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Endocannabinoid signaling in microglial cells

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The endocannabinoid signaling system (eCBSS) is composed of cannabinoid (CB) receptors, their endogenous ligands (the endocannabinoids, eCB) and the enzymes that produce and inactivate these ligands. Neurons use this signaling system to communicate with each other and Δ^9-tetrahydrocannabinol (THC), the main psychotropic ingredient of *Cannabis sativa*, induces profound behavioral effects by impinging on this communication. Evidence now shows that microglia, the macrophages of the brain, also express a functional eCBSS and that activation of CB receptors expressed by activated microglia controls their immune-related functions. This review summarizes this evidence, discusses how microglia might use the eCBSS to communicate with each other and neighboring cells, and argues that compounds selectively targeting the eCBSS expressed by microglia constitute valuable therapeutics to manage acute and chronic neuroinflammation, without inducing the psychotropic effects and underlying addictive properties commonly associated with THC.

Cannabis sativa contains over 60 phytocannabinoids, at least three of which are bioactive: THC, cannabinol (CBN) and cannabidiol (CBD) (Howlett et al., 2002; Turner et al., 1980). This plant is the most famous of the *Cannabis* family, but represents only one of the many subspecies containing bioactive cannabinoids. THC induces psychotropic effects and modifies essential physiological processes by interacting with CB receptors expressed by neurons and other cell types (Howlett et al., 2002; Huestis et al., 2001). CBN does not induce psychoactive effects, but reduce inflammatory responses by interacting with CB receptors expressed by immune (Herring and Kaminski, 1999; Perez-Reyes et al., 1973). CBD is anxiolytic, reduces blood pressure and inflammation, most likely by interacting with CB receptors expressed in the brain, vascular endothelial cells and immune cells, respectively (Campos and Guimaraes, 2008; Costa et al., 2004; Járai et al., 1999; Malfait et al., 2000; Perez-Reyes et al., 1973). Research in this field has focused on understanding the molecular mechanisms mediating the action of cannabinoids – be they endogenous, plant-derived or synthetic – in specific cell types, and has developed powerful pharmacological and genetic tools targeting the eCBSS that allowed for a better understanding of its involvement in physiological functions and pathological processes.

This review presents evidence for the existence of a functional eCBSS in one such cell type, the microglial cells, and describes how cannabinoids control their immune-related responses and role in the initiation and propagation of neuroinflammation. But before reviewing this evidence, I will summarize our current understanding of the basic components that compose the eCBSS: namely the CB receptors, the eCBs and their metabolic enzymes.

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Cannabinoid receptors

At least five subtypes of CB receptors have been identified: the two cloned receptors (CB_1 and CB_2), GPR55, and two receptors that have been pharmacologically pinpointed but remain to be identified at the molecular level.

CB_1 receptors are expressed throughout the brain by many different subtypes of neurons and at lower levels by other types of cells (Howlett et al., 2002; Matsuda et al., 1990; Tsou et al., 1998). In brain, they are most abundant in GABAergic interneurons, being between three to ten times less abundant in glutamatergic principal neurons (Kawamura et al., 2006; Uchigashima et al., 2007). A series of elegant experiments demonstrated that despite exhibiting a lower expression in glutamatergic principal neurons, selective genetic deletion of CB_1 receptors in this neuronal subpopulation blocks the classic behavioral tetrad induced by THC, namely catalepsy, locomotion impairment, analgesia and changes in body temperature, all of which are normally induced by cannabinoid-like compounds (Monory et al., 2007).

CB_1 receptors couple to $G_{i/o}$ proteins, and modulate the activity of specific ion channels and second messengers. It is likely that their acute *versus* sustained stimulation differentially modulates cell functions. For example, acute stimulation of neuronal CB_1 receptors for milliseconds to seconds inhibits presynaptic N-type calcium channels and activates inwardly rectifying potassium channels, thereby reducing neurotransmisson (Henry and Chavkin, 1995; Mackie and Hille, 1992; Mackie et al., 1995). Their sustained stimulation for minutes to hours regulates effector proteins such as PKA and Erk (Chevaleyre et al., 2007; Derkinderen et al., 2003; Marsicano et al., 2003), thereby modifying enzymatic activities and gene expression. A particularly relevant example in the context of neuroinflammation is the CB_1-mediated increase in the expression of brain derived neurotrophic factor (BDNF) in neurons, a factor known to modify synaptic plasticity and to enhance cell survival (Khaspekov et al., 2004; Marsicano et al., 2003).

CB_2 receptors couple to G_i proteins, but most likely not to G_o proteins (Glass and Northup, 1999). They share 44% protein identity with CB_1 and display a distinct pharmacological profile and expression pattern (Felder et al., 1995; Galiègue et al., 1995; Munro et al., 1993). Many laboratories have reported that healthy brain tissue does not contain CB_2 receptors, with the exception of a small population of neurons located in the brain stem (Carlisle et al., 2002; Derocq et al., 1995; Galiègue et al., 1995; Munro et al., 1993; Schatz et al., 1997; Sugiura et al., 2000; Van Sickle et al., 2005). The consistency of these reports suggests that the “resting” microglia that are present in healthy brain also lack CB_2 receptors. However, the assumption that CB_2 receptors are *never* expressed in brain is oversimplified, since the expression of this receptor can be induced in immune cells, be they resident microglia (see below) or invading immune cells. Note that a couple of reports claimed that almost all neurons present in healthy brain express CB_2 receptors, but these studies did not include critical controls, including the parallel immunostaining of the analyzed brain areas, but this time using tissue from CB_2 knockout mice to ascertain for staining specificity (Gong et al., 2006; Onaivi et al., 2008).

