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Microglia activation states and cannabinoid system: Therapeutic implications

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ABSTRACT

Microglial cells are recognized as the brain's intrinsic immune cells, mediating actions that range from the protection against harmful conditions that modify CNS homeostasis, to the control of proliferation and differentiation of neurons and their synaptic pruning. To perform these functions, microglia adopts different activation states, the so-called phenotypes that depending on the local environment involve them in neuroinflammation, tissue repair and even the resolution of the inflammatory process. There is accumulating evidence indicating that cannabinoids (CBs) might serve as a promising tool to modify the outcome of inflammation, especially by influencing microglial activity. Microglia has a functional endocannabinoid (eCB) signaling system, composed of cannabinoid receptors and the complete machinery for the synthesis and degradation of eCBs. The expression of cannabinoid receptors – mainly CB2 – and the production of eCBs have been related to the activation profile of these cells and therefore, the microglial phenotype, emerging as one of the mechanisms by which microglia becomes alternatively activated. Here, we will discuss recent studies that provide new insights into the role of CBs and their endogenous counterparts in defining the profile of microglia activation. These actions make CBs a promising therapeutic tool to avoid the detrimental effects of inflammation and possibly paving the way to target microglia in order to generate a reparative milieu in neurodegenerative diseases.

1. Introduction

Microglia represents between 5–20% of total glial cells in rodents, depending on the specific central nervous system (CNS) region (Lawson et al., 1990), and they are the primary cells that respond to potentially harmful conditions that could lead to neuronal loss, like injury or infection. Thus, microglia is the central custodians acting under the protection of the blood–brain barrier (BBB: Daneman, 2012) and is activated in neuroinflammatory conditions to moderate any potential damage to the CNS and to favor tissue repair. Indeed, data has emerged to suggest that microglia can control proliferation and differentiation of neurons, as well as the formation of new synapses in the healthy CNS (Graeber, 2010; Hughes, 2012). This dual nature as a mononuclear phagocyte and a glial cell of the CNS expands the interest in microglial ontogeny, origin, development and function in health and disease.

1.1. The origin of microglia

Long before the introduction of the term "microglia" by del Río-Hortega early in the 20th century (del Río-Hortega, 1932), there had been much discussion about the nature and the origin of these cells. Two schools of thought developed at the same time supporting both the ectodermal or mesodermal origin of microglia. The neuroectodermal origin was suggested based on the supposition that a common progenitor existed for microglia, astrocytes and oligodendrocytes (Fujita & Kitamura, 1975; Kitamura et al., 1984), a theory for which further support gathered (Hao et al., 1991; Fedoroff et al., 1997). Pioneer bone marrow chimera experiments suggested the existence of radiation-resistant local precursors that are present in the

Abbreviations: 2-AG, 2-arachidonoylglycerol; Aβ, amyloid β; ABDH, α/β-hydrolase; AD, Alzheimer's disease; AEA, anandamide; Arg-1, arginase-1; BBB, blood brain barrier; BDNF, brain derived nerve factor; CB, cannabinoid; CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; CD45, cluster of differentiation 45; CNS, central nervous system; CCL, CC chemokine ligand; CX3CR1, CX3C chemokine receptor 1; DAGL, diacylglycerol lipase; E, embryonic; EAE, experimental autoimmune encephalomyelitis; eCB, endocannabinoid; eCBSS, endocannabinoid signaling system; FAAH, fatty acid amide hydrolase; GPR, G-protein coupled receptor; IFNγ, interferon gamma; IGF-1, insulin growth factor-1; IL, interleukin; IL-1β, interleukin 1 beta; iNOS, inducible nitric oxide synthase; GPR55, G protein-coupled receptor 55; LPS, lipopolysaccharide; Mac, macrophage antigen; MAGL, monoacylglycerol lipase; MMP, matrix metalloproteinase; MS, multiple sclerosis; MPTP, 1-Methyl-4-phenyl-1,2,3,6,-tetrahydropropyridine; NO, nitric oxide; PD, Parkinson's disease; PPAR, peroxisome proliferator-activated receptor; SCI, spinal cord injury; SOCS, suppressor of cytokine signaling; TGF-β, transforming growth factor beta; TLR, Toll-like receptor; TMEV-IDD, Theiler's murine encephalomyelitis virus-induced demyelinating disease; TNFα, transforming growth factor alpha; YS, yolk sac.

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CNS prior to birth, and showed that microglia is highly resistant to radiation in contrast to other blood leukocyte populations and cannot be replaced by donor cells (Lassmann et al., 1993; Priller et al., 2001). Indeed, recent studies suggest that brain irradiation per se results in the infiltration of bone marrow derived immune cells into the CNS (Moravan et al., 2016), including CCR2^{+} macrophages (Morganti et al., 2014).

The mesodermal origin was based on morphological evidence and phenotypic features that focused on the resemblance between microglia and macrophages. For example, microglia is recognized by antisera that interact with monocyte/macrophage antigens (Hume et al., 1983; Murabe & Sano, 1983), and both microglia and macrophages express markers like CD11b, the Fc receptor and F4/80 in mouse (Perry et al., 1985; Akiyama & McGeer, 1990). Pioneering studies were eventually able to establish the myeloid nature of microglia, since mice lacking the myeloid transcription factor Pu.1 were devoid of myeloid cells and microglia (McKercher et al., 1996). Further evidence supported this hypothesis (Herbomel et al., 2001; Beers et al., 2006; Schulz et al., 2012) and therefore microglia is classified as mononuclear phagocytes that include monocyte-derived cells, dendritic cells, peripheral and CNS-associated macrophages (Prinz et al., 2011; Gomez Perdiguero et al., 2013).

Fate-mapping studies provided evidence that under homeostatic conditions, microglia originates from hematopoietic stem cells in the yolk sac (YS) during early embryogenesis (Ginhoux et al., 2010; Schulz et al., 2012; Kierdorf et al., 2013). Although a population of maternally derived committed CD45 expressing macrophages has been found in the YS at E7.5, this population progressively declines and is no longer detected at E9. Thus, it is believed that these cells could have a restrictive protective effect against intrauterine infections in the embryo (Bertrand et al., 2005). In mice, primitive hematopoiesis initiates in the YS shortly after the onset of gastrulation at E7, and before the circulatory system becomes fully established between E8.5–E10 (reviewed in Ginhoux et al., 2013). Interestingly, c-kit^{+} cells that are negative for lineage markers of mature hematopoietic cell progenitors have been found in the early YS, and these cells can differentiate into CX3CR1 microglia and Ter119^{+} erythrocytes, suggesting a common progenitor in the YS for both lineages (Kierdorf et al., 2013). Microglia progenitors arise in the blood islands of the YS around E9 (Fig. 1), and they migrate through the embryo vascular system to the brain and

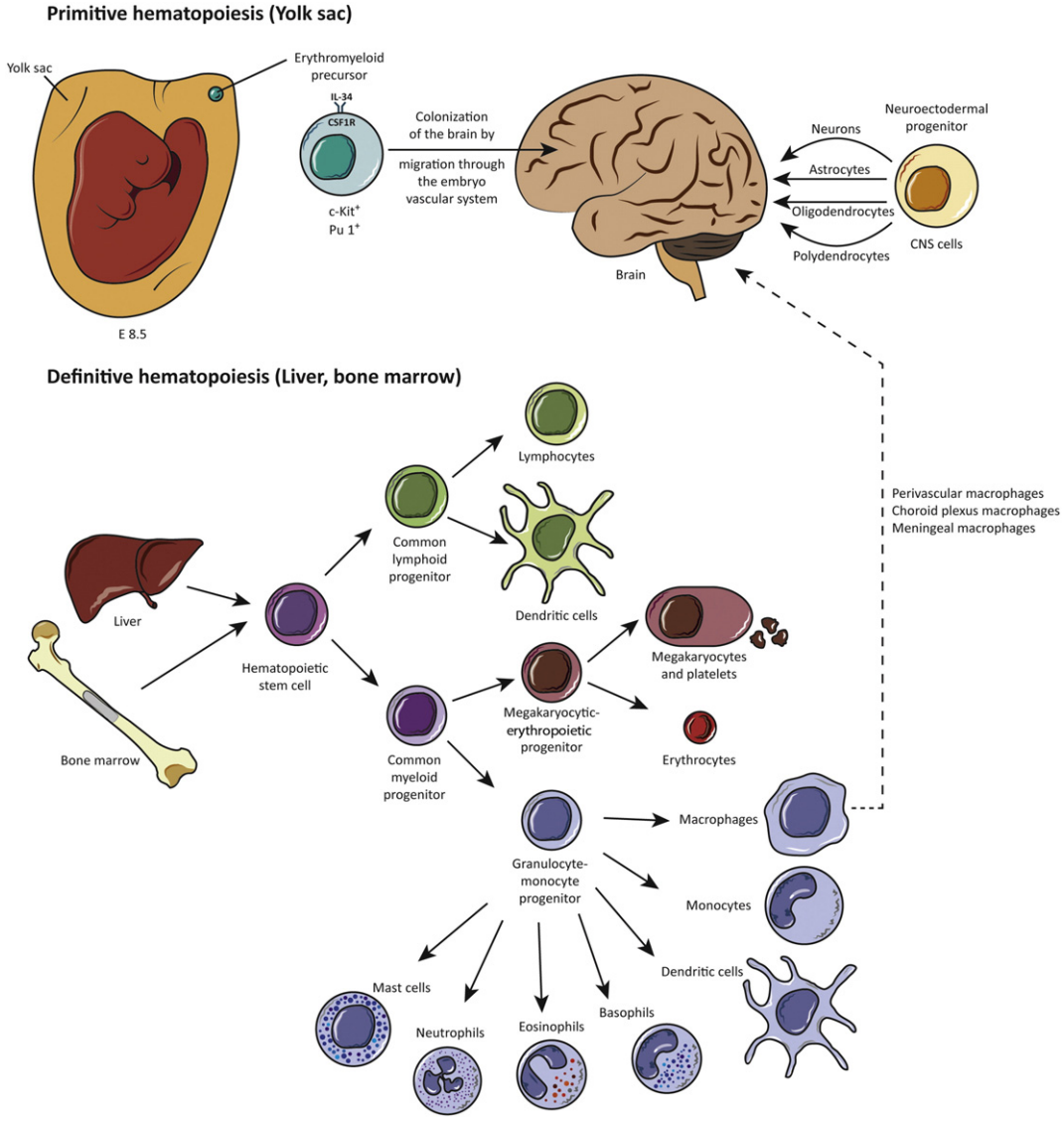

Fig. 1. Microglia derives from erythromyeloid precursors in the YS. Microglia progenitors, mainly regulated by CSF-1R and its ligand IL-34, are positive for c-kit gene and depend on the expression of the transcription factor Pu-1. They arise in the blood islands of the YS around E8.5 and migrate through the embryo vascular system to the brain and other tissues. Neurons, astrocytes, oligodendrocytes and polydendrocytes derive from neuroectodermal progenitors within the CNS. Definitive hematopoiesis is established from hematopoietic stem cells in the fetal liver and finally in the bone marrow, giving origin to both lymphoid and myeloid cells.

