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REVIEW

CB_2 receptors in the brain: role in central immune function

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Recently, it has been recognized that the cannabinoid receptor CB_2 may play a functionally relevant role in the central nervous system (CNS). This role is mediated primarily through microglia, a resident population of cells in the CNS that is morphologically, phenotypically, and functionally related to macrophages. These cells also express the cannabinoid receptor CB_1. The CB_1 receptor (CB1R) is constitutively expressed at low levels while the CB_2 receptor (CB2R) is expressed at higher levels and is modulated in relation to cell activation state. The relatively high levels of the CB2R correspond with microglia being in 'responsive' and 'primed' states, suggesting the existence of a 'window' of functional relevance during which activation of the CB2R modulates microglial activities. Signature activities of 'responsive' and 'primed' microglia are chemotaxis and antigen processing, respectively. The endocannabinoid 2-arachidonylglycerol has been reported to stimulate a chemotactic response from these cells through the CB2R. In contrast, we have shown *in vivo* and *in vitro* that the exogenous cannabinoids delta-9-tetrahydrocannabinol and CP55940 inhibit the chemotactic response of microglia to *Acanthamoeba culbertsoni*, an opportunistic pathogen that is the causative agent of Granulomatous Amoebic Encephalitis, through activation of the CB2R. It is postulated that these exogenous cannabinoids superimpose an inhibitory effect on pro-chemotactic endocannabinoids that are elicited in response to *Acanthamoeba*. Furthermore, the collective results suggest that the CB2R plays a critical immune functional role in the CNS.
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Keywords: *Acanthamoeba*; amoebic encephalitis; brain infection; cannabinoid receptors; CB2R; CB_2 receptor; CP55940; delta-9-tetrahydrocannabinol; microglia; THC

Abbreviations: ACEA, (*N*-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; 2-AG, 2-arachidonylglycerol; AIDS, acquired immune deficiency syndrome; CM, *A. culbertsoni*-conditioned medium; CP55940, (−)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol; HIV1, human immunodeficiency virus type 1; IL, interleukin; MS, multiple sclerosis; NO, nitric oxide; O-2137, 1-[4-(1,1-Dimethylheptyl)-2,6-dimethoxyphenyl]-3-methylcyclohexanol; SR141716A, (5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide hydrochloride; SR144528, ((1S-endo)-5-(4-Chloro-3-methylphenyl)-1-((4-methylphenyl)methyl)-*N*-(1,3,3-trimethylbicyclo(2.2.1)hept-2-yl)-1H-pyrazole-3-carboxamide; THC, delta-9-tetrahydrocannabinol

Introduction

To date, two unique cannabinoid receptors, one located primarily in the brain (CB_1 receptor (CB1R)) and the other in the immune system (CB_2 receptor (CB2R)), have been identified. The CB1R appears to be responsible for most, if not all, of the centrally mediated effects of cannabinoids (Compton *et al.*, 1993). This receptor has been well characterized using potent radiolabelled cannabinoid agonists that bind in the high picomolar to low nanomolar range. The CB1R is concentrated in areas of the brain that control movement, coordination, sensory perception, learning and memory, reward and emotions, hormonal function, and body temperature. This localization within the brain is consistent with the pharmacological profile of cannabinoids in that high densities are found in cerebellum, hippocampus and cerebral cortex, whereas low quantities are present in the brain stem (Herkenham *et al.*, 1991; Thomas *et al.*, 1992). The CB1R is G-protein coupled as evidenced by inhibition of adenylyl cyclase (Howlett *et al.*, 1986), inhibition of N-type calcium channels (Mackie and Hille, 1992) and increased binding of non-hydrolyzable GTPγS in the presence of cannabinoids (Sim *et al.*, 1996). In addition, the cloning of this cannabinoid receptor resulted from screening of a

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G-protein-coupled receptor library (Matsuda *et al*., 1990). It is not surprising that a cannabinoid receptor was found also in the immune system. Transcripts (that is, mRNAs) for the CB1R and CB2R have been found in spleen and tonsils (Munro *et al*., 1993; Galiègue *et al*., 1995) and other immune tissues (Munro *et al*., 1993; Bouaboula *et al*., 1996). However, in all cases reported to date, levels of message for the CB2R in immune cells exceed those for the CB1R. The distribution pattern of levels of CB2R mRNA displays major variation in human blood cell populations with a rank order of B lymphocytes > natural killer cells ≫ monocytes > polymorphonuclear neutrophils > CD8 lymphocytes > CD4 lymphocytes (Galiègue *et al*., 1995). A rank order for levels of CB2R transcripts similar to that for primary human cell types has been recorded for human cell lines belonging to the myeloid, monocytic and lymphoid lineages (Galiègue *et al*., 1995). A general similar pattern has been reported for mouse and rat (Daaka *et al*., 1995). In addition, cognate protein has been identified in rat lymph nodes, Peyer's Patches and spleen (Lynn and Herkenham, 1994). The presence of the CB2R primarily within immune cells suggests a role for this receptor in the activities attributed to these cells. However, CB1R also may be involved in cannabinoid-mediated modulation of select immune functions (Stefano *et al*., 1996; Sinha *et al*., 1998; Waksman *et al*., 1999). Recent studies support the presence of yet uncloned cannabinoid receptors, principally on the basis of pharmacological evidence of cannabinoid action in CB1R- and CB2R-deficient mice (Jaggar *et al*., 1998; Di Marzo *et al*., 2000; Breivogel *et al*., 2001). The first of these has been reported to couple to Gi/o proteins (Hajos and Freund, 2002; Offertaler *et al*., 2003), to be activated by micromolar concentrations of abnormal-cannabidiol (CBD), a synthetic analogue of CBD, and to be potentiated through cGMP and protein kinase G (Begg *et al*., 2003). A second putative non-CB_1, non-CB_2 receptor has been referred to as the 'palmitoylethanolamide receptor' because palmitoylethanolamide, an analogue of anandamide that does not bind the CB2R, causes a reduction of pain associated with inflammatory response (Calignano *et al*., 1998; Jarai *et al*., 1999) that is blocked by the CB2R antagonist ((1S-endo)-5-(4-Chloro-3-methylphenyl)-1-((4-methylphenyl)methyl)-*N*-(1,3,3-trimethylbicyclo(2.2.1) hept-2-yl)-1H-pyrazole-3-carboxamide (SR144528). The nuclear peroxisome proliferator-activated receptor-alpha (PPAR-α) has been reported as the mediator of the anti-inflammatory actions of this lipid amide (Lo Verme *et al*., 2005a, b). Finally, Breivogel *et al*. (2001) reported the existence of a 'WIN receptor' based on experiments of GTPγS binding in brain membranes from CB1R knockout mice. In their studies, anandamide and the aminoalkylindole WIN55212-2 stimulated GTPγS binding. However, the 'novel' receptor was found to exhibit a pharmacology distinctive from that of the CB1R and the CB2R. It was not activated by delta-9-tetrahydrocannabinol (THC) and the classical cannabinoid agonists (−)-cis-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cyclohexanol (CP55940) and HU210 and was blocked weakly by the CB1R antagonist (5-(4-Chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(1-piperidyl)pyrazole-3-carboxamide hydrochloride (SR141716A).

