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REVIEW

Direct suppression of autoreactive lymphocytes in the central nervous system via the CB_2 receptor

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The cannabinoid system is now recognized as a regulator of both the nervous and immune systems. Although marijuana has been used for centuries for the treatment of a variety of disorders, its therapeutic mechanisms are only now being understood. The best-studied plant cannabinoid, Δ^9-tetrahydrocannabinol (THC), produced by *Cannabis sativa* and found in marijuana, has shown evidence of being immunosuppressive in both *in vivo* and *in vitro*. Since THC binds to at least two receptors that are differentially expressed by the immune and nervous systems, it has not been possible to clearly discriminate the biological effects it exerts in the two systems. In addition, endogenous cannabinoids have also been described that bind to both receptors and exert both neuronal and immune modulatory activity. The generation of mice deficient in specific cannabinoid receptors has facilitated studies to discriminate cannabinoid-specific functions. This review focuses on the function of the cannabinoid receptor 2 (CB_2), primarily expressed in the immune system, in regulating T cell effector functions associated with autoimmune inflammation in the central nervous system (CNS).
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Abbreviations: Anandamide/AEA, arachidonoylethanolamide; 2-AG, 2-arachidonoylglycerol; CB, cannabinoid receptor; EAE, experimental autoimmune encephalomyelitis; IFN, interferon; MS, multiple sclerosis; MBP, myelin basic protein; h, T helper; THC, Δ^9-tetrahydrocannabinol

Introduction

Diseases of the nervous system are diverse and result in ~9% of all human deaths (Bergen and Silberberg, 2002). The most prevalent immune-mediated disease of the CNS is multiple sclerosis (MS) (Sospedra and Martin, 2005). In the CNS of MS patients, inflammatory plaques form, which contain immune cells that are thought to drive both demyelination and neuronal dysfunction resulting in a variety of clinical symptoms (Sospedra and Martin, 2005). Although clinically very devastating, there are no highly successful treatments available for MS. Because the cannabinoid system has been shown to be anti-inflammatory and negatively regulate immune cell functions, there is interest in cannabinoid-based therapies for MS. The cannabinoid system can modulate/regulate both neuronal and immune functions; therefore, it is not clear whether cannabinoid-based therapies that demonstrate efficacy are due to effects on the immune or nervous system or both. There is yet much to be learnt about how the cannabinoid system interacts with and regulates the immune system in a CNS disease like MS.

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In MS and its animal model experimental autoimmune encephalomyelitis (EAE), the pathogenic cell is thought to be a CD4 T cell that recognizes and mounts an immune response against 'self-antigens' in the myelin sheath (Sospedra and Martin, 2005). However, CD8 T cells have also been shown to be pathogenic in specific EAE models (Huseby *et al*., 2001; Ji and Goverman, 2007; Johnson *et al*., 2007). The autoimmune attack results in inflammatory lesions in the CNS composed of CD4 and CD8 T cells, B cells and macrophages, and is accompanied by demyelination and motor neuron defects (Sospedra and Martin, 2005). Recently, using the mouse model of MS, EAE, we have shown that the cannabinoid system directly regulates the CD4 self-reactive T cell modulating the severity of disease (Maresz *et al*., 2007). These data will be discussed.

The endocannabinoid system

The cannabinoid system is evolutionally conserved and is present in invertebrates and vertebrates (Salzet *et al*., 2000). One of the best-studied cannabinoids is Δ^9-tetrahydrocannabinol (THC), the predominant active component of *Cannabis sativa* or marijuana. The marijuana plant has been exploited