Recent studies suggest that the orphan receptor GPR55 might represent a third CB receptor (Johns et al., 2007; Lauckner et al., 2008; Oka et al., 2007; Ryberg et al., 2007; Waldeck-Weiermair et al., 2008). However, each study reported distinct – in some cases opposite – pharmacological profiles for classic cannabinoids at this receptor, most likely because of pharmacodynamic differences that such a receptor can exhibit when expressed in different cell-based systems. For example, one study claimed that the potency of CP55,940 at GPR55 lies within the nanomolar range, while another claimed that it act with a potency in the micromolar range, and yet another study claimed that it is inactive (Johns et al., 2007; Lauckner et al.,

2008; Oka et al., 2007; Ryberg et al., 2007). The inconsistency of these studies indicates that more work is required for the field to establish GPR55 as a true component of the eCBSS.

Using CB_1 knockout mouse brain tissue, several laboratories identified a unique subtype of CB receptor that inhibits glutamatergic transmission in the hippocampus, but does not control adenylyl cyclase activity (Breivogel et al., 2001; Hájos et al., 2001; Hoffman et al., 2005; Monory et al., 2002). This receptor is stimulated by the non-selective cannabinoid agonists WIN55,212-2 and CP55,940, as well as by the $TRPV_1$ agonist capsaicin (Hájos and Freund, 2002). SR141716A, which antagonizes CB_1 receptors at nanomolar concentrations, antagonizes this receptor as well, but only at micromolar concentrations (Hájos and Freund, 2002). These intriguing pharmacological results beg for the molecular identification of such receptor so that the field can directly test its relevance in the eCBSS.

Using CB_1/CB_2 receptor double knockout mice, the group of George Kunos identified the abnormal-cannabidiol (abn-CBD) receptors (also known as anandamide receptors) (Járai et al., 1999). These $G_{i/o}$ protein-coupled receptors are expressed by endothelial cells of blood vessels, increase cGMP production and regulate blood pressure (Begg et al., 2003; Offertáler et al., 2003). They are activated by abn-CBD and are antagonized by CBD and O-1918 (Járai et al., 1999; Offertáler et al., 2003). Our laboratory found that microglia also express this receptor subtype (see below) (Franklin and Stella, 2003; Walter et al., 2003). Here too, the field is waiting for the molecular identification of this receptor to test its relevance in the eCBSS.

In summary, two CB receptors, CB_1 and CB_2, have been identified at the molecular level and unambiguously shown to belong to the eCBSS. Additional receptors might also be stimulated by cannabinoids, but many basic questions, including their molecular identification and their true belonging to the eCBSS, remain open.

Endocannabinoid production, pharmacology and inactivation

In 1992, Raphael Mechoulam and coworkers identified arachidonoylethanolamide, which was named anandamide, as the first endogenous compound that binds to CB_1 receptors with high affinity (Devane et al., 1992). Anandamide is considered a *bona fide* eCB because (I) it is produced by cells in an activity-dependent manner, (II) it activates CB_1 and CB_2 receptors and (III) it is enzymatically inactivated. Depolarization- and agonist-induced increase in anandamide production was initially shown in neurons in primary culture and then in rat brains (Bequet et al., 2007; Caille et al., 2007; Di Marzo et al., 1994; Giuffrida et al., 1999; Stella and Piomelli, 2001; Walker et al., 1999). A rise in intracellular calcium concentration is often required to stimulate anandamide production and multiple calcium-dependent lipases and acyltransferases have been implicated in this process (for review, see (Di Marzo, 2008)). Whether distinct enzymatic pathways generating anandamide are recruited by different physiopathological stimuli remains unknown. Anandamide binds CB_1 and CB_2 receptors with relatively high affinity and regulates the activities of their effector proteins as a partial agonist (Bouaboula et al., 1995; Davis et al., 2003; Derkinderen et al., 2003; Felder et al., 1993; Vogel et al., 1993). Biological inactivation of anandamide occurs in two steps: a rapid uptake into cells, the molecular identity of which is still unknown, followed by intracellular hydrolysis, which is primarily mediated by fatty acid amide hydrolase (FAAH) (Cravatt et al., 2001). A large amount of data is available on FAAH: from its crystal structure to the chemical platform required for its selective inhibition (McKinney and Cravatt, 2005).

