REVIEW

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CB_1 Receptor Signaling in the Brain: Extracting Specificity from Ubiquity

Arnau Busquets-Garcia*,[1,2,4], Jaideep Bains*,[3,4] and Giovanni Marsicano*,[1,2,4]

[1]INSERM U1215, NeuroCentre Magendie, Team 'Endocannabinoids and Neuroadaptation', Bordeaux, France; [2]Université de Bordeaux, Bordeaux, France; [3]Department of Physiology and Pharmacology, Hotchkiss Brain Institute, University of Calgary, Calgary, AB, Canada

Endocannabinoids (eCBs) are amongst the most ubiquitous signaling molecules in the nervous system. Over the past few decades, observations based on a large volume of work, first examining the pharmacological effects of exogenous cannabinoids, and then the physiological functions of eCBs, have directly challenged long-held and dogmatic views about communication, plasticity and behavior in the central nervous system (CNS). The eCBs and their cognate cannabinoid receptors exhibit a number of unique properties that distinguish them from the widely studied classical amino-acid transmitters, neuropeptides, and catecholamines. Although we now have a loose set of mechanistic rules based on experimental findings, new studies continue to reveal that our understanding of the eCB system (ECS) is continuously evolving and challenging long-held conventions. Here we will briefly summarize findings on the current canonical view of the 'ECS' and will address novel aspects that reveal how a nearly ubiquitous system can determine highly specific functions in the brain. In particular, we will focus on findings that push for an expansion of our ideas around long-held beliefs about eCB signaling that, while clearly true, may be contributing to an oversimplified perspective on how cannabinoid signaling at the microscopic level impacts behavior at the macroscopic level.

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SIGNALING OF CB_1 RECEPTORS IN THE BRAIN: INTRINSIC OR EMERGING FEATURES?

Despite the ever-growing complexity of the data and the constant addition of new players, most of what is known in the brain concerning the functions of the endocannabinoid (eCB) system (ECS) refers to type 1 cannabinoid (CB_1) receptors. Therefore, in sake of brevity, this short review will mainly focus on the properties of CB_1 receptors. The reader is referred to recent papers and reviews for enlarged visions of the ECS in the central nervous system (CNS), such as, for instance, the potential importance of type 2 cannabinoid (CB_2) receptors in certain central functions (Fernandez-Ruiz *et al*, 2008; Li and Kim, 2016; Navarro *et al*, 2016; Onaivi *et al*, 2012; Ortega-Alvaro *et al*, 2011) or of non-cannabinoid receptor targets of eCBs (Di Marzo *et al*, 2002; Sigel *et al*, 2011).

CB_1 receptor is a seven transmembrane G protein-coupled receptor (GPCR), and its properties encompass a great deal of molecular, cellular and functional complexity. It is well known that similarly to many other GPCRs (Gentles and Karlin, 1999), the coding region of the cannabinoid receptor type 1 (*cnr1*) gene is intronless. This means that the expression of the *cnr1* gene will have one major RNA processing event to skip, accelerating its protein expression. This advantage may have implications related to the biological functions of the CB_1 receptors (Onaivi *et al*, 1999). Nevertheless, the presence of splice isoforms both in humans and mice (Ruehle *et al*, 2017), coming from 5′-UTR introns of the gene, and possible post-translational modifications demonstrates that CB_1 receptors can come in different flavors already at transcriptional and translational level, with potential signaling differences (Bagher *et al*, 2013; Oddi *et al*, 2017; Straiker *et al*, 2012). Besides these gene expression variables, however, a number of recent observations indicate that CB_1 receptor signaling is pleiotropic and depends on