other tissues were they differentiate into "fetal" macrophages (Naito et al., 1990). Macrophage-like cells can be found in the developing neuroepithelium as early as E8.5/E9.0, with the capacity to differentiate in vitro into microglia-like cells that express markers like F4/80, Mac1 and Mac 3 (Alliot et al., 1991). In the embryo, definitive hematopoiesis will be established from hematopoietic stem cells generated in the aorta, gonads and mesonephros (AGM) region after E8.5 (Orkin & Zon, 2008). At around E10.5, both YS and AGM-derived hematopoietic precursors will colonize the fetal liver, which represents the major hematopoietic organ for monocytes and potentially perivascular, choroid plexus and meningeal macrophages (Kumaravelu et al., 2002). Finally, after birth myeloid cells are continuously produced in the bone marrow (Fig. 1) from hematopoietic stem cells (Prinz & Priller, 2014).

The microglia population expands after birth, and the number of $CD11b^{+}F4/80^{+}$ microglia increases 20-fold between P0 and P11 in rodents by a mechanism that does not seem to involve the recruitment of peripheral myeloid cells (Ginhoux et al., 2010; Schulz et al., 2012). In this line, it has been recently demonstrated that time-controlled microglia depletion induced by tamoxifen in genetically modified mice is renewed by an internal pool, demonstrating the renovation capability of microglia without peripheral myeloid contribution (Bruttger et al., 2015). However, some bone-marrow-derived cells can infiltrate into the CNS, and assume the morphology and phagocytic capacity of microglia, mainly during inflammation or disease. Indeed, bone marrow transplants within 24 h of birth in the transcription factor Pu.1 knockout mice show that bone marrow-derived cells can contribute to the postnatal microglia population as they drive the de novo generation of these cells in this situation (Beers et al., 2006). In the adult CNS, irradiation and parabiosis studies have demonstrated that the contribution of monocytes to microglia population depends on inflammation and it requires conditions that would probably disrupt the BBB to allow bone-marrow derived cells to enter the CNS (Ajami et al., 2007; Mildner et al., 2007). A strong debate has recently arisen after it was seen that the adult brain is repopulated within 1–2 weeks after microglia depletion by cells that are assumed to be peripheral monocytes since they express CD45 and CCR2 (Varvel et al., 2012), or by local progenitors positive for the neuronal marker nestin (Elmore et al., 2014). It remains controversial as to whether these nestin expressing cells are microglial progenitors in the CNS or if they are derived from infiltrating monocytes, reopening the debate as to the origin of microglia in homeostatic conditions and disease, an issue that will hopefully be clarified in the next few years.

1.2. Microglia phenotypes: classical and alternative activation

Given the distinct developmental origin of macrophages and microglia, the different features of these two cells have begun to be considered, particularly in terms of the nomenclature and concept of microglial phenotype polarization. Traditionally, the concept of classic or alternative macrophage activation has been applied to microglia, considering the different CNS phenotypes as either the classically activated (M1-type) or the alternatively activated state (M2-type). This dichotomic nomenclature overlooks the richer spectrum of phenotypes related to functions of macrophages and microglia, associating M1 with cytotoxic properties and M2 with regeneration and repair (subtype M2a), immunoregulation (M2b) or an acquired-deactivating phenotype (M2c) (Martinez et al., 2009). The proposed phenotypes, inductors and markers for microglia are summarized in Fig. 2. Although the signals that control the polarization of M1 and M2 phenotypes can

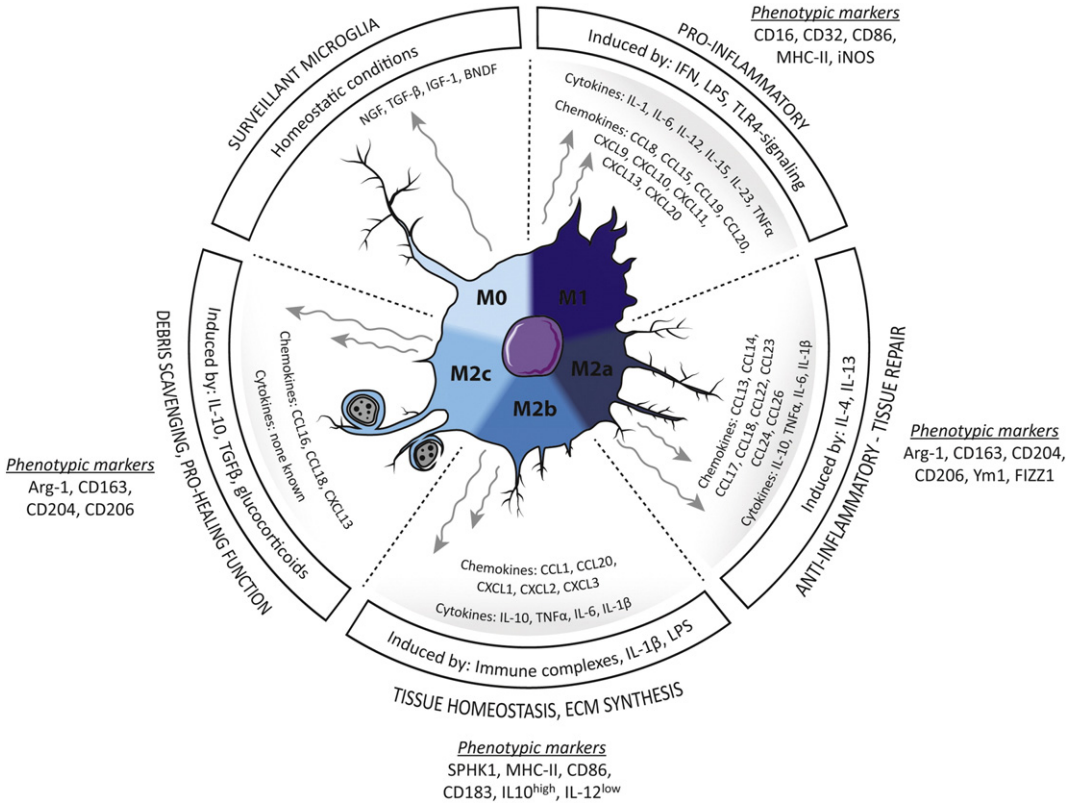

Fig. 2. Microglia phenotypes, markers and actions. Proposed morphologic changes, polarizing agents, phenotypic markers and secreted cytokines and chemokines in the M0, M1, M2a, M2b and M2c microglia phenotypes based on macrophage phenotypes. Surveillant microglia (M0 state) shows ramified morphology and releases neurotrophic factors like IGF-1 and BDNF. Classically activated microglia (M1 state) is positive for CD16/32/86, MHC-II and iNOS, shows ameboid morphology, and can be induced by pro-inflammatory signals like IFN or LPS; it releases a plethora of chemokines and pro-inflammatory cytokines as shown. Alternative microglia (M2a, M2b and M2c) can be induced by different signals like anti-inflammatory cytokines, immune complexes or glucocorticoids, respectively. These alternative phenotypes show complex ramified morphologies and many markers as shown, some of them defined by a higher ($IL\text{-}10^{high}$) or lower ($IL\text{-}12^{low}$) expression pattern, and are implicated in anti-inflammatory functions and debris scavenging. Adapted from David and Kroner (2011).

be determined in vitro (Chorr et al., 2013), in vivo studies show that the local environment of the brain can simultaneously supply M1 and M2 polarizing cues (Martinez & Gordon, 2014). Thus, the microglia response could depend on the ratios between a range of phenotypes.

Several of the signals that drive macrophages and microglia into the different activation states have been defined, and since this enables the adaptive response of innate immunity to take place, cytokines derived from T lymphocytes are among the signals implicated in the polarization of these cells. In humans, it has been shown that T lymphocytes isolated from patients with multiple sclerosis (MS) and polarized to Th1 or Th2 cells in vitro, differentially modulate human monocytes and microglia to an M1 or M2 antigen-presenting cell (APC), respectively (Kim et al., 2004). In mice, the M1 phenotype of both macrophages and microglia has typically been associated with Th1 cytokine interferon gamma (IFNγ) and bacterial lipopolysaccharide (LPS), whereas the M2a phenotype is driven by the Th2 cytokines Interleukin 4 (IL-4) and 13 (IL-13) (Prinz & Priller, 2014). Other prototypic inducers are Toll-like receptor (TLR) agonists, immune complexes and IL-1R ligands for M2b, or glucocorticoids, IL-10 and transforming growth factor-β (TGF-β) for M2c (Schmid et al., 2009).