In 1995, two laboratories identified a second endogenous ligand, 2-arachidonoylglycerol (2-AG), which is more abundant than anandamide in brain (Mechoulam et al., 1995; Sugiura et al., 1995). Many laboratories commonly measure anandamide and 2-AG levels in brain, but depending on the analytical method that is used, amounts of either anandamide or 2-AG range from nanomoles to micromoles *per* gram of protein (for review see (Muccioli and Stella,

2007)). 2-AG is produced by diacylglycerol lipase (DGL) and a rise in intracellular calcium concentration increases this production (Beltramo and Piomelli, 2000; Dinh et al., 2002; Stella and Piomelli, 2001; Witting et al., 2004a). Alternative pathways for 2-AG synthesis have been suggested (Di Marzo, 2008). 2-AG binds to CB_1 and CB_2 with lower affinity than anandamide, but stimulates the activity of effector proteins as a full agonist (Mechoulam et al., 1995; Stella et al., 1997; Sugiura et al., 2000; Sugiura et al., 1995; Walter et al., 2003). In fact, 2-AG has a higher intrinsic efficacy than anandamide, because both anandamide and 2-AG can appear to be full agonists under appropriately high levels of CB_1 (CB_2) expression and/or efficient post-receptor coupling to signal transduction cascades. My laboratory found that 2-AG might also act as a full agonist at abn-CBD receptors, one of the cannabinoid receptor that style needs to be identified at the molecular level (Franklin and Stella, 2003; Walter et al., 2003).

Similarly to anandamide, inactivation of 2-AG occurs in two steps: a rapid uptake into cells, probably involving the same unknown molecular entity responsible for anandamide uptake (Beltramo and Piomelli, 2000), followed by its intracellular hydrolysis by monoacylglycerol lipase (MGL). MGL was initially purified and its cDNA isolated and cloned from adipose tissue (Karlsson et al., 1997; Tornqvist and Belfrage, 1976). This enzyme is abundant in brain tissue, particularly at presynaptic terminals (Gulyas et al., 2004; Karlsson et al., 1997). Adenovirus-mediated expression of MGL in cultured neurons enhances 2-AG hydrolysis and reduces activity-dependent accumulation of 2-AG, suggesting that MGL constitutes a rate-limiting step of 2-AG accumulation (Dinh et al., 2002). Accordingly, pharmacological inhibition of MGL leads to 2-AG accumulation in brain tissue and enhancement of its cannabimimetic effects (Makara et al., 2005). Additional enzymes have been shown to metabolize 2-AG, including cyclooxygenases, lipooxygenases, DGL and FAAH, as well as several newly identified serine hydrolases (Blankman et al., 2007; Dinh et al., 2004; Goparaju et al., 1998; Kozak et al., 2000; Mentlein et al., 1984; Somma-Delpéro et al., 1995). Thus, 2-AG hydrolysis appears to be more complex than that of anandamide, involving more enzymes, the respective roles of which still need to be defined.

The balance between eCB production and inactivation determines the extent of eCB accumulation in tissue and resulting activation of CB receptors (Figure 1). Thus, compounds inhibiting enzymes responsible for either anandamide or 2-AG hydrolysis will lead to their respective accumulation, and partial or full activation of CB receptors, respectively. Inhibitors that lead to the accumulation of either anandamide or 2-AG have been reported. Systemic injection of the FAAH inhibitor URB597 leads to anandamide accumulation in brain without affecting 2-AG levels (Fegley et al., 2005). This selective accumulation of anandamide relieves signs of anxiety and depression in mice undergoing distress, and reduces inflammation-induced pain and autoimmune-mediated cell damage, without inducing CB_1-mediated psychotropic effects (Baker et al., 2001;Cravatt et al., 2004;Gobbi et al., 2005;Kathuria et al., 2003). Systemic injection of the MGL inhibitor URB602 leads to 2-AG accumulation in the dorsal midbrain without affecting anandamide levels. This selective accumulation of 2-AG mimics stress-induced analgesia, also without producing psychotropic effects (Hohmann et al., 2005). A recent study showed that combined inhibition of FAAH and MGL with a non-selective organophosphorus agent mimics full-blown CB_1-mediated effects, emphasizing the need to selectively inhibit either of the two eCB inactivation pathways if one desires a therapeutic approaches that lack untoward side effects (Nomura et al., 2008).

In summary, two eCBs, anandamide and 2-AG, have been extensively studied. They are produced by independent enzymatic pathways, act at CB receptors with distinct pharmacological profiles and are inactivated by uptake followed by independent hydrolysis. Evidence suggests that the selective inhibition of distinct enzymes responsible for eCB hydrolysis constitutes a promising therapeutic approach to relieve specific symptoms without producing overt side effects.