by humans since their early history and was used for centuries in Asian medicine to reduce the severity of pain, inflammation and asthma (Berdyshev, 2000). However, only recently have the mechanisms of the medicinal properties of THC begun to be understood. This understanding is largely due to the identification and cloning of two cannabinoid receptors. The CB_1 receptor is expressed primarily by neurons (Matsuda *et al*., 1990), and at low levels in cells of the immune system (Kaminski *et al*., 1992). The identification of the CB_2 receptor, which is expressed primarily by immune cells (Munro *et al*., 1993; Galiègue *et al*., 1995), provided a basis for the immunomodulatory properties of THC. The CB_2 receptor has recently been shown to be also expressed by some neurons in the brain stem (Van Sickle *et al*., 2005). Identification of arachidonoylethanolamide (anandamide, AEA) (Devane *et al*., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam *et al*., 1995; Sugiura *et al*., 1995), as two lipid ligands for the CB_1 and CB_2 receptors, initiated studies examining the role of 'endocannabinoids' in the maintenance of physiological homoeostasis and the development of certain neuronal and immune system diseases. 2-AG is produced at nanomolar amounts in the brain and spinal cord (Baker *et al*., 2001). Of the two major endocannabinoids, evidence points to 2-AG as the primary endocannabinoid agonist at the CB_2 receptor (Sugiura *et al*., 2000a). Endocannabinoid levels are regulated by synthesis and degradation; AEA is metabolized intracellularly by fatty acid amide hydrolase (Schmid *et al*., 1985) and 2-AG by monoglyceride lipase (Saario *et al*., 2004). Both enzymes result in the production of arachidonic acid, the precursor to the immune-regulatory prostaglandins and other eicosanoids. Other endocannabinoids have been described, but their biological functions are not well understood (Saario *et al*., 2004). In addition, a number of synthetic cannabinoid receptor agonists and antagonists have been developed, of which some have preferential binding to one of the two receptors. These synthetic cannabinoids have been used in many published studies.

Cannabinoid receptors are G-protein-coupled receptors that are linked to $G_{i/o}$-proteins (Berdyshev, 2000), and negatively regulate adenylate cyclase and the accumulation of intracellular cAMP (Schatz *et al*., 1997). Intracellular cAMP regulates PKA activation, which in turn has a major role in gene regulation in immune cells. The prevailing hypothesis is that the cAMP-dependent signalling positively regulates the establishment of immune responses and that cannabiniod receptor signalling can antagonize early events associated with immune cell activation (Berdyshev, 2000). In other words, signalling through the CB_2 receptor is thought to negatively regulate immune cell function. This negative regulation could contribute to the development of disease processes or be exploited for the treatment of disease.

The endocannabinoid system and the immune system

Although first described in the nervous system, it is becoming increasingly clear that the endocannabinoid system plays an important role in modulating immune function. Although underappreciated in the past, the linkage of the nervous and immune systems is becoming a well-established concept. When immunology meets the nervous system, this is termed 'neuroimmunology'. In neurological diseases with an immune component, it is difficult to discern when the immune system is regulating the nervous system and when the nervous system is regulating the immune system. Even when it is clear that one system is regulating the other, the mechanisms involved are extremely difficult to unravel. This is certainly the case with the endocannabinoid system, because its regulation is not on or off, but rather a tuning effect.

The endocannabinoid system has been speculated to modulate immune functions through the CB_2 receptor. The expression of the CB_2 receptor in haematopoietic cells has primarily been measured using reverse transcription (RT)-PCR methods, and it was reported that human haematopoietic cell populations differ in the level of CB_2 receptor expression and are ranked in the following order: B cells > natural killer cells > monocytes > polymorphonuclear neurotrophils > CD8 T cells > CD4 T cells (Galiègue *et al*., 1995). A similar CB_2 expression pattern was reported for mouse immune cells (Lee *et al*., 2001).

The immune modulatory activities of the endocannabinoids are extensive and are thought to include inhibition of T-cell proliferation, decreased cytokine production by T cells, dysregulation of the T helper (h)1/Th2 cytokine balance, perturbation of immunoglobulin production, downregulation of CD8 cytotoxic T-cell activity and impairment of macrophage function (Klein *et al*., 1991, 2004; Schatz *et al*., 1993; Condie *et al*., 1996; Berdyshev, 2000; Yuan *et al*., 2002). Most of these studies have been performed *in vitro*. Some of these immunological defects are likely due to disruptions in T-cell interactions with antigen-presenting cells (Berdyshev, 2000). In addition to these *in vitro* effects, it has been shown that administration of high-dose THC in mice resulted in decreased resistance to *Listeria monocytogenes* or *Herpes simplex* virus (Morahan *et al*., 1979). Although the list of possible immunological perturbations by the endocannabinoid system is extensive, regulation of any specific immune response that results in autoimmunity or protective immunity are not well studied or understood.