In the steady state, microglial cells have a small soma and ramifications with non-overlapping processes, and they have distinct morphological features depending on their state of activation. The term "resting" microglia has been updated in the healthy CNS to "surveillant", as they continuously monitor the nervous tissue and palpate the environment, such that the entire brain volume is examined in approximately 5 h, as suggested by two-photon studies in vivo (Nimmerjahn et al., 2005). The secretion of neurotrophic factors by microglia like nerve growth factor (NGF), TGF-β, insulin growth factor-1 (IGF-1) or brain-derived neurotrophic factor (BDNF) has been demonstrated in pathological conditions, contributing to the restoration of homeostasis (Bessis et al., 2007; Polazzi & Monti, 2010). Microglia responses are thought to be required for synaptic pruning (Paolicelli et al., 2011), phagocytosis of apoptotic neuroblasts in the dentate gyrus of the developing hippocampus (Sierra et al., 2010), and for processes like neurogenesis in the mature brain (Walton et al., 2006) or the guidance of stem cells in their migration to sites of inflammation and injury (Aarum et al., 2003). Maintaining the microglia surveillance phenotype depends on cell–cell contact with neurons, including signaling through microglial CX3CR1, CD172 or CD200R receptors upon interaction with secreted CX3CL1 by neurons, or the neuronal CD47 and CD200 proteins, respectively. In addition, soluble adhesion molecules, neurotransmission-associated inhibitors, inhibitory cytokines and their receptors can also contribute to the microenvironment's inhibitory influences for restraining microglia activation (reviewed by Ransohoff & Cardona, 2010).

In CNS pathology, the phenotypes of microglia change in response to the particular signals detected in the brain parenchyma. For example, the release of adenosine triphosphate (ATP) and the extracellular calcium waves that follows injury can activate the movement of microglial processes towards the lesion site and the polarization of these cells from ramified to activated (Davalos et al., 2005; Sieger et al., 2012). Microglia represents the innate immune response's first line of defense and they become activated to an M1-like phenotype state, which involves the expression of myeloid cell markers and morphological changes from the ramified to a phagocytic phenotype. This morphological change has been described as de-ramification, a process in which the soma enlarges, and the number and length of the process progressively diminishes until the cell acquires an amoeboid morphology (Stence et al., 2001; Perry et al., 2010; Fontainhas et al., 2011; Kettenmann et al., 2011). Microglial responses also include signaling through pattern recognition receptors (PRRs), ion channels and receptors for neurotransmitters, leading to the production of pro-inflammatory cytokines such as tumor necrosis factor (TNF) and IL-1β, chemokines like CC-chemokine ligand 2 (CCL-2), and nitric oxide (NO). Subsequently, microglia can in turn act as elements of the adaptive immune system by upregulating the expression of major histocompatibility complex (MHC) class II molecules to present antigens to T cells, and secreting other pro-inflammatory cytokines like IL-12, IL-23 or IL-6 to induce Th1 or Th17 cells (reviewed in Saijo & Glass, 2011). All these responses occur in association with inflammatory processes like trauma, ischemia–reperfusion injury or upon the detection of signs of infection in the CNS, and they are all associated with neurotoxicity in the brain.

After the onset of the classical activation of microglia, designed to eliminate a pathogen or restrain an injury, the resolution of the inflammation initiates with an anti-inflammatory and repair phase that is induced to restore tissue homeostasis and that is essential to avoid neurotoxicity and chronic inflammation. For this repair phase to occur, microglia shifts to an alternative M2 activation state, an anti-inflammatory milieu is promoted and repair genes are expressed to dampen the pro-inflammatory response. The concept of alternative macrophage activation was raised in the early 1990s, with pioneer experiments showing that IL-4, a product of Th2 lymphocytes, induced macrophages to adopt a phenotype distinct from that induced by IFN-γ (Stein et al., 1992). This alternative phenotype was characterized by reduced pro-inflammatory cytokine secretion and enhanced MHC-II antigen expression. Later reports showed that these cells also augment the expression of C-type mannose receptor 1 (CD206), as well as the production of anti-inflammatory cytokines (IL-4, IL-10, IL-13) and factors involved in repair and tissue reconstruction like growth factors (IGF-1, TGF-β), arginase-1 (Arg-1) and chitinase-3-like protein 3 (Ym-1 in rodents: Colton, 2009; Henkel et al., 2009). In vivo experiments indicated that the alternative macrophage state is associated with tissue remodeling, immunoregulation, angiogenesis, inflammatory dampening and allergy response, among others (Sica & Mantovani, 2012; Mantovani et al., 2013). Conversely, much less is known about the alternative activation of microglia in vivo and in vitro, although following the initial description of alternative microglia activation (Ponomarev et al., 2007), this phenotype has been associated in vivo with repair in models of spinal cord injury (Shechter et al., 2013), stroke (Desestret et al., 2013) and demyelination (Miron et al., 2013).

It is noteworthy that some markers of the M1 and M2 phenotypes are expressed only in macrophages and not in microglia. In fact, the capabilities of human microglia and blood-derived macrophages to express M1 and M2 markers has been elegantly addressed by PCR array experiments (Durafourt et al., 2012), showing that the capacity of microglia to adopt an M2 profile is more restricted than that of macrophages, whereas M1 markers are induced in both cell types. However, some confusion persists regarding Arg-1, one of the best characterized markers of M2 phenotype in mouse macrophages/microglia, since its appearance in human microglia remains controversial (Raes et al., 2005). Similarly, the CD206 receptor is not expressed by parenchymal microglia when compared to perivascular or choroid-plexus macrophages (Galea et al., 2005), and human M2 microglia does not express other markers like Chi313 or CD23 (Durafourt et al., 2012). In summary, the polarizing agents and the effects of alternative activation of microglia towards an M2 phenotype still need to be properly addressed, and this issue holds great promise for the treatment of neuroinflammatory diseases and CNS injury.

1.3. Acute inflammation in the central nervous system

Microglia has been recognized as the brain's intrinsic immune cells, mediating a dynamic, controlled and restricted inflammatory response to a variety of insults in the CNS. This response is referred to as neuroinflammation, and it constitutes an innate and adaptive immune response that involves a plethora of factors (including cytokines, chemokines and other immune mediators) related to some of the actions of microglia. Neuroinflammatory responses transcend the classical vision of inflammation, since they involve a coordinated response that orchestrates microglia and other CNS cells (e.g. astrocytes), involving the participation

of the BBB together with the activity of peripheral immune cells that infiltrate the CNS parenchyma (Streit et al., 2004).

Stimuli like stroke, trauma, infections or toxins can produce a rapid yet controlled activation of the immune system within the CNS (Popovich & Longbrake, 2008), a response necessary to minimize injury and to repair the damage. Such stimuli first induce an immediate yet short-lived reaction to injury, often referred to as "acute neuroinflammation", that is a limited response that involves the activation of the resident immune cells, phagocytosis, an increase in the number of microglial cells (termed "microgliosis") and release of inflammatory mediators (Tansey et al., 2007). When microglia is removed in adult mice through specific and conditional ablation, these cells repopulate from highly proliferative clusters within the CNS independently of bone-marrow-derived precursors (Bruttger et al., 2015). Whether peripheral macrophages contribute to the microglial population in the initial inflammatory response remains a matter of debate (Michell-Robinson et al., 2015). Some factors that regulate microglia population dynamics are thought to participate in this phase, like adenosine or cannabinoid receptors (Kettenmann et al., 2011). After this M1 response, required to kill any invading organism and "clean" the damaged zone, a true shift of the microglia towards an M2 phenotype is needed to kick-start the anti-inflammatory response, promote angiogenesis, begin to remove the debris, and to reconstruct the extracellular matrix (Varin & Gordon, 2009). If this shift is not prompted, then the pro-inflammatory response is perpetuated in time, leading to the production of NO and reactive oxygen species (ROS), cytokines and other factors that can damage CNS cells and tissue (Kigerl et al., 2009).

In contrast to the neuroinflammatory response that occurs after CNS injury, chronic inflammation is associated with a wide range of neurodegenerative diseases and their associated neuropathologies. Such chronic non-classical inflammatory responses imply an overactivation of microglia and can be caused by diverse factors that include neuronal death, damage, or environmental toxins (Block et al., 2007). Stress can also alter microglial structure and function inducing a pro-inflammatory phenotype of microglia through a mechanism that appears to be driven by corticosteroids and norepinephrine, two traditional stress-linked signaling molecules (reviewed in Walker et al., 2013). Indeed, a permanent inflammation has been found in the hypothalamus of mice and humans during obesity, suggesting a role of microglia in the neuronal injury in a brain area crucial for body weight control (Thaler et al., 2012). There is evidence that microglia is a key causative factor underlying chronic neuroinflammation. Defining the characteristics and the causes of deleterious microglial activation is an important focus of research in the field of neurodegenerative diseases, as well as that associated with other neurological disorders, aging (Conde & Streit, 2006; Norden & Godbout, 2013; Fenn et al., 2014; Von Bernhardi et al., 2015; Ziebell et al., 2016) and even the maintenance of chronic pain (Hains & Waxman, 2006). However, a better understanding of the dynamics of M1 and M2 microglia that are associated with both acute and chronic neuroinflammation is now forthcoming (see below).

M1 and M2 activation in the CNS has been studied intensely in relation to spinal cord injury (SCI), with several reports of microglia polarization dynamics associated with this type of insult (Kigerl et al., 2009; David & Kroner, 2011; Thawer et al., 2013). Briefly, in the initial response post-injury, the damaged tissue environment favors the upregulation of both M1 and M2 markers, and the process evolves into a predominant M1 response from day 3 onwards, with a downregulation of the M2 profile. Following the initial trauma, secondary inflammation seems to be one of the main determinants that impair regeneration and that enhance the damage to the spinal cord. Indeed, in vitro studies have shown that M1-conditioned media are neurotoxic and prevent axonal elongation, whereas M2-conditioned media encourage axonal growth after injury (Kigerl et al., 2009). In SCI the polarization of microglia towards an M2 phenotype is dependent on IL-4 signaling, which is required for the induction of Arg-1 in these cells and promotes neurite outgrowth ex vivo as well as peripheral IL-4Rα(+) myeloid cell recruitment to the CNS (Fenn et al., 2014). Another example of acute inflammation is traumatic brain injury, where microglia dynamics seem to resemble those of SCI in terms of an initial acute phase, when both phenotypes are evident, and a second phase characterized by the downregulation of M2 markers, leading to a skewed and long-term M1 profile (Hsieh et al., 2013). The importance of the polarization of microglia towards an M2 profile for the regeneration of the damaged tissue has been suggested given that anti-inflammatory treatment has a protective effect in traumatic brain injury (Yi et al., 2008). Accordingly, aged mice with an impairment of M2 response display larger lesion sites (Kumar et al., 2013).