Microglial cells

Microglia derive from the monocytic lineage, entering the developing brain before the establishment of a functional blood-brain barrier and distribute uniformly throughout the parenchyma (Streit, 2001). In healthy brain, microglia display a “resting” phenotype responsible for the continuous immune surveillance of their environment (Fetler and Amigorena, 2005; Raivich, 2005). Studies carried out with GFP-expressing microglia suggest that thorough surveillance of the CNS environment is linked to the rapid extension and retraction of the microglial cellular processes, which occurs without evident movement of their cell bodies and is independent of neuronal activity (Davalos et al., 2005; Nimmerjahn et al., 2005; Wu and Zhuo, 2008). When neural cells are damaged, neighboring “resting” microglia rapidly change their phenotype and behavior, a process referred to as “microglial cell activation”. The molecular steps involved in this activation process implement distinct cellular functions aimed at repairing damaged neural cells and eliminating toxins and pathogens from the area (Garden and Moller, 2006). Damaged cells release chemoattractants that both increase the overall motility (*i.e.* chemokinesis) and stimulate the directed migration (*i.e.* chemotaxis) of microglia, the combination of which recruits the microglia much closer to the damaged cells (Trapp et al., 2007). Microglia express membrane receptors that recognize toxins, pathogens, and molecules released by damaged cells, including fractalkine, ATP, glutamate and eCBs (see below). Depending on the combination and extent of stimulation of such receptors, expression of specific genes is induced and their respective products tailor the phenotype of microglia toward becoming pro-inflammatory (also referred to as a M_1 phenotype) or anti-inflammatory (also referred to as a M_2 phenotype) (Garden and Moller, 2006; Gordon, 2003). Thus, pharmacological tools that target the molecular mechanisms controlling microglial cell migration and phenotype may be therapeutically useful for diseases associated with excessive pro-inflammatory responses. Ideally, such tools would prevent the accumulation of pro-inflammatory microglia while enhancing the recruitment of anti-inflammatory microglia at injury sites. The evidence summarized below suggests that compounds targeting the eCBSS expressed in microglia might represent such pharmacological tools.

Microglial cells express functional cannabinoid receptors

Whether resting microglia found in healthy brain parenchyma express CB_1 and/or CB_2 receptors has not been addressed directly, but one can presume – as stated at the beginning of this review – that these cells do not express CB_2 receptors (because the mRNA encoding for these receptors is undetectable in healthy brain tissue) (Carlisle et al., 2002; Derocq et al., 1995; Galiègue et al., 1995; Griffin et al., 1999; McCoy et al., 1999; Munro et al., 1993; Schatz et al., 1997; Sugiura et al., 2000). On the other hand, a rapidly growing number of studies performed on both microglial cells in culture and activated microglia found in diseased brain tissue suggest that CB_2 receptor expression may be up-regulated as part of their activation process.

Microglial cells in primary cultures are intrinsically activated or “primed” because of the procedure involved in transferring these cells into culture (Becher and Antel, 1996). Multiple laboratories have shown that “primed” microglia prepared from human, rat or mouse tissue express CB_2 receptors (Carlisle et al., 2002; Facchinetti et al., 2003; Klegeris et al., 2003; Ramirez et al., 2005; Rock et al., 2007; Walter et al., 2003). The expression level of these receptors can be further up- or down-regulated by certain pathogens and cytokines (Carayon et al., 1998; Derocq et al., 2000; Gardner et al., 2002; Lee et al., 2001; Rodríguez et al., 2001; Waksman et al., 1999). For example, lipopolysaccharide (LPS) reduces CB_2 receptor expression in primed microglia and combinations of GM-CSF plus IFNγ increase it (Carlisle et al., 2002; Maresz et al., 2005) Although IFNγ activates microglia toward an M_1 phenotype, this cytokine does not significantly affect CB_2 receptor expression in primed microglia

(Carlisle et al., 2002; Maresz et al., 2005). Several rodent microglial cell lines, which intrinsically exhibit high rates of cell proliferation, also express CB_2 receptors (Carrier et al., 2004; Walter et al., 2003) In summary, CB_2 expression by microglia in culture is well accepted since this result was found by many independent laboratories that use different *in vitro* models.

Depending of the neuropathology or type of brain insult, the phenotype of activated microglia will vary, as well as the presence or not of CB_2 receptors. One remarkable example is found in rat spinal cord, where neuropathic pain up-regulates CB_2 receptor in microglia, but chronic inflammatory pain does not (Zhang et al., 2003). Note that this study used *in situ* hybridization to detect the presence of CB_2 receptor mRNA in microglia, most likely because of the well-known difficulty of obtaining antibodies that unambiguously recognize CB_2 receptor protein in tissue. This latter point is noteworthy because many studies used polyclonal antibodies to claim CB_2 receptor expression by activated microglia in diseased tissue, but these studies often lack the required control of parallel immunostaining of similar tissue isolated from CB_2 knockout mice. One study reported CB_2 receptor expression by perivascular microglia in healthy human brain tissue (Nunez et al., 2004). This particular subpopulation of microglia has a higher turn-over rate with circulating peripheral monocytes compared to the microglia residing in brain parenchyma. The specificity of the antibodies that were used in this study is more difficult to assess because of the obvious lack of knockout tissue control, but if this result holds true, it would imply that healthy human brain constitutively expresses CB_2 receptors (not on neurons but on perivascular microglia). Activated microglia found in brain tissue from patients suffering from Alzheimer's disease, multiple sclerosis (MS) and amyotrophic lateral sclerosis (ALS) also express CB_2 receptors, especially when these cells are associated with the plaques that accumulate in these diseases (Benito et al., 2003; Benito et al., 2007; Yiangou et al., 2006). One study also reported CB_2 receptor expression by activated microglia found in a simian model of AIDS dementia (Benito et al., 2005).