*Correspondence: Dr A Busquets-Garcia, INSERM U1215, NeuroCentre Magendie and Université de Bordeaux, Team 'Endocannabinoids and Neuroadaptation', 146, rue Leo Saignat, 33077 Bordeaux, France; Tel: +33 5 5757 3756; Fax: +33 5 5757 3751 E-mail: arnau.busquets-garcia@inserm.fr, Dr J Bains, Department of Physiology and Pharmacology, Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Drive NW, Calgary, AB, Canada T2N4N1; Tel: +14032207585; Fax: +14032832700. E-mail: jsbains@ucalgary.ca, Dr G Marsicano, INSERM U1215, NeuroCentre Magendie and Université de Bordeaux, Team 'Endocannabinoids and Neuroadaptation', 146, rue Leo Saignat, 33077 Bordeaux, France; Tel: +33 5 5757 3756; Fax: +33 5 5757 3751. E-mail giovanni.marsicano@inserm.fr
[4]These authors contributed equally to this work.
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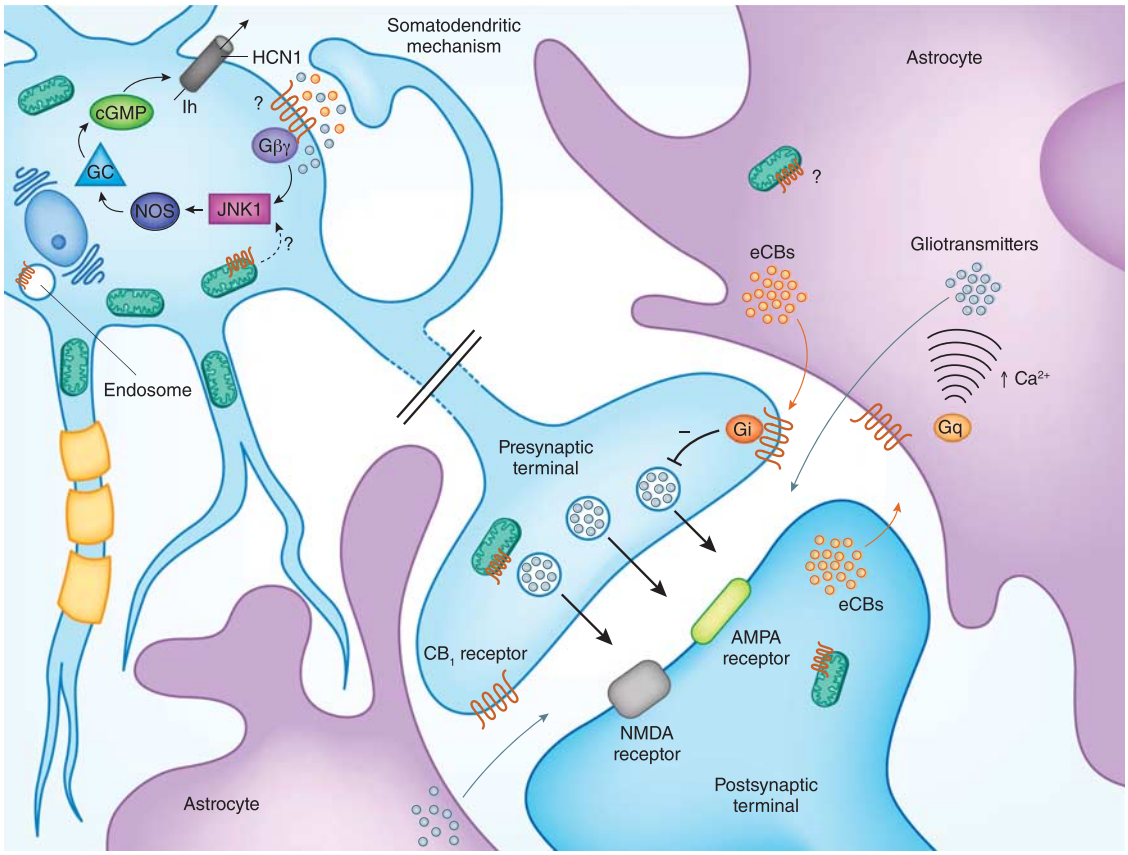

Figure 1. Schematic view of potential localizations of CB_1 receptors at the synapse. CB_1 receptors are present at both presynaptic terminals and postsynaptic compartments of neurons and on astrocytes, exerting different impacts at the tripartite synapse. Whereas the presynaptic plasma membrane localization is long recognized, new evidence points to the presence of CB_1 at mitochondrial membranes of both presynaptic and somatodendritic compartments of neurons, although their specific functions are still to be fully determined. The presence of CB_1 at postsynaptic plasma membranes is possible, but no direct anatomical evidence for this exists so far. Endosomal CB_1 expression has been also proposed by different studies. CB_1 (and possibly $mtCB_1$) receptors are present in astrocytes, where they control astroglial synaptic functions. For additional information see the main text.