In both types of injury it is assumed that the predominance of an M1 microglial activation profile is due to the high levels of pro-inflammatory cytokines in the damaged tissue, such as TNFα or IFNγ, perpetuating inflammation and hindering regeneration. Several strategies have been studied to avoid this situation. As such, and beside the treatment with rosiglitazone to induce an anti-inflammatory response, beneficial effects of alternative microglial activation have been achieved in SCI after mesenchymal stem cell transplantation (Nakajima et al., 2012), intravenous injection of substance P (Jiang et al., 2012), and injection of granulocyte colony-stimulating factor (GCSF: Guo et al., 2013). Finally, in another acute neuroinflammatory scenario like the ischemic reperfusion injury that follows stroke, an M1 profile is also favored in the damaged tissue beyond the initial ischemic area, propagating cell death (Hu et al., 2012). In this situation, the absence of some of the signals that drive M2 polarization has detrimental effects since mice lacking either IL-10 or IL-4 developed increased infarct volumes (Xiong et al., 2011; Perez-de Puig et al., 2013). In summary, in these situations of insult or injury, the balance between the M1 and M2 microglial profile is essential to precisely control damage and avoid undesirable side effects like chronic inflammation or neurotoxicity.

1.4. Chronic inflammation in the central nervous system: the perspective of the M2 > M1 phenotype to promote regeneration

In contrast to acute neuroinflammation, the term "chronic neuroinflammation" is used to define a scenario of a long-standing and often self-perpetuating inflammatory response. This includes a long-term activation of microglia with the sustained release of pro-inflammatory mediators and chemokines, as well as increased oxidative and nitrosamide stress (Tansey et al., 2007). Rather than fullfilling a protective role like acute neuroinflammation, the prolonged nature of this type of response is most often detrimental and associated with CNS damage and a compromised BBB that enhances the infiltration of peripheral macrophages into the CNS, further maintaining inflammation (Rivest, 2009). Neurodegenerative diseases could be related to other non-chronic CNS inflammatory diseases such as atherosclerosis, in terms of an M1 activation state of macrophages leading to a persistent low-grade inflammatory component rather than a strong, self-limited response potentially associated with infection or injury (Saijo & Glass, 2011). As such, the role of microglia in neurodegenerative diseases has been recently reviewed (Perry et al., 2010; Ransohoff & Cardona, 2010), and studies suggest that neuroinflammatory responses may begin prior to significant loss of neuronal populations (Frank-Cannon et al., 2009).

Alzheimer's Disease (AD), Parkinson's Disease (PD) and MS are some of the neurodegenerative disorders with neuroinflammatory component, in which the profile of microglia seems to be an essential cue to assess disease evolution, its consequences and possible pharmacological approaches (Fig. 3). Although much effort has been made to decipher some of the mechanisms that control microglial activation in acute neuroinflammatory responses, much will still be needed to understand the pathological mediators produced by microglia in the context of neurodegenerative diseases and the environmental factors

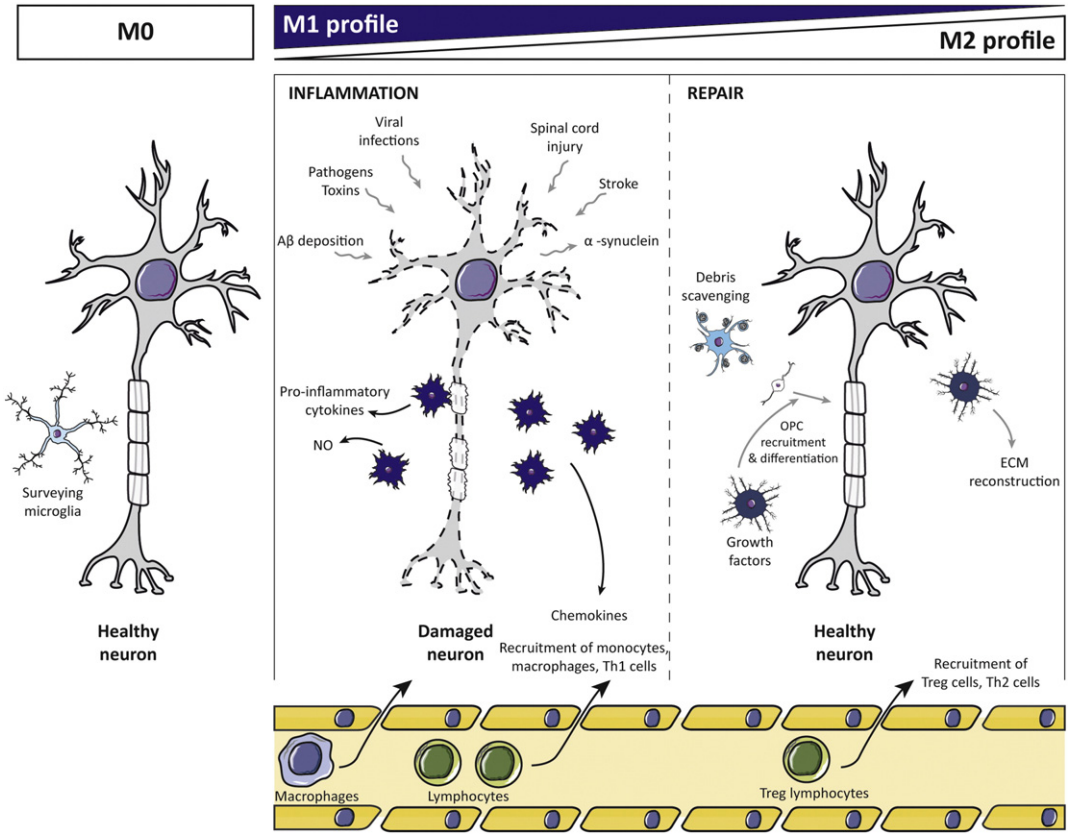

Fig. 3. Reprogramming microglia phenotype to treat neuroinflammation. Under homeostatic conditions, surveillant microglia (M0 profile) is monitoring the nervous system, and its function depends on cell–cell contact with neurons and secreted factors to inhibit microglia activity. In neuroinflammation, microglia detects signals like viral infections, toxins, Aβ deposition, α-synuclein, injury or stroke, and is activated towards an M1 profile to secrete inflammatory mediators and chemokines, participating in the recruitment of monocytes, macrophages and Th1 cells that amplify the inflammatory response and contribute to the damage of neurons. The perspective of the M2 > M1 phenotype to promote regeneration is based on the hypothesis that M2 microglia could promote neuroprotective and reparative effects in the CNS. Through secretion of growth factors, reconstruction of the extracellular matrix, debris scavenging and generation of pro-healing functions together with the recruitment of Treg and Th2 cells, the M2 profile could be beneficial in the restoration of homeostasis within the CNS.

that microglial cells find in damaged tissue, so that an effective therapeutic approach can be adopted in terms of safety, timing and efficacy.

1.4.1. Alzheimer's disease

In the late 1980s microglia was reported to localize near amyloid plaques in AD brains (McGeer et al., 1988), and evidence has appeared that links neuroinflammation and AD (reviewed in Akiyama et al., 2000; McAlpine et al., 2009). Indeed, several reports highlight the inflammatory nature of amyloid β (Aβ), as it can bind to several innate immune receptors that are present in microglia (Cherry et al., 2014). This led to the postulation of the "inflammatory cascade hypothesis of AD", whereby Aβ deposition could induce neuroinflammation, which in turn generates more Aβ and sets off a vicious circle that promotes both neurodegeneration and inflammation (Karran et al., 2011). In terms of microglial phenotypes, an elegant study illustrated that APP/PS1 mice, a transgenic mouse model of AD, shift from an M2 profile early on in the disease towards an M1 response after 18 months of pathogenesis, which is concomitant with a downregulation of the M2 marker Ym1 and upregulation of inflammatory factors (Jimenez et al., 2008). This situation might indicate that in AD, and probably related to age, an increased inflammatory milieu combines with a loss of responsiveness of microglia in terms of M2 induction, which could be due to an age-dependent decrease in IL-4α receptor levels in these cells (Fenn et al., 2012). Another scenario is evident from in vitro experiments, since the M1 polarization of microglia inhibits the phagocytosis of Aβ (Koenigsknecht-Talboo & Landreth, 2005), whereas stimuli like IL-4 or IL-10 that promote an M2 profile effectively block the inhibition of phagocytosis by LPS (Michelucci et al., 2009). Thus, it would seem that the inflammatory response in terms of classical activation may impair the ability of microglia to remove Aβ. Acute LPS treatment has also been reported to reduce Aβ load in APP transgenic mice (DiCarlo et al., 2001; Herber et al., 2004) and that ablation of inducible nitric oxide synthase (iNOS) has protective effects in terms of plaque formation in APP/PS1 mice (Nathan et al., 2005). However, contradictory reports have also been published (Wilcock et al., 2008), such that the role of inflammatory cytokines in the impairment of Aβ clearance remains somewhat controversial. Conversely, the polarization of microglia towards an alternative activation state in AD models has been reported to be beneficial. For example, after intracerebral injection of IL-4 and IL-13 in APP23 mice (Kawahara et al., 2012), or after intrahippocampal injection of adeno-associated virus (AAV) carrying an IL-4 sequence in the APP/PS1 model (Kiyota et al., 2010), there is a reduction in Aβ plaques concomitant with improved cognition that could reflect a dampened pathology. In humans, the situation becomes complicated since microglia of AD patients shows a mixed profile of alternative and classical activation. Microarray analysis of post-mortem brain samples from AD patients shows an up-regulation of pro-inflammatory mediators like IFN-γ and IL-1β, together with an increase in MHC-II (Colangelo et al., 2002; Blalock et al., 2004), and a significant increase in the mRNA levels of TNFα, Arg-1 or CD206, while the levels of iNOS and IL-1β remained unchanged (Colton et al., 2006). Together, the overall discrepancies from in vitro results, in vivo models of AD and the analysis of brain tissue from AD subjects, suggest

a complicated environment in the CNS that affects the response of microglia to Aβ plaque and their clearance.