Microglia are CB2R-expressing resident macrophages in the CNS

There is increasing evidence that the CB2R, in addition to its linkage to immune cell activities at peripheral sites, plays a functionally relevant role in immunity in the CNS. This role appears to be exerted primarily through microglia, a resident population of cells in the brain, spinal cord and retina that is morphologically, phenotypically and functionally related to macrophages (Streit *et al*., 1988; Dickson *et al*., 1991; Ling and Wong, 1993; Gehrmann *et al*., 1995; Gehrmann, 1996; Aloisi *et al*., 1998; Stoll and Jander, 1999). The function of 'quiescent' microglia in normal brain is not well understood, but in pathological conditions, they play an active role as immunoeffector/accessory cells. Microglia migrate and proliferate during and after injury and inflammation (Leong and Ling, 1992; Kreutzberg, 1995, 1996; Benveniste, 1997a). Once activated, they produce various cytokines including interleukin (IL)-1, IL-6 and tumour necrosis factor-α (Giulian *et al*., 1986; Reid *et al*., 1993; Benveniste, 1997b), and express major histocompatability complex (MHC) class I and II antigens and the complement receptor, CD11/CD18 complex. Microglia, also, are phagocytic and, upon activation, can process antigens and exert cytolytic functions. Paradoxically, these cells not only play a role in host defense and tissue repair in the CNS (Streit *et al*., 1988; Perry, 1990), but also have been implicated in nervous system disorders, such as Multiple Sclerosis (MS) (Matsumoto *et al*., 1992), Alzheimer's disease (Rogers *et al*., 1988), Parkinson's disease (McGeer *et al*., 1988) and acquired immune deficiency syndrome (AIDS) dementia (Dickson *et al*., 1991; Merrill and Chen, 1991; Spencer and Price, 1992). Neural histological features of AIDS dementia complex include diffuse leukoencephalopathy of the white matter, which is accompanied by severe loss of myelin and sparing of fibres (Kleihues *et al*., 1985). Discrete areas of demyelination with hypertrophied astrocytes, which also contain microglia, blood-derived macrophages and multinucleated giant cells, are observed. The multinucleated giant cells have been reported to constitute syncytia of macrophages or microglia, which are productively infected with the human immunodeficiency virus type 1 (HIV1) and are a histopathological hallmark of subacute encephalitis in HIV1-infected brains (Koenig *et al*., 1986; Michaels *et al*., 1988) and spinal cords (Eilbott *et al*., 1989; Maier *et al*., 1989). Thus, it has been proposed that in AIDS dementia, macrophages and microglia are the predominant cell types, which are infected and produce HIV1 (Koyanagi *et al*., 1987; Kure *et al*., 1990). Manifestations of AIDS dementia indicate that direct infection by the HIV probably does not account for CNS dysfunction. Tumours, such as extranodal primary malignant B cell lymphomas (Snider *et al*., 1983; Gray *et al*., 1988) and cerebral deposits of Kaposi's sarcoma resulting from metastases of lung foci (Gorin *et al*., 1985; Gray *et al*., 1988), have been shown to occur either before or at the time of patient seroconversion and before onset of immune suppression (Perry, 1990). Cerebrovascular complications, such as vasculitis and haemorrhages within cerebral tumour areas or in areas of demyelination, also have been shown to occur before onset of immune suppression. These observations

suggest that inflammatory cells and their products are actively involved in these histopathological events.

The CB2R is differentially expressed by microglia

Microglia, consistent with other immune cells, undergo maturation, differentiation and activation, processes, which are characterized by differential gene expression and acquisition of correlative distinctive functional capabilities (Adams and Hamilton, 1984; Hamilton *et al*., 1986; Hamilton and Adams, 1987). Peritoneal macrophages, macrophage-like cells and microglia can be driven sequentially in response to multiple signals from 'resting', to 'responsive', 'primed' and 'fully' activated states, a process that mimics events *in vivo* (Figure 1). Using this *in vitro* model, it has been shown that levels of CB2R mRNA and protein are modulated differentially in relation to cell activation state (Carlisle and Cabral, 2002). The CB2R is not detected in 'resting' cells, is present at high levels in 'responsive' and 'primed' cells, and is identified at greatly diminished levels in 'fully' activated cells. In contrast, the CB1R is present in microglia at relatively low levels and is expressed constitutively in relation to cell activation state. These observations suggest that the CB2R is expressed 'on demand' and that the modulation of CB2R levels is a feature common to cells of macrophage lineage as they participate in the inflammatory response. Furthermore, the relatively high levels of CB2R recorded for microglia when in 'responsive' and 'primed' states suggest that these cells exhibit a functionally relevant 'window' during which they are most susceptible to the action of cannabinoids. Finally, since the kinetics of CB1R and CB2R expression by microglia are distinctive, activation of the two receptors by endogenous and/or exogenous cannabinoids may result in disparate functional outcomes.