In summary, CB_2 receptor up-regulation in activated microglia may occur as a result of specific neuroinflammatory responses, and this up-regulation most likely depends on the combination of toxins, pathogens, cytokines and molecules encountered by the microglia. However, many of the reports claiming the presence of CB_2 receptors in activated microglia will have to be confirmed with more reliable tools and – when possible – the systematic use of CB_2 knockout tissue and cells.

Determining how CB_2 receptor regulates microglial cell function is often guided by the vast literature available on the role of the eCBSS in peripheral monocytes and macrophages. Typical immune functions that are assessed include: cell migration, cell proliferation, cytokine and free radical release, and phagocytosis. Stimulation of CB_2 receptors by 2-AG increases Erk activity in monocytes, resulting in increased cell migration (Bouaboula et al., 1996; Derocq et al., 2000; Jordà et al., 2002; Klemke et al., 1997). A similar 2-AG response has been replicated in BV-2 cells, a widely used mouse microglial cell line (Dirikoc et al., 2007; Eljaschewitsch et al., 2006; Walter et al., 2003). Multiple lines of evidence show that local eCB production is increased in response to tissue damage (and allied increase in intracellular calcium) (for review, see (Stella, 2004)). When considering local increases in eCB levels with the fact that 2-AG increases microglial cell migration, it is tempting to propose that one role of the eCBSS is to recruit microglia and peripheral monocytes at lesion sites (Figure 2). Note that stimulation of CB_2 receptors also increases microglial cell proliferation (Carrier et al., 2004), while reducing their ability to release detrimental factors, including TNFα and free radicals (Eljaschewitsch et al., 2006; Ramirez et al., 2005). Thus, the overall result of stimulating CB_2 receptors in microglia would be that higher numbers of anti-inflammatory (or less harmful) microglia will accumulate at lesion sites. While this hypothesis is testable, a major outstanding question is whether stimulation of CB_2 receptors expressed by microglia also enhances their beneficial

properties, including the release of trophic factors, such as of BDNF, and their ability to eliminate cell debris through silent phagocytosis (Coull et al., 2005; Green and Beere, 2000).

A fascinating series of studies showed that stimulation of CB_2 receptors expressed by microglia reduces HIV-1 expression in these cells (Peterson et al., 2004). A molecular mechanism that could be responsible for this effect is the CB_2-mediated down-regulation of CCR5, a chemokine receptor subtype involved in the docking and entry of HIV-1 into microglial cells (Rock et al., 2007). Thus, an additional function of the eCBSS in microglia might be to temper viral-induced activation of microglia, which has major therapeutic relevance in the context of AIDS dementia.

Anandamide also binds to CB_2 receptors, where it acts as a partial agonist or antagonist (Gonsiorek et al., 2000; Griffin et al., 2000; Shire et al., 1996). Understanding how anandamide interacts with CB_2 receptors expressed by microglia is important since neuropathologies are frequently associated with increases in both 2-AG and anandamide (Baker et al., 2001; Berger et al., 2004; Ferrer et al., 2003; Franklin et al., 2003; Hansen et al., 2001; Marsicano et al., 2003; Panikashvili et al., 2001; Schäbitz et al., 2002; Witting et al., 2004b). Thus, depending on the relative quantities of 2-AG and anandamide that accumulate in specific brain areas, their competition at CB_2 receptors will determine the extent to which CB_2 receptors will regulate microglial cell behavior and phenotype.

CB_1 receptors are expressed by microglial cells in culture prepared from mollusk, mouse and rat, but not human (Carlisle et al., 2002; Facchinetti et al., 2003; Klegeris et al., 2003; Molina-Holgado et al., 2002; Sinha et al., 1998; Stefano et al., 1996; Waksman et al., 1999; Walter et al., 2003). How these receptors regulate microglial cell function is controversial. For example, CP55,940 acting at CB_1 receptors increases nitric oxide (NO) production from mollusk microglia (Stefano et al., 1996), but this ligand inhibits the LPS-induced release of NO from rat microglia (Waksman et al., 1999). The latter effect of CP55,940 on NO production is only partially blocked by micromolar concentrations of SR141716A, which calls into question the true involvement of CB_1 receptors in this response (Stefano et al., 1996; Waksman et al., 1999). My laboratory has revisited the role of CB_1 receptors in microglia by using BV-2 cells and found that WIN55212-2 at 1 μM does not affect basal release of NO, nor does it modulate the LPS/IFNγ-induced production of NO (Franklin et al., 2003). Other reports have shown that only high concentrations of cannabinoids affect microglial cell function. For example, only micromolar concentrations of the three most commonly used synthetic cannabinoid agoinsts, CP55,940, WIN55,212-2 and HU210, regulate cytokine release from cultured microglia (Facchinetti et al., 2003; Puffenbarger et al., 2000). This effect is not stereoselective and only partially blocked by micromolar concentrations of CB receptor antagonists (Facchinetti et al., 2003; Puffenbarger et al., 2000). Could it be that the relevance of CB_1 receptors in regulating microglial cell function is difficult to assess because these receptors are expressed at low levels and are predominately located in the intracellular compartments of these cells (Walter et al., 2003)? Could variable responses to CB_1 agonists on microglial cells in cultures be due to changes in the expression of this receptor linked to culture conditions (changes that might not occur *in situ*)? Or is it that microglia express orphan CB receptors and that only high concentrations of commonly used synthetic cannabinoids stimulate these receptors? Supporting this third possibility, my laboratory found pharmacological evidence for the presence of abn-CBD receptors in BV-2 cells (Franklin and Stella, 2003; Walter et al., 2003). Clearly, the presence and function of CB_1 and orphan CB receptors in microglia requires further investigation.