1.4.2. Parkinson's disease

There is evidence that numerous activated microglia accumulate in the proximity of degenerating neurons in the substancia nigra (SN) of patients with PD or some of its syndromes (McGeer et al., 1988; Langston et al., 1999; Imamura et al., 2003), extending to other brain areas like hippocampus, putamen, cingulate and temporal cortex, among others (Imamura et al., 2003). In familiar PD, α-synuclein is one of the most prevalent pathological genes altered and the mutated protein can form aggregates that constitute the Lewy bodies (Dauer & Przedborski, 2003; Sanchez-Guajardo et al., 2013). It has been demonstrated in vivo that aggregated α-synuclein released from dying or dead dopaminergic (DA) neurons activates microglia towards an M1 phenotype, associated with increased production of pro-inflammatory cytokines and reactive oxygen species (ROS) (Zhang et al., 2005, 2007a, 2007b; Gao et al., 2008; Reynolds et al., 2008; Lee et al., 2010). Likewise, microglia develops into a more reactive M1 phenotype in vitro when α-synuclein is overexpressed (Rojanathammanee et al., 2011), and α-synuclein deficiency dampens the phagocytic capacity of microglia while increasing inflammatory mediators after LPS stimulation (Austin et al., 2006). In PD, microglial activation might be initiated by environmental toxins, pathogens or misfolded proteins. The selective loss of neurons in the SN of PD patients might make this structure particularly vulnerable to inflammatory insult and, indeed, glutathione deficiency and high iron concentrations establish a dangerous redox equilibrium (Block et al., 2007). Some of the factors that can be released by damaged DA neurons and activate microglia are matrix metalloproteinase 3 (MMP3: Kim et al., 2005) and a complex containing melanin, lipid components and peptides, named neuromelanine (Zecca et al., 2003). Specifically, the environmental toxin 1-Methyl-4-phenyl-1,2,3,6,-tetrahydropropyridine (MPTP) has been shown to indirectly activate microglia as a result of the damage imparted on DA neurons by blocking the electron transport chain of the mitochondria (Dauer & Przedborski, 2003). In neuron–glia cultures, an active metabolite of MPTP, 1-methyl-4-phenylpyridinium (MPP^+), induces an increase in MMP 3 together with an increase in microglial activation that leads to DA cell neurodegeneration. Indeed, the response to MPTP as a model of PD is reduced in terms of microglia activation and DA neurodegeneration in MMP3 knockout mice (Kim et al., 2005). Little is known about the dynamic changes of microglia in PD, impeding effective treatments that modify the M1/M2 balance from being applied. For example, MPTP intoxication induces an M1 profile, as reflected by the release of pro-inflammatory mediators, and the activation of NAPDH oxidase and NF-κB pathways (Liberatore et al., 1999; Wu et al., 2003). However, no change in the expression of M2 markers is evident in a mouse model of PD that overexpress human α-synuclein, such as an increase in anti-inflammatory cytokines IL-4, IL-13, or in Arg-1 (Theodore et al., 2008). Further studies of PD pathology must be performed so that a beneficial manipulation of the microglial phenotypes can be achieved, as proposed for AD.

1.4.3. Multiple sclerosis

The onset and progression of MS has also been linked to microglia, although the complex immune nature of this disease makes it difficult to fully understand the role of the classic or alternative phenotypes of microglia in terms of any detrimental or beneficial effects, and possible therapeutic treatments. MS can be considered as a diverse set of diseases, from relapsing and remitting to progressive secondary in the most severe form, in each of which microglia and immune responses that compromise autoantigen Th17, Th1 or B cells, and even monocytes, are involved (Saijo & Glass, 2011). It is likely that the heterogeneous inflammatory responses and the cells responsible for the different pathological phases of MS could create diverse scenarios in the CNS for microglia to adopt a specific phenotype. However, it is clear that persistent inflammation is one of the causes for the demyelination and neurodegeneration observed in the human disease and, indeed, elevated levels of inflammatory cytokines can be found in MS patients (Amor et al., 2010).

It has already been indicated that IFNγ is a potent inducer of the M1 microglial phenotype and thus the secretion of inflammatory mediators by immune cells could be one of the mechanisms underlying the neuroinflammation of MS patients. Nonetheless, post-mortem analysis of MS tissues indicated that myeloid cells in active lesions could have an intermediate activation status since they express markers for M1 and M2 phenotypes (Vogel et al., 2013). The importance in the time-dependent dynamics between the M1 and M2 profiles of microglia emerge from mouse models of MS, such as the toxic demyelinating cuprizone and lysolecithin models, experimental autoimmune encephalomyelitis (EAE) and Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD).

A mixture of microglia phenotypes was observed in the corpus callosum in the cuprizone model during de- and re-myelination states (Voss et al., 2012), whereas lysolecithin injection in the same brain area is accompanied by a switch from the M1 to M2 phenotype at the initiation of the remyelination (Miron et al., 2013). In the EAE model, an elegant pharmacogenetically inducible in vivo model of microglia blockade (CD11b-HSVTK mice) was generated to show that inhibition of microglia activation by ganciclovir treatment delays EAE onset, producing less severe clinical symptoms, and no significant myelin or axonal damage (Heppner et al., 2005). Controversially, the lack of the cytokine IL-4 exclusively in the CNS impairs M2 microglial polarization and exacerbates EAE symptoms (Ponomarev et al., 2007), while transduction of IL-4 with a viral vector reverses these effects (Shaw et al., 1997; Furlan et al., 1998). Along similar lines, the anti-inflammatory cytokine IL-10 also has favorable effects in terms of demyelination and CNS inflammation when injected intraperitoneally in EAE mice through adult neural stem cells engineered to express this cytokine (Yang et al., 2009).

TMEV-IDD represents an important mouse model for primary progressive MS, with an acute neuroinflammatory component that drives the progression of the chronic demyelinating phase (Pullen et al., 1994). There is some evidence in vitro that TMEV infection of microglia provokes a switch from an anti-inflammatory to the pro-inflammatory profile (Gerhauser et al., 2012). Interestingly, a recent study defined the dynamic changes of M1/M2 polarization in the course of the demyelinating and chronic phase of TMEV-IDD (Herder et al., 2015). As such, there appears to be a dominant M1 profile in the spinal cord of infected mice during the demyelination phase of the disease, followed by a mixed M1/M2 phenotype of microglia/macrophages associated with the chronic neuroinflammatory response. These results highlight the complex inflammatory milieu associated with neurodegenerative diseases that course with chronic neuroinflammation, establishing a scenario in which an M1 profile could be continuously induced, dampening the neuroprotective and reparative effects of M2 microglia.

2. Cannabinoid system

Evidence is accumulating that CBs might represent a promising tool to modify microglial activity and profiles in order to achieve benefits for pathologies with an inflammatory component including neurodegenerative and psychiatric diseases (Pacher et al., 2006; Tanasescu & Constantinescu, 2010; Suárez-Pinilla et al., 2014; Lisboa et al., 2016). In vivo and in vitro models of inflammation have shown that some of the beneficial effects of CBs in diverse pathological states may be mediated by their immunomodulatory actions (Arévalo-Martín et al., 2008; Maroof et al., 2013). Microglia expresses a functional endocannabinoid signaling system (eCBSS), and some reports have related the expression of cannabinoid CB2 receptors to the activation state of microglia, as observed in macrophages (Carlisle et al., 2002; Walter et al., 2003;

Martín-Couce et al., 2012; Mecha et al., 2015). Indeed, upregulation of CB2 receptors has been associated with a restoration of tissue homeostasis in pathological neuroinflammatory conditions (Miller & Devi, 2011). These results highlight the potential of CBs to improve the disease consequences of chronic neuroinflammation through the modulation of microglial M1 and M2 activation/polarization, as will be discussed below.

2.1. Overview: ligands and receptors of the endocannabinoid system

CBs are defined as components of the *Cannabis sativa* plant, and other compounds that bind and activate cannabinoid receptors. Beside the popularity of *C. sativa* as a recreational drug, their ability to cause medicinal effects has been widely recognized as early as the third millennium B.C. in China (Mechoulam, 1986). When considering CBs as therapeutic agents, it is important to contemplate the eCB system as a whole, including the entire machinery for the synthesis and degradation of eCBs, the receptors and the intracellular pathways they interact with, each element of which offers a novel molecular target for pharmacotherapy. Indeed, studying these elements will provide new insights into the mechanisms underlying the beneficial effects of plant-derived phytocannabinoids. A complete list of the machinery involved in the synthesis, degradation and actions of cannabinoids can be found in Fig. 4. Among the eCB ligands, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are lipids synthesized in the CNS by neurons and glia. These eCBs are released into extracellular space which allows them to activate CB receptors, and are in turn inactivated by their uptake and ensuing hydrolysis (Pacher et al., 2006; Mechoulam & Parker, 2013). The CB receptor ligands are derived from free arachidonic acid and their chemical structure is eicosanoid. The first eCB, N-arachidonoyl ethanolamide, was isolated from pig brain and named AEA. Various lipases and hydrolases can provoke the release of AEA from its membrane precursor, including N-acyl phosphatidylethanolamine phospholipase D (NAPE-PLD) (Liu et al., 2003; Simon & Cravatt, 2006; Di Marzo, 2011). AEA activates the CB1 receptor and the CB2 receptor as a partial agonist, and following its uptake it is inactivated by fatty acid amide hydrolase (FAAH)-mediated hydrolysis and other enzymes like COX-2 (Cravatt & Lichtman, 2003; Fowler, 2012). The other main eCB, 2-AG, is released from its membrane precursor by phospholipase C (PLC) and the α and β diacylglycerol lipases (DAGLs) isoforms (Stella et al., 1997; Bisogno et al., 2003; Murataeva et al., 2014). Nevertheless, the vast majority of 2-AG is synthesized via diacylglycerol lipase α (Tanimura et al., 2010). 2-AG activates CB1 and CB2 receptors as a full agonist and after uptake, it is hydrolyzed by monoacylglycerol lipase (MAGL), and to a lesser extent by α,β-hydrolase-6 (ABHD6) and α,β-hydrolase-12 (ABHD12) (Freund et al., 2003; Litchman et al., 2010; Pertwee et al., 2010). Beside the aforementioned CB1 and CB2 receptors, eCBs and phytocannabinoids can also interact with other receptors like the transient receptor potential cation channel subfamily V member 1 (TRPV1), abnormal cannabidiol (abn-CBD) receptors, G protein-coupled receptor 55 (GPR55), (Pertwee et al., 2010), and peroxisome proliferator-activated receptors like PPARγ and PPARα (O'Sullivan, 2007; O'Sullivan & Kendall, 2010), among others. Interestingly, the pattern of regulation of GPR55 following microglia activation is very similar to that of CB2 and GPR55 mRNA is downregulated after LPS treatment similarly to that observed with CB2, suggesting that the level of expression of these receptors is concomitantly controlled by the state of activation of microglia that might be involved in neuroinflammation (Pietr et al., 2009).