Consistent with observations suggestive of a functionally relevant 'window' for CB2R expression, a number of studies have indicated that activities attributed to 'fully' activated microglia are not susceptible to cannabinoid-mediated action that is linked to the CB2R. Waksman *et al*. (1999) reported that the production of inducible nitric oxide (iNO), a potent inflammatory mediator that is released from microglia and macrophage-like cells upon their 'full' activation, was inhibited by cannabinoids in a mode that was linked, at least in part, to the CB1R. The cannabinoid receptor high-affinity binding enantiomer CP55940 exerted a concentration-dependent (0.1–8 μM) inhibition of nitric oxide (NO) release from neonatal rat microglia subjected to activation with interferon-γ in concert with bacterial lipopolysaccharide, which far exceeds the binding and agonist activity at either of the CB receptors. In contrast, a minimal inhibitory effect on iNO production was exerted by the lower affinity binding paired enantiomer CP56667. These results implicated a cannabinoid receptor as linked functionally to the inhibition of iNO production, since the binding affinity of the paired enantiomers has been shown to correlate with bioactivity *in vivo* and *in vitro* (Compton *et al*., 1993; Felder *et al*., 1995), and enantiomeric selectivity is a characteristic feature of receptor-mediated cellular activity. To confirm the NO release data, the effect of the paired cannabinoid enantiomers on the activity of nicotinamide adenine dinuleotide phosphate-diaphorase was determined since its proportional intracellular activity correlates with that of NO synthase in neuronal cells (Hope *et al*., 1991). Consistent with the NO data, a differential inhibition of nicotinamide adenine dinuleotide phosphate-diaphorase activity in rat microglia was effected by CP55940 versus its paired enantiomer CP56667. Pretreatment of microglia with the $G_{\alpha i}/G_{\alpha o}$ protein inactivator pertussis toxin, cyclic AMP reconstitution with the cell-permeable analogue dibutyryl-cAMP or treatment with the $G_{\alpha s}$ activator cholera toxin, resulted in reversal of the CP55940-mediated inhibition of NO release. Finally, functional studies performed with the CB1R-selective antagonist SR141716A (Rinaldi-Carmona *et al*., 1994) resulted in a reversal of the CP55940-mediated inhibition of iNO production. Collectively, these immune pharmacological results supported a functional linkage between the CB1R and cannabinoid-mediated inhibition of iNO production by neonatal rat microglia. Puffenbarger *et al*. (2000) extended these studies on the effects of cannabinoids on 'fully' activated microglia and indicated that the inhibition of the inducible expression of pro-inflammatory cytokines was exerted through a non-CB_1, non-CB_2 receptor process. Exposure of neonatal rat cortical microglia to THC resulted in reduced amounts of lipopolysaccharide-induced mRNAs for IL-1α, IL-1β, IL-6 and tumour necrosis factor-α. Of

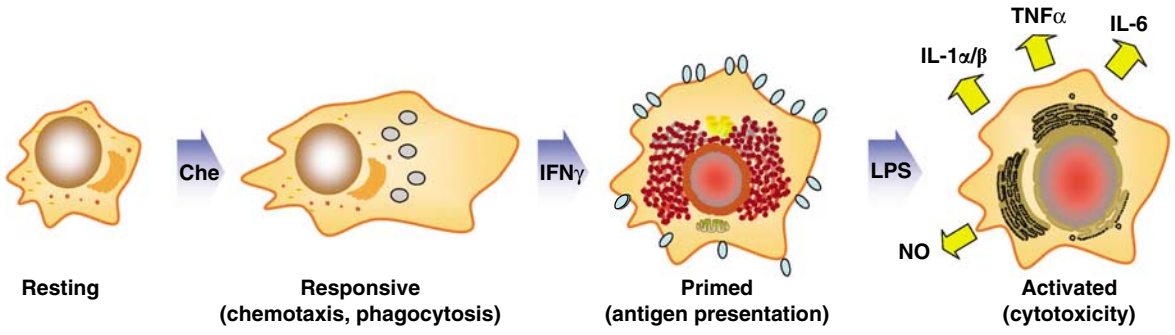

Figure 1 *In vitro* model of macrophage/microglial multi-step activation. Peritoneal macrophages, macrophage-like cells and microglia can be driven sequentially in response to multiple signals from 'resting' to 'responsive', 'primed' and 'fully' activated states, a process that mimics events *in vivo*. Each of these states is characterized by differential gene expression and acquisition of correlative distinctive functional capabilities (modified from: Adams and Hamilton, 1984; Hamilton *et al*., 1986; Hamilton and Adams, 1987).

these cytokine mRNAs, the response of that for IL-6 was exquisitely sensitive to THC treatment. Similarly, exposure of microglia cells to the putative endogenous cannabinoid (endocannabinoid) anandamide before lipopolysaccharide treatment resulted in a decrease in cytokine mRNA levels, but not to the same extent as that caused by THC; however, when methanandamide, the non-hydrolyzable analogue of anandamide, was tested, its ability to inhibit cytokine mRNA expression was comparable to that of THC. Exposure of microglia to either of the paired enantiomers CP55940 or CP56667 resulted in similar inhibition of lipopolysaccharide-induced cytokine mRNA expression. A comparable inhibitory outcome was obtained when the paired enantiomers levonantradol and dextronantradol were employed. Neither the CB1R-selective antagonist SR141716A nor the CB2R-selective antagonist SR144528 (Rinaldi-Carmona *et al*., 1998) was able to reverse the inhibition of cytokine mRNA expression by levonantradol. Collectively, the absence of stereoselectivity in the inhibition of cytokine mRNA expression and the inability of either the CB1R or CB2R antagonists to block the inhibitory effect of levonantradol demonstrated that while cannabinoids had the capacity to modulate levels of pro-inflammatory cytokine mRNAs in neonatal rat microglia, the inhibition of cytokine mRNA expression is apparently mediated neither through the CB1R nor through the CB2R.