Microglial cells produce and inactivate endocannabinoids

Microglial cells in culture produce anandamide and 2-AG, with ionomycin and millimolar concentrations of ATP selectively increasing 2-AG production (Carrier et al., 2004; Walter et al., 2003). The molecular mechanism underlying ATP-induced 2-AG production involves the activation of $P2X_7$ ionotropic receptors, which are highly permeable to calcium and induce sustained rises in intracellular calcium that directly increase DGL activity while inhibiting MGL activity (Witting et al., 2004a). This inverse sensitivity of DGL and MGL to calcium constitutes an original and efficient modality for sustained increased production of 2-AG by microglia, a modality recently extended to neurons (Maccarrone et al., 2008).

Two pieces of evidence suggest that microglia likely constitute the main cellular source of eCBs measured under neuroinflammatory conditions. First, microglial cells in culture produce approximately 20-fold more eCBs than neurons and astrocytes in culture (Walter et al., 2002; Walter et al., 2003). Second, 2-AG accumulation measured in experimental autoimmune encephalomyelitis (EAE)-inflamed brains from $P2X_7$ knockout mice is significantly lower than that measured in EAE-inflamed brains from wild-type mice mouse brains, which is remarkable when considering that $P2X_7$ receptors are only expressed by activated microglia (Witting et al., 2006).

Microglia efficiently inactivate both anandamide and 2-AG. Accordingly, primary microglia in culture express FAAH and MGL (Witting et al., 2004a). My laboratory found that BV-2 cells express a novel 2-AG hydrolyzing activity that is pharmacologically distinct from MGL and FAAH (Muccioli and Stella, 2008). Furthermore, a screen for novel inhibitors of eCB hydrolysis identified several compounds that differentially reduce MGL, FAAH and the novel 2-AG hydrolyzing activity expressed by BV-2 cells (Muccioli and Stella, 2008). A recent elegant study that was performed by the laboratory of Ben Cravatt (which used a functional proteomics approach) led to the identification of novel enzymes capable of hydrolyzing 2-AG (Blankman et al., 2007), one of which might be responsible for 2-AG hydrolysis by BV-2 cells. This result is noteworthy because the existence of a novel, pharmacologically distinct 2-AG hydrolyzing activity expressed by microglia opens promising therapeutic avenues. Indeed, the chemical platform required to selectively inhibit this novel enzyme will likely be different from the one required to selectively inhibit MGL and FAAH. It is also likely that selective inhibition of this novel enzyme expressed by microglia will lead to different cannabimimetic effects *in vivo* compared to those induced by selective FAAH and MGL inhibitors. Directly testing these possibilities will require the molecular identification and characterization of this novel enzyme expressed by microglia, as well as the identification of specific inhibitors of its activity.

Conclusions

The studies outlined in this review were carried out over the last decade and convincingly show that microglia express many of the components required for functional eCB paracrine and autocrine signaling. Microglia express CB_2 receptors and non-psychotropic CB_2 agonists regulate their immune-related functions, including migration, proliferation and cytotoxin release. Microglia produce large amounts of eCBs that most likely contribute to the long-lasting increase in eCB levels measured under neuropathological conditions. Such sustained increases in eCB production may contribute to the orchestration of a defense mechanism typified by the accumulation of anti-inflammatory microglia at lesion sites. Thus, the pharmacological stimulation of CB_2 receptors, as well as the pharmacological inhibition of eCB hydrolysis, in microglia should result in boosting this defense mechanism, a working hypothesis that has already been tested in some mouse models of neurodegeneration (Bilsland et al., 2006; Lastres-Becker et al., 2003).

In closing, I would like to highlight an exception to this pattern that is found in mice undergoing EAE. In this case, neural cell damage does not lead to a pronounced increase in 2-AG at lesion sites (Witting et al., 2006) (Maresz et al., 2005) even though CB_2 receptors on microglia are functional (synthetic CB_2 agonists confine EAE-induced lesions (Arévalo-Martín et al., 2003)). This lack of increase in 2-AG production could be due to IFN-γ released by T-cells invading the CNS. Indeed, we found that IFN-γ disrupts the functionality of purinergic $P2X_7$ receptors expressed in microglia (the main receptor subtype involved in ATP-mediated increase in eCB production) (Witting et al., 2006; Witting et al., 2004a). IFN-γ also down-regulates the expression of DGL, the enzyme responsible for producing 2-AG in microglia (Witting et al., 2006; Witting et al., 2004a). Accordingly, we found that induction of EAE in $P2X_7$ knockout mice results in even lower 2-AG levels at lesion sites and more pronounced cell damage than in wild type mice (Witting et al., 2006). Thus, the pro-inflammatory properties of IFN-γ, which play a central role in both EAE and MS pathogenesis, may also disrupt what one could refer to as the "help me" signal carried by 2-AG, while not affecting the functionality of CB_2 receptors expressed by microglia. If this working hypothesis holds true, it would provide strong support for the use of both non-psychotropic CB_2 agonists and inhibitors of 2-AG hydrolysis as medicine to treat MS patients.