In terms of the classical cannabinoid receptors, CB1 is abundantly expressed by many neurons throughout the brain, and at much lower level in the immune and other peripheral tissues (Matsuda et al., 1990; Tsou et al., 1998; Howlett et al., 2002). CB1 receptors are $G_{i/o}$ protein-coupled receptors that modulate the activity of several plasma membrane proteins and intracellular signaling pathways in neurons in a time-dependent fashion. It has been reported that the activation of neuronal CB1 receptors for seconds reduces neurotransmission by inhibiting presynaptic N-type calcium channels and by activating inwardly rectifying potassium channels (Mackie & Hille, 1992; Mackie et al., 1995). On the other hand, activation of these receptors for minutes to hours induces changes in gene expression that produce neuroprotective effects, for instance via BDNF (Marsicano et al., 2003). CB2 receptors are also $G_{i/o}$ protein-coupled (Munro et al., 1993), although they are expressed distinctly and have a different pharmacological profile relative to CB1 receptors. Although CB2 receptors are primarily expressed by immune cells (Galiègue et al., 1995), they are also expressed in microglia, astrocytes, oligodendrocytes, progenitor neural cells and even neurons (Molina-Holgado et al., 2002; Benito et al., 2007; Brusco et al., 2008). These two CB receptors have been well characterized and they possess different physiological properties. Thus, CB2 is that which is mainly associated with the anti-inflammatory and immunomodulatory activity of CBs (Miller & Stella, 2008), not at least because it is prominently expressed in cells of the

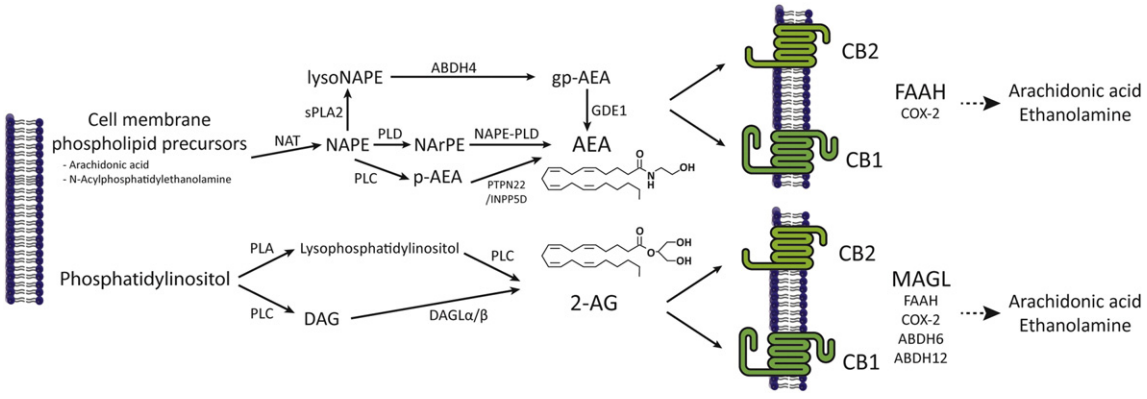

Fig. 4. Main routes of synthesis and degradation of endocannabinoids. eCBs are lipids synthetized on demand from membrane precursors due to specific lipase activities like NAPE-PLD or DAGLα/β. AEA is mainly degradated by FAAH, whereas 2-AG is mainly degradated by MAGL, giving arachidonic acid and ethanolamide as final products. NAT: N-acetyltransferase, NAPE: N-acyl phosphatdyletanolamide sPLA2: soluble phospholipase A2, LysoNAPE: lyso-N-acylphosphatidylethanolamine, ABDH4: α/β hydrolase domain containing protein 4, AEA: anandamide, PLC: phospholipase C, PLD: phospholipase D, NArPE: N-arachidonoylphosphatidylethanolamine, p-AEA: phospho-anandamide, gp-AEA: glycerophosphoanandamide, NAPE-PLD: N-acyl phosphatidylethanolamine phospholipase D, PTPN22: protein tyrosine phosphatase nonreceptor type 22, INPP5D: inositol 5-phosphatase, GDE1: glycerophosphodiester phosphodiesterase 1, FAAH: fatty acid amide hydrolase, COX2: cyclooxygenase 2, PLA: phospholipase A, DAG: diacylglycerol, DAGL: diacylglycerol lipase, 2-AG: 2-arachidonoylglycerol, MAGL: monoacylglycerol lipase, ABDH6: α/β hydrolase domain containing protein 6, ABDH12: α/β hydrolase domain containing protein 12.

immune system. Here, we will focus in the specific actions of cannabinoids in the immune system and, specifically, in microglia.

2.2. Cannabinoids and inflammation: immune modulation

Details about the structure and function of the CB receptors and their endogenous ligands are currently being elucidated in cells of the immune system and microglia. CB1 receptors are expressed beyond the CNS, in the spleen, tonsils and peripheral blood leukocytes (Bouaboula et al., 1993; Galiègue et al., 1995), and its mRNA is expressed by cells of the immune system as follows: B cells > natural killer (NK) cells > polymorphonuclear neutrophils > CD8Tcells > monocytes > CD4Tcells (Massi et al., 2006). By contrast, the CB2 receptor is quite predominant in the immune system, with a level of expression that appears to be 10–100 times greater than that of CB1, principally in B cells > NK cells, monocytes, polymorphonuclear neutrophils and T cells (Galiègue et al., 1995). In addition, CB2 is also expressed in dendritic cells (Matias et al., 2002). The levels of CB receptors are closely related to the activation state of immune cells and, for example, CB1 receptors are upregulated in T cells through a mechanism that involves IL-4 (Börner et al., 2008). Studies on mouse peritoneal macrophages suggest that CB2 can be influenced by immune modulators (Carlisle et al., 2002), and its activation includes migration of immune cells inside or outside the CNS, as well as alterations in cytokine release (Cabral & Staab, 2005). CB receptor stimulation generates rapid and transient bursts in adenylate cyclase (AC) activity, mainly down-regulating cAMP formation and its ensuing signaling (Koh et al., 1995). These actions reflect the effects on complex cellular regulation cascades, since natural cannabinoids can act as inverse AC agonists or antagonists in some circumstances (Bayewitch et al., 1996).

The bias in the balance between the two types of Th cells mediated by cannabinoids is of particular interest, enhancing Th2 and suppressing Th1 responses through a mechanism that involves both CB1 and CB2 receptors (Yuan et al., 2002). Cannabinoid blockade of Th1 associated cytokine pathways like TNF-α, IL-1, IL-2, IL-6 or IL-12 (reviewed by Croxford & Yamamura, 2005) and the potentiation of Th2 cytokine pathways, potentially opens the way for CB-based therapies. Indeed, Th1 suppression produces promising results in experimental models of experimental arthritis and EAE (Tanasescu & Constantinescu, 2010). Other effects of cannabinoids may affect their number, proliferation, migration, Igs production or isotype switching of B cells (Croxford & Yamamura, 2005), as well as proliferation and cellular cytolytic activity of NK cells (Massi et al., 2006).

Interestingly, the relation between CBs and macrophages is bidirectional, paving the way for studies into eCBs and microglia. Macrophages express both cannabinoid receptors, although CB2 is predominant (Sinha et al., 1998), and their activation influences the release of inflammatory mediators like pro-inflammatory cytokines or NO (Burnette-Curley & Cabral, 1995; Berdyshev et al., 2001), as well as inhibiting their phagocytic capacity and suppressing antigen presentation to T cells (Sacerdote et al., 2005). All these effects are dependent on the activation state of macrophages, and they are maximal in primed and minimal in resting or fully activated states (Tanasescu & Constantinescu, 2010). Moreover, macrophages can synthesize eCBs, such as AEA and 2-AG, which can in turn modulate cell differentiation and immune responses through CB and non-CB dependent mechanisms (Massi et al., 2006). Beside CB1 and CB2 receptors, other receptors have been implicated in the cannabinoid-mediated alteration of the immune system, including PPARγ (Rockwell et al., 2006), GPR55 (Pertwee, 2012) and adenosine A2 A receptors (Ribeiro et al., 2012). Remarkably, several reports assess a cross-talk between the eCBSS and TLR signaling. For example, CB1 regulates LPS-induced inflammation in adipocytes and mediates LPS-induced fever response, whereas LPS inhibits 2-AG hydrolysis in the liver/spleen and activation of eCBSS diminishes the inflammatory response mediated by TLR in peripheral blood mononuclear cells, macrophages and microglia (reviewed in Fitzpatrick & Downer, 2016).

In terms of the immune system, cannabinoids are well established as modulators and they fulfill important roles in its regulation, homeostasis and disease. Thus, CBs have promising therapeutic implications in a variety of conditions that include allergic asthma, gut and liver disease, rheumatic disease, atherosclerosis, and neurodegenerative diseases.

2.3. Microglia and cannabinoid system

As for macrophage-like cells, the pattern of microglial maturation, differentiation and activation is also under the regulatory influence of the CB system. There is evidence that microglia has a complete eCBSS, revealing possible novel functions of eCBs in autocrine or paracrine signaling, and in the control of neuroinflammation. Indeed, these cells produce large amounts of eCBs, and under neuropathological conditions they could be responsible for the long-lasting increase in eCB levels measured in the CNS.