One caution regarding the interpretation of immunological *in vitro* experiments is that fetal calf serum contains high levels of AEA and other *N*-acylethanolamides (Berdyshev *et al*., 2000). In addition, many natural and synthetic cannabinoids bind to both the CB_1 and CB_2 receptors, making it impossible to clearly assess the role of each receptor in the immune processes measured. The generation of CB_1 and CB_2 cannabinoid receptor knockout mice have facilitated studies examining the regulation of immune responses by the endocannabinoid system. In the study describing the $CB_2^{-/-}$ mouse, the immune modulatory function of the CB_2 receptor on macrophages was confirmed by showing that THC failed to inhibit T-cell IL-2 production in the presence of $CB_2^{-/-}$ macrophages (Buckley *et al*., 2000). Thus, the $CB_2^{-/-}$ mouse is an excellent model to study the role of the CB_2 receptor in immune responses.

Cannabinoids and MS

There is increasing evidence that a major physiological function of the endocannabinoid system is in the regulation of neuroinflammation. CNS inflammation occurs in a variety of clinical situations including Alzheimer's disease, ischaemia and traumatic brain injury (Walter and Stella, 2004). However, none of these are considered to be immune-mediated diseases. One of the best-studied immune-mediated diseases of the CNS is MS (Sospedra and Martin, 2005). MS is a human autoimmune disease of the CNS that results in focal areas of inflammation that contain immune cell infiltrates and areas of demyelination (Sospedra and Martin, 2005). The prevailing view is that MS is initiated by CD4 T cells of the Th1 subtype. These Th1 T cells are so characterized because they produce the proinflammatory cytokine interferon (IFN)-γ. Th1 cell production of IFN-γ and other proinflammatory cytokines, such as tumour necrosis factor-α and granulocyte–macrophage-colony stimulating factor are thought to drive the inflammatory process in MS. In MS, these Th1 cells have specificity to 'myelin' antigens expressed by oligodendrocytes. These 'self-antigens' include myelin basic protein (MBP), myelin oligodendrocyte protein and proteolipid protein.

Individuals with MS often undergo a dramatic life alteration. The symptoms of MS can be associated with any function of the CNS, since the immune cell-mediated inflammation can occur virtually in any area of the CNS, including the spinal cord. In addition, it is becoming clear that neuronal dysfunction and even neuronal loss are present even early in the MS disease course (Sospedra and Martin, 2005). One of the most prevalent symptoms of MS is the loss of motor function. This can occur to such an extent that individuals with MS have trouble walking and holding objects. Since MS most often strikes women between the ages of 20 and 40 years (Sospedra and Martin, 2005), life alterations often include a change in employment status and a reduced inability to care for children. Because there is no cure for MS and treatments are limited, much research needs to be done on the immunological mechanisms involved in the pathogenesis of MS.

Because of the psychotropic effects of CB_1 receptor signalling, one of the more controversial treatments for MS is the smoking of marijuana or the administration of THC or other components of *C. sativa*. Clinical studies have suggested beneficial effects of cannabinoids in MS symptoms of neuronal in nature including spasticity, pain, tremor and nocturia (Pertwee, 2002). However, a recent clinical trial examining the effect of THC on the control of spasticity concluded that no beneficial effect occurred (Zajicek *et al.*, 2003). Also, a more recent 2004 review of all clinical trials that did not include the Zajicek trial (Zajicek *et al.*, 2003), examining the use of cannabis in individuals with MS, concluded that the results were equivocal (Killestein *et al.*, 2004). Since these trials primarily examined neuronal effects, it is still unclear as to whether immune modulation by cannabinoids would be beneficial in MS.