Chemotaxis as a signature activity of 'responsive' microglia

Signature activities of macrophage-like cells when in 'responsive' and 'primed' states of activation, states associated with the early inflammatory response, are chemotaxis and antigen presentation, respectively. Chemotaxis is the ability of cells to migrate in response to a stimulus and is distinctive from stimulus-independent random cellular motion (Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Chemotaxis differs from chemokinesis, a stimulus-dependent cellular motility whereby cells exhibit enhanced random motion that is dependent on a chemo-stimulant (Becker, 1977; Keller *et al*., 1978). Thus, chemotaxis is a process in which cell motility is directed towards a concentration gradient of chemo-stimulant (Harris, 1953, 1954; Jin and Hereld, 2006; Kehrl, 2006). In this chemotactic process, macrophage-like cell interaction with chemoattractants not only initiates a rapid and directed movement but also is associated with a complex array of cellular events that includes changes in ion fluxes, alterations in integrin avidity, production of superoxide anions and secretion of lysosomal enzymes (Murdoch and Finn, 2000). 'Classical' chemoattractants include bacterial-derived N-formyl peptides, the complement fragment peptides C5a and C3a, and lipids such as leukotriene B_4 and platelet-activating factor (Schiffmann *et al*., 1975; Goldman and Goetzl, 1982; Hanahan, 1986; Gerard and Gerard, 1994). Chemokines represent a second group of chemoattractants. These 8- to 17-kDa molecular mass range cytokines are selective for leucocytes *in vitro* and elicit accumulation of inflammatory cells *in vivo* (Baggiolini *et al*., 1994, 1997; Kim, 2004; Le *et al*., 2004). As in the case for cannabinoid receptors, the specific effects of chemokines on target cells are mediated by G-protein-coupled receptors (Murdoch and Finn, 2000; Charo and Ransohoff, 2006). Ligation of chemokines with their cognate receptors initiates a series of signal transductional events that results in regulation of leucocyte trafficking in inflammation, tissue injury, tumour development and host response to infection (Charo and Ransohoff, 2006).

Several studies have documented that cannabinoids affect the migratory activities of macrophages and macrophage-like cells. Stefano *et al*. (1998) reported that acute exposure to anandamide resulted in transformation of macrophages from an amoeboid and motile state to that of a rounded and non-motile conformation. These investigators proposed that the transforming events were linked to the CB1R since the CB1R-selective antagonist SR141716A blocked the transformation. Sacerdote *et al*. (2000) demonstrated that *in vivo* and *in vitro* treatment of rat peritoneal macrophages with CP55940, a full agonist at both CB1R and CB2R, resulted in decreased migration *in vitro* to the peptide formal-methionyl-leucine-phenylalanine. It was indicated, however, that while both the CB1R and CB2R were involved in this process, the cannabinoid-mediated effect was linked primarily to the CB2R. The chemotactic response of mouse macrophages to formal-methionyl-leucine-phenylalanine also has been shown to be decreased by CBD (Sacerdote *et al*., 2005), a cannabinoid that binds weakly to the CB2R. The CB2R antagonist SR144528 prevented this decrease, suggesting a functional linkage to the cognate receptor. Walter *et al*. (2003) found that the endocannabinoid 2-arachidonylglycerol (2-AG) triggered migration of microglia and that the CB2R was involved in this effect. Franklin and Stella (2003) demonstrated that arachidonylcyclopropylamide, an agonist selective for the CB1R, induced a dose-dependent increase in migration of mouse microglial cell line BV-2. However, while the arachidonylcyclopropylamide-induced response was blocked by pertussis toxin pretreatment consistent with the involvement of a $G_{i/o}$-protein-coupled receptor, the CB1R antagonist SR141716A did not prevent the arachidonylcyclopropylamide-mediated migration. In contrast, two antagonists of the CB2R (SR144528 and cannabinol) as well as two antagonists of 'abnormal-CBD-sensitive' receptors (O-1918 and CBD) prevented the response. Based on these collective results, Franklin and Stella (2003) suggested that CB2Rs and 'abnormal-CBD-sensitive' receptors regulated the migration of microglial-like cells. Stella and co-workers extended these studies and showed that P2X7 ionotropic receptors played a key role in controlling the production of 2-AG by microglia (Witting *et al*., 2004). Recently, Raborn *et al*. (in press) demonstrated that THC and CP55940 mediated inhibition of mouse peritoneal macrophage chemotaxis to the chemokine RANTES/CCL5 and that this event was linked to the CB2R. In these studies, the CB2R-selective ligand O-2137 (1-[4-(1,1-Dimethylheptyl)-2,6-dimethoxyphenyl]-3-methylcyclohexanol) exerted a robust inhibition of chemotaxis, while the CB1R-selective ligand (*N*-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) had a minimal effect. The CP55940-mediated inhibition was reversed by the CB2R-selective antagonist SR144528 but not by the CB1R-selective

antagonist SR141716A. In addition, THC treatment had a minimal effect on the chemotactic response of peritoneal macrophages from CB2R knockout mice. Collectively, the preponderance of data that has been obtained indicates that cannabinoids act through the CB2R to alter macrophage migration with exogenous cannabinoids, such as THC exerting inhibitory effects and endocannabinoids such as 2-AG eliciting an opposite stimulatory effect. Furthermore, the studies of Raborn *et al*. (in press) indicate that THC and CP55940 can transdeactivate migratory responsiveness to the chemokine RANTES/CCL5, suggesting that signaling through the CB2R leads to 'cross-talk' with chemokine receptors. Thus, the CB2R may be a constituent element of a network of G-protein-coupled receptor signal transductional systems, inclusive of chemokine receptors, that act coordinately to modulate macrophage migration.

It has been shown that the CB2R also is involved in cannabinoid-mediated inhibition of processing of select antigens by macrophages. Antigen processing and presentation constitute a complex set of events. The activation of helper/inducer CD4+ T cells requires their physical contact with another cell type called an antigen-presenting cell, which is partially due to the specificity of the T-cell antigen receptor, a plasma membrane protein. Unlike antibodies, the T-cell receptor does not bind to antigen alone, but rather the receptor recognizes a complex composed of the antigen and MHC class II molecules that are expressed at the surface of antigen-presenting cells (Schwartz, 1985). CD4+ T cells are usually specific for protein antigens, not carbohydrates or lipids. The form of the antigen in the complex with MHC class II molecules is a peptide fragment, not the native antigen (Guillet *et al*., 1987). In addition, these antigens usually are not synthesized by the antigen-presenting cells but are exogenous proteins (Germain, 1986). This process is distinctive from that which occurs for cytotoxic CD8-positive T cells, which recognize peptide antigens that can be derived from endogenous proteins in the context of MHC class I molecules on antigen-presenting or infected target cells. Important functions of antigen-presenting cells are internalization of antigen, proteolytic cleavage of antigen into peptides, formation of the peptide-class molecule complex and expression of the complex at the cell surface (Germain, 1986). These series of events comprise antigen processing. Once the complex is expressed at the cell surface, the process is referred to as antigen presentation. Several steps in antigen processing have been characterized. Soluble protein antigens are internalized by antigen-presenting cells through endocytosis, which is either nonspecific or receptor mediated (Unanue and Allen, 1987). Of the various types of antigen-presenting cells, only macrophages internalize particulate antigens by phagocytosis (Unanue and Allen, 1987). Other phagocytic cells lack MHC class II molecules and do not function as antigen-presenting cells. Regardless of the mode of antigen uptake, intracellular antigen enters an acidic organelle, where antigen processing probably occurs. Treatment of antigen-presenting cells with acidotropic agents, such as chloroquine, ammonium chloride and monensin (McCoy and Schwartz, 1988), that neutralize intracellular acidic pH eliminate antigen processing (Seglen, 1983). Cathepsins, acid proteases, within the acidic organelles are thought to cleave the antigens. Various protease inhibitors prevent antigen processing by antigen-presenting cells, depending on the antigen (Van der Drift *et al*., 1990). Thus, the interference by cannabinoids such as THC with any one of these steps could result in impaired antigen processing.