As described for macrophages, the expression of CB receptors in microglia is closely related to their activation profile and, thus, their phenotype. In healthy brain tissue, the expression of CB1 and CB2 receptors has not been directly addressed (Stella, 2009) and since only trace amounts of CB2 mRNA can be found (Ehrhart et al., 2005; Maresz et al., 2005), it is assumed from macrophage studies that microglia does not express cannabinoid receptors in the resting state. In culture, microglia is found to be in a "primed" state (intrinsically activated), probably induced by the procedures involved in setting up the cultures (Becher & Antel, 1996). CB1 is the most controversial receptor in terms of CBs effects on microglia. CB1 has been detected in microglia cultures from mouse, rat and mollusk but not humans, producing different effects like the increase (mollusk) or decrease (rat) in NO production by microglia (Stefano et al., 1996; Waksman et al., 1999). By contrast, cultured microglia obtained from mouse, rat or human tissues, as well as the rodent microglial cell line BV-2, express detectable amounts of CB2 receptors (Carlisle et al., 2002; Facchinetti et al., 2003; Klegeris et al., 2003; Walter et al., 2003; Ramirez et al., 2005; Mecha et al., 2015). Interestingly, classic studies have shown that LPS reduces CB2 receptor expression in primed microglia in vitro (Carlisle et al., 2002), whereas combinations of GM-CSF (granulocyte macrophage colony stimulating factor) plus IFNγ increase the expression of this receptor (Maresz et al., 2005). Hence CB2 expression appears to change with the activation state of these cells. We recently found that when microglia is polarized towards an M1 phenotype following LPS stimulation, both CB1 and CB2 receptors are downregulated, whereas they are both upregulated in M2 microglia polarized with IL-4 + IL-13 or TGF-β (Mecha et al., 2015). This divergent response to pro-inflammatory and anti-inflammatory signals at the level of CB receptors is thus related to microglial phenotypes.

Under neuroinflammatory conditions in vivo, CB2 expression can be found in activated microglial cells associated with neuritic plaques in AD brains (Benito et al., 2003), as well as in activated microglia in the spinal cord after neuropathic but not inflammatory pain (Zhang et al., 2003). In addition, CBs have a wide range of effects on microglia that include: (i) enhancing proliferation by activating CB2 receptors (Carrier et al., 2004); (ii) influencing cell migration (Walter et al., 2003; Eljaschewitsch et al., 2006; Dirikoc et al., 2007); (iii) increasing the beneficial properties of microglia, such as BDNF release or the induction of phagocytosis (Gokoh et al., 2007; Tolón et al., 2009); and (iv) diminishing detrimental factors like free radicals or pro-inflammatory cytokines (Ramirez et al., 2005; Spittau et al., 2013; Lu et al., 2015; Ma et al., 2015; Malek et al., 2015; Wen et al., 2015).

Since the majority of the in vitro studies reveal that high concentrations of CBs are needed to influence immune-related activities, it was suggested that these compounds also could act through other receptors, with TRPV1, GPR55 or PPARγ as obvious candidates (Bernardo et al., 2005; Kim et al., 2006a, 2006b; Pietr et al., 2009). Finally, new elements

in the eCB system have been proposed, like N-arachidonoyl glycine (NAGly, synthesized primarily from AEA via FAAH-dependent pathway) and the GPR18 receptor. These novel factors may be playing a role in inducing phenotypic switches and/or directing microglial migration (McHugh et al., 2010). Beside microglia, other players like neurons and astrocytes and their eCBSS cannot be discarded during neuroinflammatory responses. Remarkably, recent reports state that DAGLα and MAGL are preferentially expressed in neurons compared to astrocytes or microglia (Viader et al., 2016) and the transcellular metabolism of 2-AG is a cooperative process between astrocytes and neurons (Viader et al., 2015). Indeed, the selective depletion of MAGL in astrocytes attenuates LPS-induced neuroinflammation (Grabner et al., 2016).

2.4. Microglia synthesizes endocannabinoids

In the CNS, eCBs are produced by both neural (neurons and glia) and immune cells (Salzet et al., 2000; Freund et al., 2003), and they appear to fulfill a key role in neuroimmune networks (García-Ovejero et al., 2013; Hernangómez et al., 2014). The machinery to synthesize and inactivate eCBs appears to be present in microglia which can synthesize eCBs on-demand in response to increases in intracellular calcium (Hillard, 2000), as witnessed by the 2- to 3-fold increase of 2-AG after exposure to calcium ionophore (Walter et al., 2003; Carrier et al., 2004). The rat RTMGL1 microglial cell line synthesizes 2-AG and AEA in basal conditions (Carrier et al., 2004), and macrophages can produce eCBs following LPS stimulation (Di Marzo et al., 1999; Liu et al., 2006). Indeed, microglia produces approximately 20-fold more eCBs than astrocytes and neurons in vitro (Walter et al., 2003) and, thus, it has been suggested that these cells could constitute the main cellular source of eCBs in neuroinflammatory conditions (Stella, 2009). Controversially, we recently demonstrated that cultured rat M2a microglia selectively increases their 2-AG synthesis in a time-dependent manner, whereas M2c microglia augments AEA synthesis (Mecha et al., 2015). This observation suggests an scenario in which stimuli related to anti-inflammatory and repair mechanisms activate 2-AG and AEA synthesis in order to promote the autocrine activity of these lipid messengers (on the producing cell) or their paracrine effects (on local microglia). The synthesis of eCBs by different stimuli in microglia is depicted in Fig. 5 in which we show that eCB levels increase under neuroinflammatory conditions and, in particular, there is an increase in 2-AG in the brain of EAE mice (Witting et al., 2006), whereas 2-AG synthesis diminishes significantly in P2X purinoceptor 7 (P2X7) knockout mice. Since the P2X7 receptor is only expressed by activated microglia, these data support the hypothesis that the synthesis and actions of eCBs are closely related to the activation state of microglia and their M1/M2 profile. On the other hand, the inactivation of eCBs in microglia depends on the activity of the enzymes that degrade them, mainly FAAH and MAGL (Witting et al., 2004) but also the serine hydrolase ABDH6 (Blankman et al., 2007; Marrs et al., 2010). Hence, selective inhibition of eCB degradation to enhance eCB signaling and their beneficial effects in neuroinflammation may represent a promising therapeutic possibility.

2.5. Endocannabinoids drive the acquisition of alternative phenotype in microglia

Since M2 microglia upregulates the expression of eCB receptors, the increase in the production of eCBs could in turn activate CB1 or CB2 receptors and specific eCB signaling cascades, amplifying the M2 profile. Indeed, we have shown that the exposure of rat or human microglia to low concentrations of 2-AG and AEA increases the expression of the M2 marker Arg-1, together with an increase in other markers of alternative phenotype like suppressor of cytokine signaling 3 (SOCS3; Mecha et al., 2015). These data support the hypothesis that the eCBSS plays a role in the modulation of immune-related responses in microglial cells by inducing an M2 phenotype. This amplification of the alternative profile may be an attempt to moderate the persistency of an excessive activated state that could prevent the resolution of inflammation and become deleterious by expanding the damage. In keeping with these results, 2-AG treatment polarizes macrophages towards an alternative phenotype in EAE mice (Lourbopoulos et al., 2011), ameliorating the acute and chronic phases of the disease. Moreover, the blockade of CB1 and CB2 receptors with selective antagonists dampens the acquisition of an alternative phenotype in cultured microglia stimulated with IL-4 and IL-13 (Mecha et al., 2015). Results obtained from $CB2^{-/-}$ mice highlight the importance of CB2 signaling in cultured microglia, since these cells do not polarize towards an M2 phenotype, have alterations in morphology and their phagocytic capacity is compromised. Similarly, CB2 activation by JWH-015 attenuates the CD40 mediated inhibition of microglial phagocytosis of $A\beta_{1-42}$ peptide (Ehrhart et al., 2005), and CB2 receptors have been implicated in dectin-1-mediated macrophage phagocytosis (Shiratsuchi et al., 2008).

Based on these results, we propose the following hypothetical scenario following injury or inflammation in the brain. The initial response involves M1 microglia carrying out crucial tasks to defend the brain parenchyma, although they can switch from a primed to an M2 profile to avoid disturbances of brain homeostasis through a mechanism that involves eCBs production and signaling in an autocrine or paracrine manner. Indeed, the selective release of 2-AG or AEA in response to cytokine stimuli is important, given that the CNS local

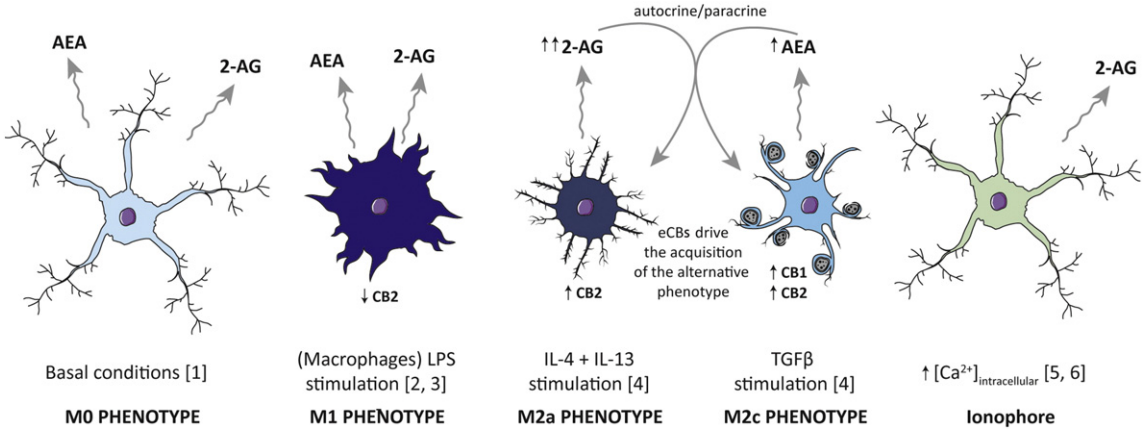

Fig. 5. Microglia phenotype and the synthesis of endocannabinoids. Summary of the reported synthesis of eCBs in microglia in vitro when stimulated with different polarizing agents, as well as under resting conditions and following calcium ionophore stimulation. First, microglia synthetizes 2-AG and AEA under basal conditions. It has been described that after LPS stimulation, M1 macrophages can increase the synthesis of the two main eCBs and downregulate the receptor CB2. Selectively, M2a and M2c microglia synthetize 2-AG and AEA, respectively, increase the expression of the receptor CB2, and drive the acquisition of an alternative phenotype. When treated with a calcium ionophore, microglia also synthesizes 2-AG.

microenvironment is constantly changing in response to inflammation or injury and thus the response of microglial cells must be dynamic to adapt to those changes. Indeed, eCBs are synthesized in vitro in LPS-stimulated cells and under basal conditions (Di Marzo et al., 1999; Carrier et al., 2004; Liu et al., 2006) but, as alternative activation states are not acquired in these circumstances, microglial cells not only require eCB production but also the aforementioned upregulation of CB receptors in order to induce M2 polarization. The activation of eCBSS through CB2 receptors produces an anti-inflammatory profile in human macrophages and foam cells (Chiurchiù et al., 2014). Therefore, the evidence presented here sustains the idea that pro- or anti-inflammatory signals in microglial cells can modulate the eCBSS in a neuroinflammatory scenario, selectively affecting some of its components. In summary, anti-inflammatory stimuli could first alter the cannabinoid machinery to favor an increase in the synthesis of 2-AG and AEA, and to enhance the production of CB receptors, mainly CB2. Subsequently, the eCBs released by microglia could act in an autocrine or paracrine manner, mediating specific actions through CB receptors that are critical for the expression of markers of alternative phenotypes and for phagocytosis. This situation reflects the smooth and coordinated effects of CBs on the phenotypic changes of microglia, together with their influence on phagocytosis, proliferation and migration. Such phenomena could underlie the protective and immunomodulatory effects of eCBSS in the control and restoration of homeostasis in response to CNS inflammation.