EAE and cannabinoids

The best-studied animal model of MS is EAE in mice. EAE recapitulates much of the MS clinical disease, including the presence of inflammatory lesions, demyelination and neuronal dysfunction/loss. As with MS, the inflammatory lesions primarily consist of T cells and macrophages. EAE can be induced by the immunization of susceptible mouse strains with myelin antigens emulsified in complete Freund's adjuvant in combination with the injection of *Bordetella pertussis* toxin. EAE induced by this active immunization protocol begin to exhibit clinical disease starting around day 14. The severity of clinical disease steadily increases over about a week, beginning as tail weakness and then progressing as an ascending paralysis. Depending on the strain of mouse used, the mice will exhibit a chronic disease course or undergo spontaneous recovery. Some mouse strains will also relapse. A second method of EAE induction is the adoptive transfer of activated myelin-specific T cells into sublethally irradiated hosts. In this adoptive transfer model, the mice exhibit signs of clinical EAE starting at day 7. The clinical progression and recovery is very similar to that observed with active induction.

Unlike with MS, treatment of EAE with cannabinoids revealed a clear therapeutic benefit (Walter and Stella, 2004). The general consensus from a number of studies is that administration of either plant derived (THC) or synthetic cannabinoids with selectivity for the CB_1 and/or CB_2 receptors reduced clinical signs of EAE, including spasticity and tremor and paralysis (Lyman *et al.*, 1989; Wirguin *et al.*, 1994; Achiron *et al.*, 2000; Baker *et al.*, 2001). In a chronic relapsing EAE model, the use of selective antagonists for the CB_1 and CB_2 receptors demonstrated a role for both receptors in controlling spasticity, with evidence for the CB_1 receptor being the main target (Baker *et al.*, 2000). Several studies have examined the effect of cannabinoid treatment using the Theiler's murine encephalomyelitis virus model of MS. In this model, mice are intracerebrally injected with the virus and the mice develop clinical signs, similar to EAE, about 30 days later. In these studies, synthetic cannabinoids were effective in decreasing clinical disease (Arévalo-Martín *et al.*, 2003; Croxford and Miller, 2003). Two additional studies showed that the administration of an inhibitor of the putative endocannabinoid transporter also reduced TMEV clinical disease (Mestre *et al.*, 2005; Ortega-Gutierrez *et al.*, 2005). Similarly, in a chronic relapsing model of EAE, spasticity was also ameliorated by a competitive endocannabinoid uptake inhibitor and by a selective inhibitor of fatty acid amide hydrolase (Baker *et al.*, 2001). Mechanisms that have been suggested for the suppression of EAE via the cannabinoid system include induction of encephalitogenic T-cell apoptosis and reduced migration of immune cells into the CNS (Ni *et al.*, 2004; Sánchez *et al.*, 2006). Thus these cumulative studies suggest that the cannabinoid system could be manipulated for the treatment of MS. However, since the above studies did not use endogenous cannabinoid receptor ligands, it is unclear how the 'endocannabinoid' system functions in regulating CNS inflammation.

CB_1 receptor in regulating EAE

One of the drawbacks in using either natural or synthetic cannabinoids to study the functions of the cannabinoid system is that they all exhibit some level of binding affinity

for both the CB_1 and CB_2 receptors. The same is true for the CB receptor antagonists. Thus, the strategy of administering cannabinoids to wild-type mice to modulate EAE does not allow a clear distinction between CB_1 and CB_2 receptor-mediated effects. Functional differentiation between the two receptors became possible upon the creation of knockout mice lacking either receptor. Mice deficient in the CB_1 receptor were susceptible to EAE induction; however, they did not recover from paralysis to the same extent as the wild-type controls and they exhibited greater neurodegeneration (Pryce *et al*., 2003). These data provided further evidence that the CB_1 receptor regulated neuroinflammation associated with EAE. To distinguish between CB_1 receptor effects mediated by the immune versus nervous system, Dr D Baker and colleagues generated conditional knockout mice using lineage-specific promoters specific for T cells or neurons (Maresz *et al*., 2007). Using THC to inhibit EAE, it was shown that mice which were completely deficient of the CB_1 receptor were refractory to its effects. Mice with a CB_1 receptor deficiency in T cells were responsive to THC inhibition of EAE; whereas, mice lacking neuronal CB_1 receptor were not. These cumulative data demonstrate that THC mediates its effects through the CB_1 receptor expressed by neurons and that T-cell CB_1 receptor expression is dispensable for the regulation of EAE.