Rationale for cannabinoid-based therapies relies on a unique characteristic of these compounds: their curative properties do not overlap with currently available medicines and thus cannabinoids constitute a novel therapeutic platform. Yet, in order to gain broad public support, cannabinoid-based therapies will need to "kick to the curb" the drug of abuse stigma by remaining devoid of THC-related adverse effects, which include psychotropic effects, memory impairment, anxiety, weight gain and potential addiction. Based on our understanding of the dependence that can be developed by patients using morphine as a painkiller, it is now important to rapidly increase our understanding of the medicinal potential of targeting the eCBSS so that we can exploit their desirable properties while trying to avoid grim scenarios.

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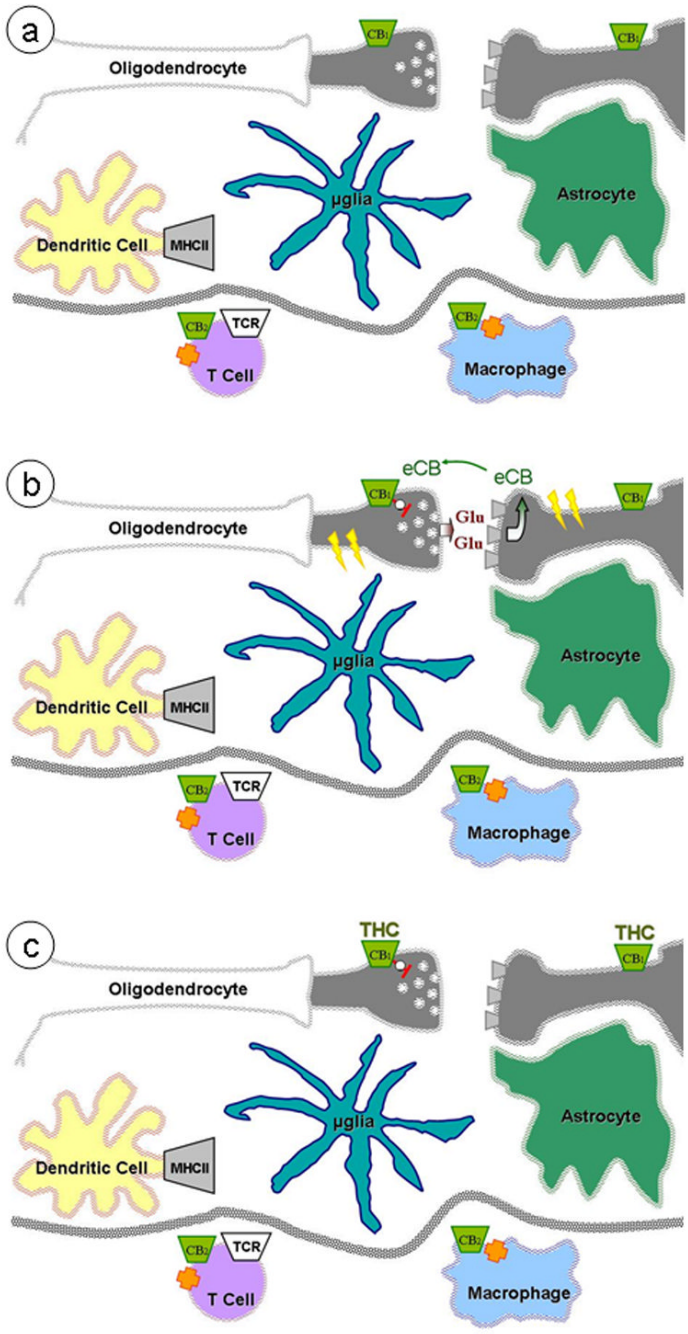

Figure 1. Endocannabinoid signaling in healthy brain

(a) CB_1 receptors are expressed by neurons and CB_2 receptors by peripheral immune cells. (b) Neuronal depolarization and neurotransmitter release (*e.g.* glutamate, Glu) leads to post-synaptic rise in calcium, which increases endocannabinoid (eCB) production. eCB act as retrograde signals onto presynaptic CB_1 receptors, reducing neurotransmitter release. (c) Δ^9-tetrahydrocannabinol (THC) acts as a high-affinity partial agonist at CB_1 receptors, impinging on this eCB signaling.

