzed by FAAH. 2-AG is synthesized from arachidonic acid-containing diacylglycerol (DAG) by diacylglycerol lipase after release it is taken into cells where it can be hydrolyzed by several enzymes, with MAGL being the major one (24). Due to endocannabinoids' "on demand" release and their known mode of inactivation, a new strategy being pursued by researchers is to inhibit the enzymes responsible for the hydrolysis of endocannabinoids in order to raise the initial neuroprotective response to a therapeutic level (93). Inhibition of the AEA hydrolytic enzyme FAAH has been shown to be protective in animal models of various diseases, including MS (93). While treatment with exogenous 2-AG has been reported in EAE (124), the effect of increasing the level of this endocannabinoid by inhibiting its hydrolytic enzymes has not been reported. In this thesis work we showed preliminary studies in which EAE animals are treated with the inhibitors for MAGL and ABHD6. Our results suggest that inhibition of ABHD6 which is thought to account for a small portion of 2-AG hydrolysis in brain lowers clinical scores of EAE animals more efficiently than of the MAGL inhibitor. A higher increase of 2-AG may lead to desensitization of CB1 receptors, as described earlier, which may help explain our

preliminary results. Further studies into the effect of ABHD6 inhibition may lead to the use of this strategy for the development of therapeutics.

**Concluding remarks**

Extracts from *Cannabis Sativa* have been used for hundreds of years for both medicinal and recreational purposes but not until fairly recently did scientists begin to understand their chemical structures and functions. The isolation of cannabinoid receptors in the early 1990s, provided a platform for the elucidation of biological functions of their binding ligands. This opened a field that seemed to have been dormant and led to increased research on the therapeutic effects of cannabinoids. To date cannabinoids have been shown to possess clinically beneficial effects in an array of different conditions, which include neurodegenerative diseases (6; 10; 59; 115; 203; 207), pain (158), traumatic brain injury (154; 199), ischemia (146) and cancer (68). The idea that cannabinoids can be used to treat MS seems promising as it not only helps with symptom management, but it has also been shown to reduce the inflammatory and neurodegenerative aspects of the disease. As we understand more about MS and how the cannabinoid system interacts with it, we will be better equipped to offer hope to those patients who suffer from the disease.

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