McCoy *et al*. (1999) examined the effect of THC on the processing of intact lysozyme by macrophages. It was demonstrated that THC impaired the ability of a macrophage hybridoma to function as an antigen-presenting cell based on its ability to secrete IL-2 upon stimulation of a soluble protein antigen-specific helper T-cell hybridoma. THC exposure significantly reduced the T-cell response to the native form of the antigen after pretreatment of the macrophages with nanomolar drug concentrations. However, THC did not affect IL-2 production when the macrophages presented a synthetic peptide of the antigen to the T cells, suggesting that the drug interfered with antigen processing, not peptide presentation. The cannabinoid inhibition of the T-cell response to native lysozyme was stereoselective consistent with the involvement of a cannabinoid receptor. That is, the bioactive CP55940 diminished T-cell activation, whereas the inactive stereoisomer CP56667 did not. The macrophage hybridoma expressed mRNA for the CB2R but not for the CB1R, whereas the T cells expressed an extremely low level of mRNA for the CB2R. The CB1R-selective antagonist SR141716A did not reverse the suppression caused by THC, demonstrating that the CB1R was not responsible for the drug's inhibitory effect. In contrast, the CB2R-selective antagonist SR144528 completely blocked the THC suppression of the T-cell response. These collective results implicated the macrophages as the target of cannabinoid inhibition of antigen processing in a mode that was linked functionally to the CB2R.

Cannabinoids modulate chemotaxis of microglia

The cumulative results of immune pharmacological studies implicate the CB2R as playing a relevant functional role in the early inflammatory process by macrophages and macrophage-like cells, namely chemotaxis and antigen-processing functional attributes of these cell types when in 'responsive' and 'primed' states. Since microglia constitute a resident population of macrophages in the brain, exhibit phenotypic and functional properties of macrophages, and express the CB2R at maximal levels when in 'responsive' and 'primed' states, a 'window' of functional relevance for the CB2R comparable to that for macrophages at peripheral sites may be operative. That is, antigen processing and/or chemotaxis by these cells may be particularly susceptible to cannabinoids in a mode linked to activation of the CB2R. To address this possibility, we have employed *in vivo* and *in vitro* rodent models of Granulomatous Amoebic Encephalitis, a chronic progressive infection of the CNS that is caused by *Acanthamoeba culbertsoni* (*A. culbertsoni*). *A. culbertsoni* is a free-living amoeba that can infect both immune-competent and immune-suppressed individuals (Martinez, 1993; Marciano-Cabral and Cabral, 2003) and has two morphologic forms as part of its life cycle, a trophozoite and a dormant cyst. The

trophozoite is the invasive form of this protozoan. The portal of entry of *A. culbertsoni* may be the nasal passages, the lower respiratory tract, open wounds or ulcers in the skin, or any mucosal or serosal surface (Martinez, 1993). For brain infections, trophozoites are thought to enter either by the olfactory neuroepithelial route following the nerve pathway from the nasal mucosa to the olfactory bulb or by haematogenous spread from a primary site of infection, such as a cutaneous lesion (Martinez, 1993; Marciano-Cabral and Cabral, 2003). Once in the brain, amoebae may be destroyed by immune effector cells, such as microglia. Alternatively, amoebae may cause a subacute infection that is characterized by encystment and establishment of a chronic state associated with granuloma formation. The formation of granulomas around amoebae is thought to play a role in limiting dissemination. Although the incubation period for *Acanthamoeba* spp. infections is unknown, several weeks may be necessary to establish clinical signs. The relatively prolonged course of neuropathological events associated with Granulomatous Amoebic Encephalitis in rodent animal models affords the opportunity to investigate the outcome of infection with sublethal levels of *Acanthamoeba* as well as the characterization of the cellular elements within the brain whose functional activities against this protozoan may be affected by cannabinoids. Utilizing a $(B_6C_3)F_1$ mouse model of Granulomatous Amoebic Encephalitis in which trophozoites were introduced through the intranasal route to mimic a natural route of infection in humans, we demonstrated that THC exacerbated *Acanthamoeba*-induced neuropathogenesis. Mice treated with THC exhibited higher mortalities from infection with *Acanthamoeba* as compared to similarly infected vehicle control mice (Marciano-Cabral *et al*., 2001). Serial frozen sections of brain from vehicle-treated infected mice processed for immunofluorescence for colocalization of amoebae and macrophage-like cells utilizing hyperimmune rabbit polyclonal *anti-Acanthamoeba* (Marciano-Cabral *et al*., 2000) and rat monoclonal anti-Mac-1 (anti-CD11b/CD18) antibody were found to contain few amoebae (Cabral and Marciano-Cabral, 2004). In contrast, numerous *Acanthamoeba* were detected in brain sections from infected animals treated with THC. Staining of paired serial sections with anti-Mac-1 antibody demonstrated that Mac-1+ cells in vehicle-treated animals were abundant in focal areas of infected brain tissue. However, these focal areas contained few amoebae. In contrast, foci in brain tissue from infected, THC-treated mice were replete with amoebae but contained few Mac-1+ cells. Comparable results were obtained when paraffinized brain sections were subjected to haematoxylin and eosin staining (Figure 2, unpublished data). For vehicle-treated mice, numerous foci of individual amoebic trophozoites surrounded by clusters of cells that resembled microglia morphologically were observed. Assessment of replicate sections using isolectin B_4, a marker for microglia, indicated that cells clustering around amoebae were predominantly microglia. In contrast, for THC-treated mice, individual amoebic trophozoites were dispersed in the olfactory lobe and frontal areas of the brain in the absence of immune cell aggregates. The paucity of Mac-1+ cells at focal sites of *Acanthamoeba* infection in the brain of mice treated with THC suggests that these immune cells either do not migrate to infected areas or are selectively targeted by the *Acanthamoeba* and destroyed.