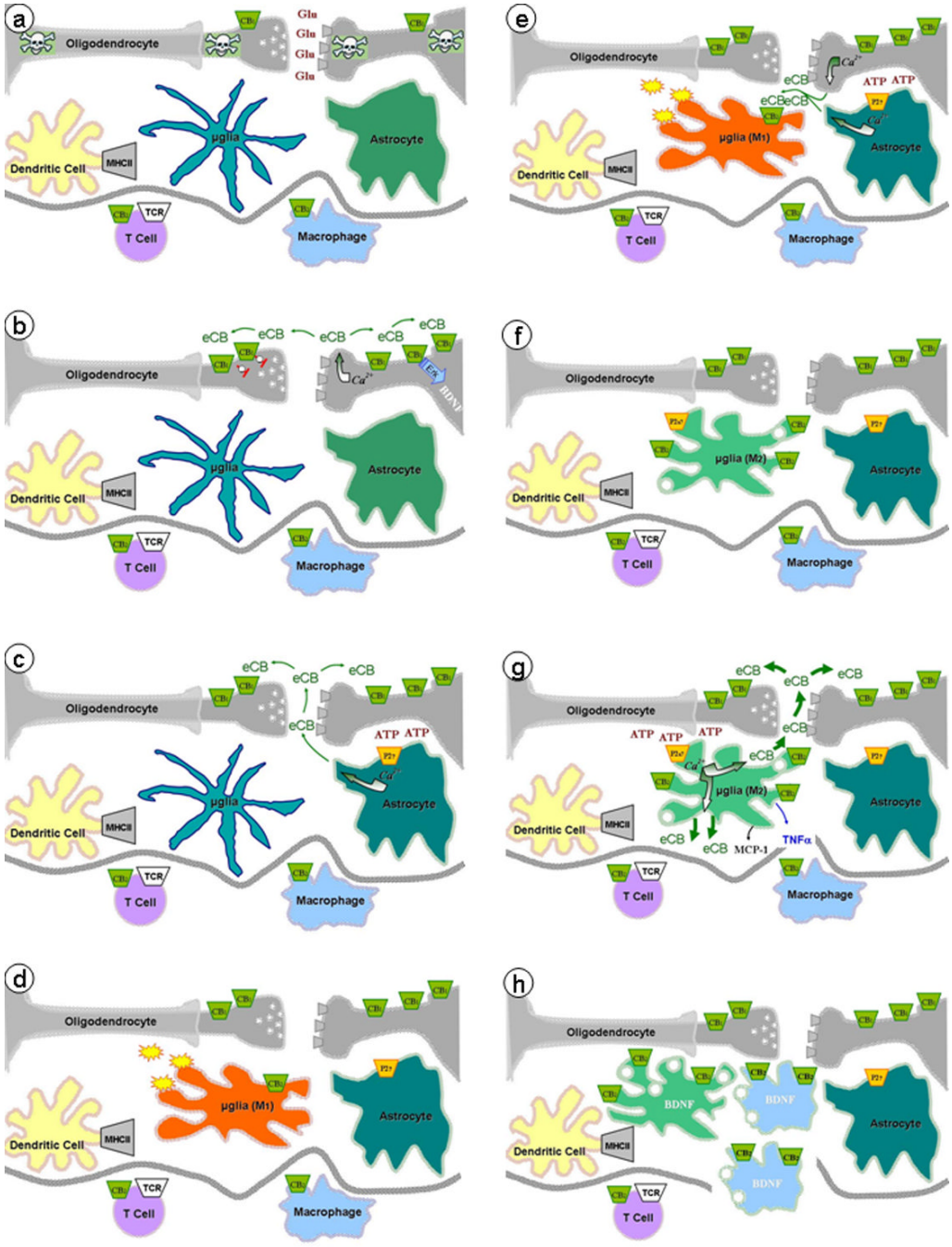

Figure 2. Endocannabinoid signaling in diseased brain

(a) Neurons damaged by injury, toxins or pathogens release large amounts of Glu, (b) resulting in strong neuronal depolarization and Glu receptor activation and sustained rise in post-synaptic calcium and enhanced eCB production. CB_1 receptor expression is up-regulated in damaged neurons. eCB acting at pre-synaptic CB_1 receptors reduce neurotransmitter release and at post-synaptic CB_1 receptors increase Erk activity and allied gene expression (*e.g.* BDNF). (c) ATP, released from damaged cells, stimulates purinergic receptors expressed by astrocytes and enhances eCB production, which may participate in stimulating pre- and post-synaptic CB_1 receptors. (d) Neuronal damage is associated with microglial cell activation (M_1, proinflammatory phenotypes), resulting in free radicals and toxin release, as well as up-

regulation of CB_2 receptor expression. (e) eCB produced by damaged neurons and stimulated astrocytes act on CB_2 receptors expressed by microglial cells, (f) leading to a switch in their phenotype (M_2, anti-inflammatory phenotype) and further up-regulation of CB_2 and $P2X_7$ receptor expression. (g) ATP released by damaged cell enhances the abundant and sustained production of eCB from microglia, which participates in stimulating pre- and post-synaptic CB_1 receptors, (h) as well as in recruiting peripheral monocytes/macrophages (in concert with the chemokine MCP-1 and the cytokine TNFα). The overall result is to limit the propagation of cell damage and favor cell repair (*e.g.* through the release of BDNF).