3. Targeting microglia: therapeutic implications of cannabinoids in neuroinflammation

Immune cells and microglia appear to express the entire machinery that constitutes a functional eCBSS and it is widely recognized that CBs inhibit neuroinflammation, as it has been mentioned before. Hence, the inflammatory process can be pharmacologically controlled by CBs to avoid tissue damage and disease by regulating some of the many events involved, including: (i) limitation of immune trafficking into the CNS acting at the BBB level (Mestre et al., 2009, 2011); reducing brain immune reactivity (Arévalo-Martín et al., 2003); and (ii) affecting microglia/macrophages responses through modulation of their activation profile towards an anti-inflammatory and reparative one (Lourbopoulos et al., 2011; Mecha et al., 2015). Beside their immunomodulatory actions, CB ligands and inhibitors of MAGL are neuroprotective in AD models in vitro and in vivo (Iuvone et al., 2004; Ramirez et al., 2005; Piro et al., 2012) and PD (Nomura et al., 2011). CBs appear to ameliorate MS symptoms in animal models and in the EAE model, since Δ^9Tetrahydrocannabinol (THC) administration to guinea pig and rats avoids the clinical development of the disease when given prior to inoculation, whereas it delays and reduces the symptoms when administered after inoculation, as well as diminishing spinal cord inflammation (Lyman et al., 1989). THC administered daily prior to the onset of EAE symptoms is also associated with lower incidence and shorter mean duration of EAE, but not a lower mean severity of disease (Wirguin et al., 1994). The administration of eCBs, or selective inhibitors of AEA re-uptake (AM374) and hydrolysis (AM404: FAAH inhibitor), which in turn enhances the endogenous levels of AEA and possibly 2-AG, ameliorates spasticity in the chronic model of EAE (Baker et al., 2001). Administration of 2-AG also ameliorates the acute and chronic phase of the disease in EAE, accompanied by a reduced axonal pathology and polarization of microglia/macrophages towards an M2 phenotype (Lourbopoulos et al., 2011). In this line, treatment with the ABDH6 inhibitor WWL70, which in turn increases 2-AG levels in the brain, ameliorates EAE clinical signs, T cells infiltration and microglia activation through a mechanism that involves CB2 receptors as necessary partners, since these therapeutic effects are absent in EAE mice when co-administered with a CB2-antagonist or in CB2$^{-/-}$ mice (Wen et al., 2015).

In the TMEV-IDD model, there is growing evidence that enhancing eCBSS by exogenous administration of CBs or by its pharmacological modulation has therapeutic effects (Arévalo-Martín et al., 2003; Ortega-Gutiérrez et al., 2005; Centonze et al., 2007; Stella, 2009; Mestre et al., 2011). Moreover, the neuroprotective and anti-inflammatory effects of cannabinoids have been confirmed in other CNS diseases and models of injury. In AD models, CB receptor agonists reduce nitrite levels and the migration of microglia in vitro, while preventing cytokine gene expression and learning deficits in vivo (Martin-Moreno et al., 2011). In addition, CBs increase β-amyloid clearance across the choroid plexus (Martin-Moreno et al., 2012). In the same line, a CB2 selective agonist termed AM1241 delays motor impairment in a model of amyotrophic lateral sclerosis (ALS: Kim et al., 2006a, 2006b). The involvement of CBs in neuroprotection and immunomodulation was also evident in models of stroke, since CB2 activation has been shown to diminish the cerebral infarct volume and to improve motor function likely associated with a reduction in leukocyte rolling and adhesion to the BBB (Zhang et al., 2007a, 2007b). Indeed, the implication of the receptor CB2 is evident in stroke as CB2$^{-/-}$ mice develop a larger cerebral infarct area and worse neurological function compared to wild type mice (Zhang et al., 2009). Other reports also highlight the importance of CBs receptor activation for the therapeutic treatment of stroke (Murikinati et al., 2010; Tuma & Steffens, 2012) and ischemic injury (Fernández-López et al., 2006). In PD, controversial results associated with CB signaling showing detrimental or beneficial effects in the disease outcome have been reported. CB1 receptors are highly expressed in areas like striatum, basal ganglia and substantia nigra pars reticulata, antagonizing D1 and D2 receptor mediated behaviors in medium spiny neurons (Martín et al., 2008). The existence of striatal CB1/D2 receptor heteromers explaining the antagonistic CB1/D2 receptor interactions has been also demonstrated (Marcellino et al., 2008). Recent data suggest that CB1 receptors could be therapeutic targets to regulate the imbalance of glutamatergic and GABAergic neurons in PD (Heumann et al., 2014). Although CB1$^{-/-}$ mice display less severe dyskinesia (Perez-Rial et al., 2011) an indecisive effect of CB1 antagonism has been suggested depending on the animal species. Given the immunomodulatory actions of CB2 agonists, pharmacological effects of CBs in various models of PD include anti-inflammatory, antioxidant and neuroprotective actions (reviewed in More & Choi, 2015), which could in turn improve the motor disability and brain function in PD. Besides the above mentioned effects on the polarization of microglia towards a reparative phenotype, the immunomodulatory effects of CB2 activation include the enhanced release of anti-inflammatory factors like IL-10 (Correa et al., 2010), while decreasing or even inhibiting pro-inflammatory ones such as IL-1β, IL-6, TNFα, CCR2 or iNOS (Puffenbarger et al., 2000; Facchinetti et al., 2003; Ramirez et al., 2005; Racz et al., 2008; Lu et al., 2015; Ma et al., 2015; Malek et al., 2015). Alternatively, CBs also exert immunomodulatory effects on cells of the immune system, like macrophages, T and B cells, shifting the cytokine balance towards an anti-inflammatory profile (Rom & Persidsky, 2013). In summary, and based on the plethora of immunomodulatory actions of CBs in microglia and in the immune system, these compounds show huge potential for pharmacological manipulation during the neuroinflammatory process in order to avoid chronic activation of the system. Moreover, they could promote a reparative environment so that the balance is restored to a more homeostatic state.

Our effort is aimed at establishing consensus views on the relevance of the eCS for human CNS health and disease, as well as to highlight new challenges and therapeutic approaches. Regarding neuroinflammatory conditions and microglia the potential application of eCS-related drugs include CB2 agonists (rodent models of neuroinflammatory diseases), FAAH and MAGL inhibitors (MS models, EAE and TMEV-IDD), as well as inhibitors of eCB uptake (results from TMEV-IDD). Some disorders related to neuroinflammation trigger a protective upregulation of CB2 receptors that when activated can ameliorate the symptomatology of these diseases (PD and MS). This raises the possibility that using a

partial cannabinoid receptor agonist may display a greater benefit-to-risk ratio than a full cannabinoid receptor agonist (Pertwee, 2012). There are cannabis-derived phytocannabinoids with biological activity and therapeutic potential in animal models of CNS diseases with an inflammatory component. Interestingly, a near 1:1 ratio of cannabidiol and THC (Nabiximols; Sativex®) has beneficial effects for MS patients to alleviate neuropathic pain spasticity and other symptoms. It has been found too that Sativex® shows efficacy in animal models of MS, EAE and TMEV-IDD as a disease modifying drug (Feliú et al., 2015; Moreno-Martet et al., 2015) with a reduction of microglial activity. The information presented in this review has come almost entirely from preclinical research. Therefore there is now a need for clinical trials designed to verify the efficacy and safety of the best drugs selected for potential strategies to treat neuroinflammatory disorders. To date the therapeutic exploitation of eCS-based medicines seems still behind our scientific knowledge of eCB signaling with questions that still await an answer.

4. Summary and conclusions

Microglial cells play a crucial role in neuroinflammation. The ontogeny and functions of these cells make them indispensable to maintain homeostasis in the CNS, responding to the changing environment by polarizing rapidly and dynamically to acquire specialized inflammatory or reparative phenotypes in the CNS parenchyma milieu. Between the classic and alternative states of activation, surveillant microglia can adopt a wide spectrum of changes in morphology, expressing distinct mediators that allow them to efficiently kill, phagocyte and reconstruct tissue, returning the CNS to its normal state. A new layer of complexity has been added through the evidence that CBs and their endogenous counterparts, the eCBs, can modulate the activity and profiles in an anti-inflammatory sense. This makes them a promising therapeutic tool to avoid the detrimental effects of chronic inflammation, and to promote a protective and reparative scenario in neurodegenerative diseases. The evidence presented in this review shows that eCBs constitute a defense mechanism that prevents the propagation of neuroinflammation and its consequences. When increased endogenously or administered exogenously, these compounds can apparently exert therapeutic effects by targeting microglia, alleviating disease symptoms and modifying the progression of neuroinflammatory and neurodegenerative diseases.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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