CB_2 receptor in regulating T-cell effector function in the CNS

Using a similar knockout strategy, we investigated the role of the CB_2 receptor on both nervous and immune tissue in regulating EAE disease. This was accomplished by inducing EAE in CB_2 receptor-deficient mice ($CB_2^{-/-}$) (Buckley *et al*., 2000). These experiments utilized $CB_2^{-/-}$ mice bred onto the B10.PL background (H-2^u) and encephalitogenic T cells bearing a transgene with specificity for MBP in the context of I-A^u (Dittel *et al*., 1999). When EAE was induced by the immunization with the Ac1-11 encephalitogenic peptide comprising the first 11 amino acids of MBP with the first residue acetylated, $CB_2^{-/-}$ mice were shown to be more susceptible to disease induction than wild-type littermates (Maresz *et al*., 2007). In addition, EAE disease in the $CB_2^{-/-}$ mice was more severe, and the mice failed to exhibit recovery from clinical signs as observed in wild-type mice (Maresz *et al*., 2007). When EAE was induced in $CB_2^{-/-}$ mice by the adoptive transfer of wild-type encephalitogenic T cells no change in disease parameters was observed as compared to wild-type mice (Maresz *et al*., 2007). In contrast, when EAE was induced in wild-type mice with CB_2 receptor-deficient encephalitogenic T cells, the mice exhibited disease that was statistically and significantly more severe, with a higher rate of mortality and complete lack of recovery, as compared to disease induced with wild-type T cells (Maresz *et al*., 2007). These cumulative data indicate that the cannabinoid system, via the CB_2 receptor on the autoreactive T cells, regulates the extent of the immune response in the CNS. These data also indicate that CB_2 receptor expression by CNS tissue nor by endogenous immune cells is important for the regulation of CNS autoimmunity.

We explored further the mechanism of the CB_2 receptor regulation of EAE. We found that T cells deficient in the CB_2 receptor exhibited enhanced effector functions in the CNS during EAE. We found that $CB_2^{-/-}$ T cells were more prevalent in the CNS during EAE indicative of their higher rates of proliferation and reduced level of apoptosis (Maresz *et al*., 2007). Increased numbers of T cells is consistent with more severe clinical EAE disease. These T cells were also shown to be producing increased levels of the proinflammatory cytokines IFN-γ and macrophage-colony stimulating factor (Maresz *et al*., 2007). No changes in these T-cell effector parameters were observed in the spleen, indicating that a CNS-specific regulation of T-cell effector function via the CB_2 receptor occurs during EAE, and by inference MS. The CB_2 receptor-specific regulation of T-cell proliferation and cytokine production was confirmed *in vitro* in the $CB_2^{-/-}$ encephalitogenic T cells using the CB_2 receptor agonist JWH-133 (Huffman *et al*., 1999).

Finally, we investigated the extent to which the cannabinoid system regulates T-cell effector function in the CNS. When we reduced the number of encephalitogenic T cells used to induce EAE from 1×10^6 to 0.5×10^6 cells, EAE was either prevented or was very mild following transfer of wild-type T cells. In contrast, 0.5×10^6 $CB_2^{-/-}$ T cells was sufficient to induce disease in 100% of the mice, and resulted in a chronic disease course without signs of recovery (Maresz *et al*., 2007). In B10.PL mice, 1×10^6 wild-type encephalitogenic T cells do not typically induce disease unless the recipient mice have been irradiated prior to the T-cell adoptive transfer. However, we found that the irradiation was dispensable when EAE was induced with $CB_2^{-/-}$ T cells (Maresz *et al*., 2007). This finding is particularly interesting since we have shown that irradiation reduces spinal cord 2-AG content (Maresz *et al*., 2005), which would result in reduced CB_2 receptor activation and could be part of the mechanism by which irradiation enhances the induction of EAE. Similarly, the CB_2 receptor is coupled to G proteins of the $alpha_i$ subclass, so its signalling is *B. pertussis* toxin sensitive (Munro *et al*., 1993). EAE induction by immunization is enhanced by the treatment of mice with *B. pertussis* toxin (Munoz *et al*., 1984); thus, perhaps part of the mechanism of the toxin is to reduce CB_2 receptor signalling on T cells.