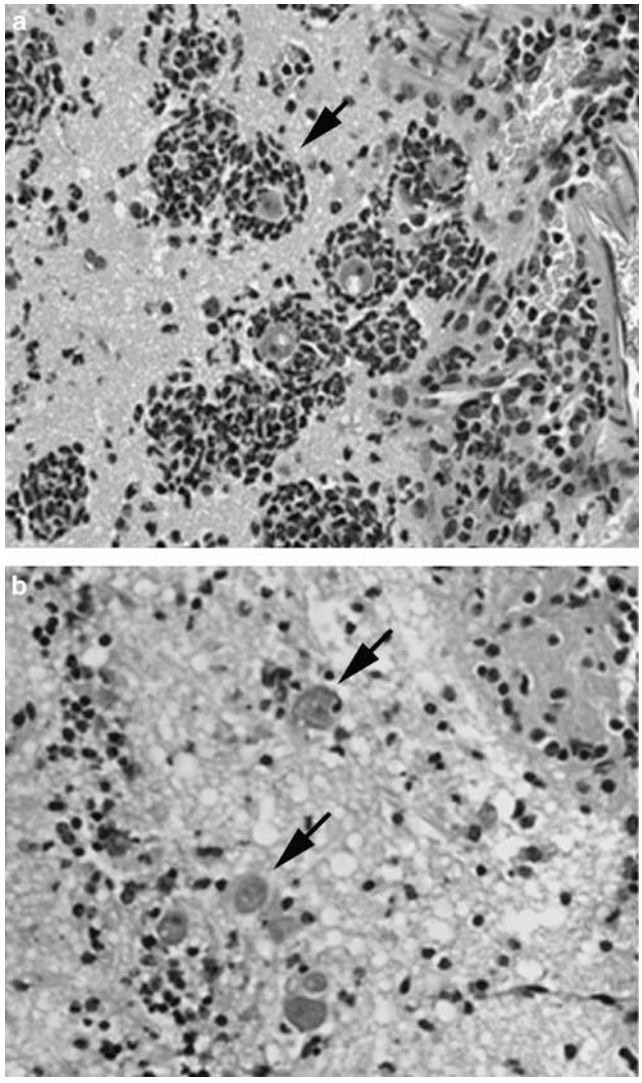

Figure 2 THC downregulates accumulation of macrophage-like cells at focal sites of *Acanthamoeba* in mouse brain. $(B_6C_3)F_1$ mice were treated once intraperitoneally with THC ($25\,mg\,kg^{-1}$) or vehicle (ethanol:emulphor:saline, 1:1:18), inoculated intranasally with 3 LD_{50} of *A. culbertsoni*, killed and the brains were removed. Paraffin sections were stained with haematoxylin and eosin. (**a**) Section from vehicle-treated mouse depicting accumulation of macrophage-like cells around *Acanthamoeba* (arrow). (**b**) Section from THC-treated mouse depicting *Acanthamoeba* in the brain in the absence of macrophage-like cell accumulation (arrows). THC, delta-9-tetrahydrocannabinol.

To determine whether THC exerted a direct effect on microglia, and to assess for a role of the CB2R in this process, *in vivo* studies were complemented with those *in vitro*. *Acanthamoeba* were maintained in culture for 24 h to generate an *Acanthamoeba*-conditioned medium (CM) that harbours proteases and other factors released from amoebae that serve as chemotactic stimuli for attracting microglia. THC treatment of neonatal rat cerebral cortex microglia *in vitro* resulted in a significant inhibition of the migratory response to CM (Figure 3, unpublished data). Experiments

performed with THC were replicated using CP55940, the full agonist at the CB1R and CB2R. Again, treatment of microglia with CP55940 resulted in a significant concentration-related decrease in migration in response to CM. The concentration-related inhibitory effect of THC and CP55940 on the migratory response of neonatal rat cerebral cortex microglia to CM implicated a role for a cannabinoid receptor. Thus, to obtain insight as to the cannabinoid receptor linked to the inhibitory effect, microglia were treated with compounds exhibiting selective high affinity binding to the CB1R or the CB2R antecedent to assessment of the migratory response (Figure 4, unpublished data). Treatment of microglia with the highly selective CB2R ligand O-2137 resulted in a profound and significant inhibition in the migratory response to CM. In contrast, the CB1R-specific ligand ACEA exerted a minimal inhibitory effect on the microglial migratory response to CM. To confirm the data indicating that activation of the CB2R with a cannabinoid receptor selective ligand exerted a major inhibitory effect on the migratory response to CM, cannabinoid receptor agonist–antagonist experiments were performed (Figure 5, unpublished data). Treatment of microglia with the CB1R antagonist SR141716A did not block the inhibitory effect of CP55940. In contrast, treatment of microglia with the CB2R-specific antagonist SR144528 resulted in a reversal of the inhibitory effect of CP55940 indicating that the cannabinoid-mediated inhibition of the CM-stimulated microglial response was linked, at least in part, to the CB2R.

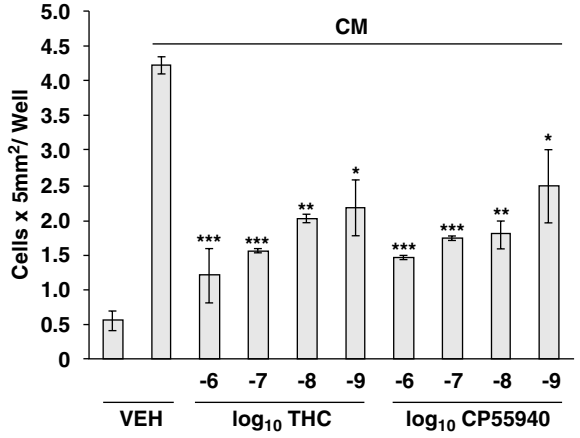

Figure 3 THC and CP55940 inhibit chemotaxis of microglia. Microglia were isolated from neonatal Sprague–Dawley rats and purified as described (Waksman *et al*., 1999), treated (3 h) with cannabinoid or vehicle (0.01% ethanol) and assessed (2 h) for migration against CM. The CB1R/CB2R partial agonist THC has a $K_i = 46$ nM at the CB2R, while the potent full agonist CP55940 has a $K_i = 0.9$ nM at the CB2R. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. $n = 3$/group. CB1R, CB_1 receptor; CB2R, CB_2 receptor; CM, *Acanthamoeba*-conditioned medium; THC, delta-9-tetrahydrocannabinol.