several additional factors, such as its cellular and subcellular localization. In this section, we will discuss the heterogeneity of CB_1 receptor signaling in the CNS. Specifically, we will address the differential signaling properties of CB_1 receptors present in different brain cells and in different subcellular locations (Figure 1). In addition, we will underline how the ECS is endowed with specific regulatory mechanisms at cellular level.

Coupling Between CB_1 Receptors and G Proteins: Cell-Type Specificity

The brain is the most heterogeneous organ of the body and arguably the most complex biological system in nature. This is an acquired concept that is reported in all textbooks dealing with the issue. Nevertheless, for obvious limits of deep knowledge and difficulties in conceptualizing a 'machine' that is continuously changing, textbooks often tend to provide 'frozen' pictures of the cellular processes of brain cells and of their interactions. In other words, neuroscientists are aware that the brain is a complex and ever changing system, but its molecular elements are seen as 'static' bricks whose intrinsic properties combined together give rise to the complex phenomena allowing the brain to do all the beautiful things it does. In this perspective, receptors, channels, neurotransmitters, and all the molecular 'bricks' of the brain would have few or many intrinsic functions, and the complexity would rise from the combination of such individual modules. Thus, for instance, receptors were generally considered to induce the same effects in the cells expressing them. CB_1 receptors have been classically described to activate or inhibit a series of intracellular cascades and we tend to assign these effects to the receptor at the different locations where it is present. Recent advances, however, suggest that CB_1 receptors seem to have only a few 'intrinsic' signaling properties, but their effects largely 'emerge' from specific temporal and spatial constraints. For instance, CB_1 receptors can regulate different G proteins in brain cells, and this ability seems to largely depend on the 'context' (cell type, subcellular location, cellular functional state, and so on) where they are activated. Far from providing a fully comprehensive account of CB_1 receptor signaling in the brain, the next section will provide some key examples to argue for the 'emerging' properties of CB_1 receptors.

Different Cells, Different G Protein Machinery?

CB_1 receptors likely have the highest expression of any GPCR in the brain, with amounts of protein comparable to NMDA and $GABA_A$ receptors (Freund *et al*, 2003; Herkenham *et al*, 1990; Howlett *et al*, 2002; Piomelli, 2003). They are present in many different cell types, but their levels of expression are astonishingly variable amongst different locations. Thus, cortical GABAergic interneurons contain prodigious levels of CB_1 receptor protein, whereas cortical glutamatergic neurons have much lower levels of these receptors. By contrast, expression levels are relatively low in hypothalamic regions (Wittmann *et al*, 2007), but functional studies indicate that both glutamate and GABA neurons express CB_1 receptors at similar levels (Wamsteeker and Bains, 2010a). Astroglial cells and, possibly, other glial cells likely contain even lower amounts of the protein (Han *et al*, 2012; Mato *et al*, 2009; Navarrete and Araque, 2008). Other neuronal types, such as noradrenergic, cholinergic, serotonergic, possibly dopaminergic, and others, also contain low-to-moderate levels of CB_1 receptor protein (Marsicano and Kuner, 2008). If the signaling of CB_1 receptors was an intrinsic property, one would expect that the levels of agonist-induced recruitment of G proteins are proportional to the levels of expression. However, this does not seem to be the case. Early studies showed that brain regions containing relatively low levels of CB_1 receptor, such as the hypothalamus, display higher levels of cannabinoid-dependent signaling than regions expressing much higher levels of the protein (Breivogel *et al*, 1997). More recent studies using conditional mutant mice lacking CB_1 receptor expression in specific neuronal subpopulations indicate that within the same brain region (hippocampus), the G protein activation by CB_1 receptors expressed in glutamatergic neurons is much stronger than the one induced in GABAergic interneurons (Steindel *et al*, 2013). Thus, deletion of the receptor from cortical glutamatergic neurons (Glu-*CB_1*-KO mice) only slightly reduces agonist binding and protein expression (<10–20%), but it decreases G protein activation (as measured by GTPgamma-binding assays on tissue extracts) by ~50%. Conversely and surprisingly, deletion of the *CB_1* gene in forebrain GABAergic neurons (GABA-*CB_1*-KO mice) strongly reduces the amount of protein in the hippocampus (more than 90%), but it induces a lower decrease of G protein activation than in Glu-*CB_1*-KO mice (Steindel *et al*, 2013). This indicates a much higher efficacy of G protein-dependent signaling of CB_1 receptors in hippocampal glutamatergic neurons than in neighboring GABAergic interneurons. The reasons of these cellular differences are not known at the moment. As also discussed below, the different abundance of CB_1 receptor protein in hippocampal GABAergic *vs* glutamatergic neurons can induce different stoichiometric relationships between the receptor and G proteins, thereby changing the efficiency of coupling. However, these data indicate that G protein-coupling efficacy is clearly not an intrinsic property of CB_1 receptors, but it is an emerging feature, depending on the cell type or subcellular compartment where they are expressed. Importantly, these processes can explain the huge cell-type-specific plethora of behavioral effects induced by cannabinoid drugs, as we will see below. Moreover, as described below, G protein-independent signaling (eg, arrestins) (Raehal and Bohn, 2014; Turu and Hunyady, 2010; Rozenfeld and Devi, 2008) can also be found and are important to consider when talking about CB_1 receptor signaling.