Concluding remarks

The mechanism by which central cannabinoid receptors modulate immune function is not definitively known, although it is likely to be the product of a number of interacting systems that result in the inhibition of the generation of effector Th1 cells that can enter the CNS to trigger disease. To examine the role of the CB_2 receptor in the regulation of CNS autoimmunity, we utilized CB_2 receptor knockout mice on the B10.PL background so that we could utilize the MBP-TCR tg mouse to specifically study the role of the CB_2 receptor on encephalitogenic T cells. Our primary finding is that the effector functions of autoreactive T cells are strongly repressed by the CNS microenvironment through the CB_2 receptor, without the need for exogenously

added cannabinoid agonists. These effects were CNS-specific, as similar alterations in T-cell effector function were not observed in the spleen. These data extend our previous report that encephalitogenic T cells in the CNS express the CB_2 receptor during EAE (Maresz *et al.*, 2005), and strongly suggest that the presence of CB_2 receptors on T cells exerts an immunosuppressive effect when the T cells have migrated into the CNS. We also found that CB_2 receptor expression by all body tissues, including the CNS, and haematopoietic cells other than the encephalitogenic T cells is not required for the receptor's immunosuppressive function. For the CB_1 receptor, expression by neurons, but not T cells, was required for its regulation (Maresz *et al.*, 2007).

Of interest is the CNS specificity of the cannabinoid-based regulation of EAE, as demonstrated by the observation that in contrast to the profound difference in the CNS, the proliferation, apoptosis and cytokine production of $CB_2^{-/-}$ T cells in the spleen were not different from wild type. One likely explanation for this finding is that the spleen expresses lower concentrations of the endogenous agonist ligands for the CB_2 receptors than the CNS. Two arachidonate derivatives, anandamide and 2-AG are proposed to serve as endogenous activators of the CB_1 and CB_2 receptors (Klein, 2005). 2-AG has higher efficacy at the CB_2 receptor than anandamide (Sugiura *et al.*, 2004), and is present at higher concentrations than anandamide in the CNS (Patel *et al.*, 2005) and is likely more important physiologically. The amount of 2-AG in the rodent brain is estimated between 3 and 10 $nmol\,g^{-1}$ tissue while the content in spleen is 3- to 10-fold lower (Sugiura and Waku, 2000). These data suggest that CB_2-mediated inhibition would play a more important role in the regulation of T-cell function in the brain than in the spleen and are consistent with our finding that splenic T cells were unaffected by the lack of CB_2 receptors. Interestingly, total plasma levels of 2-AG are 0.012 $nmol\,ml^{-1}$ or 12 nM (Sugiura and Waku, 2000), which is significantly less than its estimated K_D for the CB_2 receptor (1 μM) (Shoemaker *et al.*, 2005) and suggests that the T-cell CB_2 receptor is not activated when the cells are circulating in the blood but become activated when the cells migrate into tissues with high 2-AG content, such as the CNS. It is our hypothesis that CB_2-mediated suppression of T-cell function is an important component of the mechanism by which the CNS is immune-protected.

These studies are theoretically in agreement with the suggestions of others that cannabinoid receptor agonists would be beneficial for the treatment of MS in humans (Pertwee, 2002). Alternative approaches would be to enhance CB_2 receptor-mediated immune-suppression by increasing endocannabinoid content in the brain (Ortega-Gutierrez *et al.*, 2005) or by upregulating CB_2 receptor expression on T cells. If cannabinoid-based strategies can reduce or control CNS inflammation this has the potential to retard the progression of MS.

Our cumulative data indicate that the CB_2 receptor plays a prominent role as an inhibitory receptor in activated encephalitogenic T cells. We suggest that T cells encounter CB_2 receptor ligand at high concentrations in the CNS microenvironment, which actively suppresses their function. Thus, we propose that an important function of the CNS cannabinoid system is to maintain an anti-inflammatory microenvironment, so that entrance of activated T cells into the CNS generated during autoimmunity and infections does not lead to tissue damage.

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Conflict of interest

The author states no conflict of interest.

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