The mode by which THC and other exogenous cannabinoids such as CP55940 inhibit the chemotactic response of microglia to *Acanthamoeba* remains to be defined. However, it is known that *Acanthamoeba* produce proteases, phospholipases and other factors (Marciano-Cabral and Cabral, 2003) that may act on phospholipids in microglial membranes, generating cleavage products (Cabral, 2005). It is postulated that bioactive lipid mediators thus generated include the endocannabinoid 2-AG that serves to drive chemotaxis by autocrine and/or paracrine activation of the CB2R. The exogenous cannabinoid THC may inhibit this chemotactic response by superimposing a signal transductional activation of the CB2R. That is, THC could inhibit the synthesis and/or release of 2-AG or, alternatively, by virtue of its relative long half-life as compared to that of 2-AG, preclude this endocannabinoid from ligating to the CB2R. A proposed model of the role of the CB2R in modulation of the microglial chemotactic response to *Acanthamoeba* is shown in Figure 6.

Conclusion

The CNS is a complex arena that consists of a diverse group of cell types, including neurons, oligodendrocytes, microglia and astrocytes. While astrocytes are the predominant cell type of the CNS, microglia are the resident macrophages of the brain and provide the first line of defense against injury,

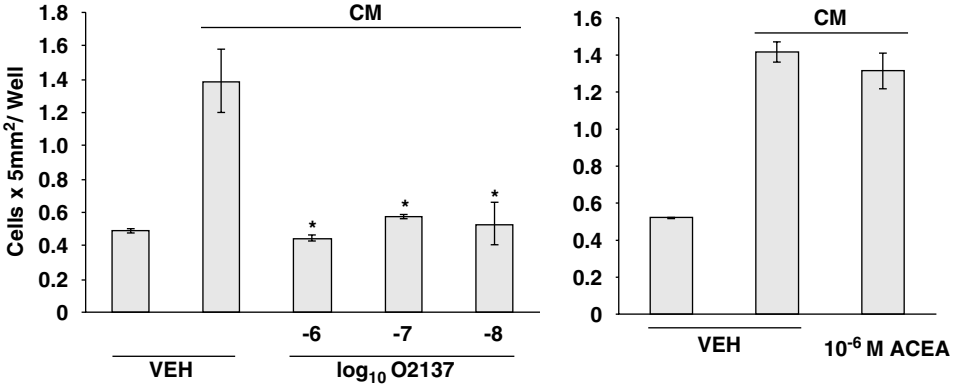

Figure 4 The CB2R, but not the CB1R, agonist inhibits chemotaxis of microglia. Microglia were treated (3 h) with cannabinoid or vehicle (0.01% ethanol) and assessed (2 h) for migration against CM. O2137: CB1R $K_i = 2700$ nM, CB2R $K_i = 11$ nM; ACEA CB1R $K_i = 1.4$ nM, >1400-fold selectivity over the CB2R. $^*P<0.05$. $n = 3$ per group. ACEA, (*N*-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide; CB1R, CB_1 receptor; CB2R, CB_2 receptor; CM, amoeba-conditioned medium.

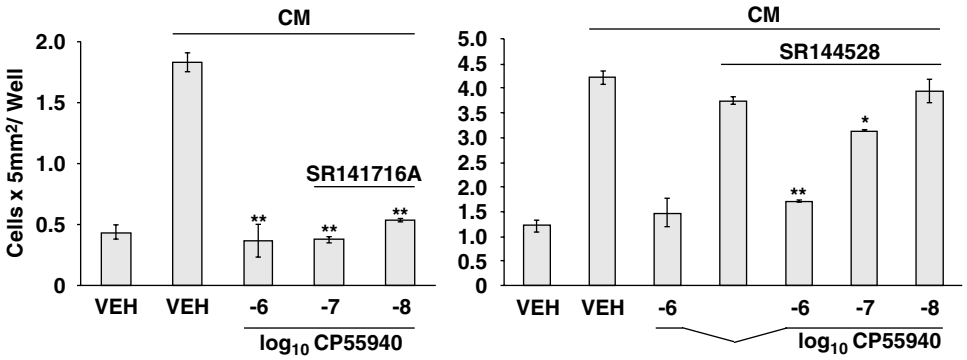

Figure 5 The CB2R antagonist reverses CP55940-mediated inhibition of chemotaxis of microglia. Microglia were treated (1 h) with antagonist (10^{-6} M) or vehicle (VEH), treated (30 min) with CP55940 or vehicle and assessed (2 h) for migration against CM. *$P<0.05$, **$P<0.01$. $n=3$ per group. CB2R, CB_2 receptor; CM, amoeba-conditioned medium.

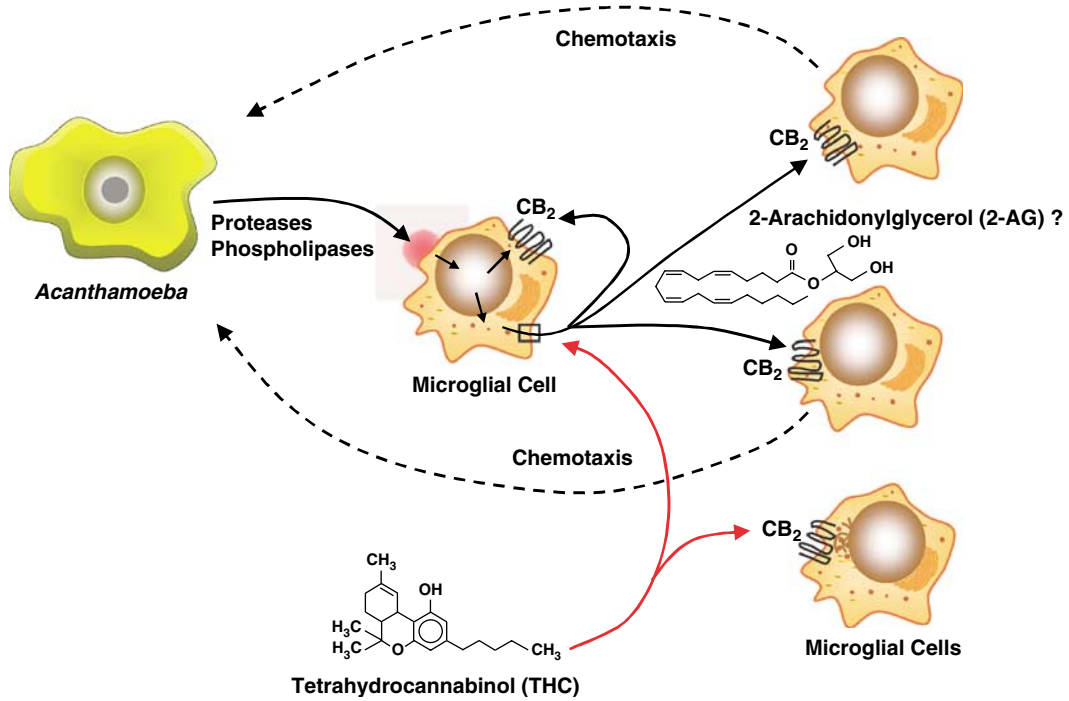

Figure 6 Model of role of CB2R in modulation of microglial chemotactic response to *Acanthamoeba*. *Acanthamoeba* elicit proteases, phospholipases and other factors that serve to generate cleavage products of phospholipids in microglial membranes through the action of phospholipases. It is postulated that bioactive lipid mediators thus generated include the endocannabinoid 2-AG that serves to drive chemotaxis of microglia by autocrine and/or paracrine activation of the CB2R. The exogenous cannabinoid THC may inhibit this chemotactic response by superimposing its effect on 2-AG by inhibiting its synthesis and/or release or by exerting a relatively long-lasting ligation to the CB2R. 2-AG, 2-arachidonylglycerol; CB2R, CB_2 receptor; THC, delta-9-tetrahydrocannabinol.

assault and/or infection. These myeloid lineage cells also play an important role in remodelling and regeneration of the CNS. The combined cellular functions of both astrocytes and microglia form the innate immune system of the CNS. Although the CNS is a highly sophisticated network of checks and balances, it possesses a vulnerability to a multitude of neurodegenerative and neuroinflammatory processes. Some of these include Alzheimer's disease, Parkinson's Disease, MS, amyotrophic lateral sclerosis and HIV-associated dementia. The pathological hallmark of these diseases is chronic inflammation induced by persistent cell activation and elicitation of proinflammatory mediators (that is, NO, cytokines and chemokines). Studies have shown that microglia and microglia-derived cells are the major cell types responsible for this neuroinflammation. For example, during the first stages of brain inflammation, microglia-derived macrophages play a key role, and their presence is the consequential step in the neurodegeneration that follows (Ashton *et al.*, 2007). Studies performed using immunohistochemistry have demonstrated that activated microglia are detected in senile plaques of Alzheimer's disease patients (Ramirez *et al.*, 2005). In addition, activated microglia, depicted by a change from a ramified morphology to an amoeboid morphology, have been detected in the spinal cords of MS and amyotrophic lateral sclerosis patients immediately following death (Yiangou *et al.*, 2006).

During activation, microglia upregulate an array of cell-surface receptors that may be critical in microglial regeneration and/or degeneration of the CNS. Included among these are immunoglobulin (Ig) superfamily receptors, complement receptors, toll-like receptors, cytokine/chemokine receptors, opioid receptors and cannabinoid receptors. Microglia have been found to express both the CB1R and CB2R *in vitro* (Carlisle and Cabral, 2002; Carrier *et al*., 2005) and to produce the endocannabinoids 2-AG as well as anandamide in lesser quantities (Carrier *et al*., 2004). Thus, these cells appear to harbour a fully constituted system of endogenous cannabinoid ligands and cognate receptors. Activation of the CB2R on these cells appears to promote migration and proliferation. Walter *et al*. (2003) demonstrated that 2-AG induced migration of microglia and that this occurred through the CB2R and abnormal-CBD-sensitive receptors, with subsequent activation of the extracellular signal-regulated kinase 1/2 signal transduction pathway. These investigators also demonstrated that microglia expressed the CB2R at the leading edge of lamellipodia, consistent with their involvement in cell migration.

There is accumulating evidence that the CB2R is also expressed in the CNS. Van Sickle *et al*. (2005) reported the presence of CB2R mRNA and protein in brainstem neurons. Furthermore, the CB2Rs were found to be activated by the cognate agonist 2-AG and by elevated endogenous levels of endocannabinoids that also signal through the CB1R. In addition, Fernandez-Ruiz *et al*. (2007), using a variety of neurodegenerative disease models, reported the expression of the CB2R in microglia, astrocytes and neuron subpopulations. This expression of the CB2R *in vivo* apparently is attributed, in large measure, to microglia. In several neurodegenerative diseases, upregulation of microglial CB2R has been observed (Zhang *et al*., 2003; Benito *et al*., 2005, 2007; Maresz *et al*., 2005; Yiangou *et al*., 2006; Ashton *et al*., 2007). In addition, CB2R-positive microglia have been identified dispersed within active MS plaques and localized in the periphery of chronic active plaques (Benito *et al*., 2007).

The collective findings refute the concept that the only cannabinoid receptor that has a functionally relevant role in the CNS is the CB1R. The current data indicate that the CB2R can also be present in the CNS and that its expression is associated with a variety of inflammatory processes. This expression is manifest primarily when microglia are in 'responsive' and primed' states of activation, signature activities of which include cell migration and antigen processing. It has been proposed that the role of the CB2R in immunity in the CNS is primarily that of anti-inflammatory (Carrier *et al*., 2005). In this context, this receptor has the potential to serve as a therapeutic target for appropriately designed CB2R-specific ligands that could act as anti-inflammatory agents in MS and other neuropathological processes. For example, in Theiler's virus infection of mouse CNS, an animal model for human MS, it was demonstrated that the synthetic cannabinoids WIN55,212-2, ACEA and JWH-015 improved neurological deficits, and reduced microglial activation, MHC class II expression and T-lymphocyte infiltration (Arevalo-Martin *et al*., 2003). Thus, selective targeting of the CB2R could lead to ablation of neuropathological processes while minimizing psychotropic effects that could be exerted by activation of the CB1R.

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Conflict of interest

The authors state no conflict of interest.

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