The design logic for why certain cell types (eg, cortical GABAergic interneurons) express high levels of CB_1 receptors and yet exhibit low-efficiency signaling through G proteins remains elusive. One hypothesis is that the pool of CB_1 receptors in these cells might function as a sort of reservoir that is available to be rapidly used in certain conditions. Complementary to this idea, it is interesting to note that CB_1 receptors were shown several years ago to be able to 'sequester' $G_{i/o}$ proteins (Vasquez and Lewis, 1999) making them unavailable to other GPCRs expressed in the same cells. This could theoretically explain why large amounts of the receptor are present, but 'silent' in certain cell types. However, if these *in vitro* results apply also to *ex vivo* or *in vivo* conditions remains to be explored. The presence of CB_1-interacting proteins could have a role in the cell-specific modulation of cannabinoid signaling. For instance, CB_1 receptors have been proposed to form homodimers (Wager-Miller *et al*, 2002) and heterodimers with other GPCRs (Hudson *et al*, 2010), including, for instance, dopamine D2 (Kearn *et al*, 2005; Khan and Lee, 2014), opioid (Hojo *et al*, 2008), orexin (Perrey *et al*, 2014), serotonergic (Vinals *et al*, 2015), or CB_2 receptors (Callen *et al*, 2012). These potential physical interactions are obviously cell-type-specific, and can modify the signaling of different pools of CB_1 receptors. In this sense, two recent studies showed how (i) heteromers between CB_1 receptors and adenosine A_{2A} displays a striking G protein-coupling signaling profile where the stimulation of both receptors reduces the downstream signaling (Moreno *et al*, 2017); (ii) the formation of heteromers between CB_1 receptors and D2 receptors changes the agonist-mediated CB_1 receptor signaling and coupling (Bagher *et al*, 2016). More research is needed to better understand the functional impact of the formation of homomers or heteromers between CB_1 receptors and other brain receptors in physiology and pathology.

Besides GPCR homo- or heterodimerization, other CB_1-interacting proteins have been identified (Smith *et al*, 2010), which can greatly determine differential eCB signaling in the brain. For instance, the cannabinoid receptor-interacting protein 1A (CRIP1A) has been proposed as one of the examples of interacting proteins that can modify CB_1 receptor signaling (Blume *et al*, 2017; Niehaus *et al*, 2007). This can have important functional implications, because CRIP1A is present in specific brain cell types (Guggenhuber *et al*, 2015; Smith *et al*, 2015). For instance, CRIP1A is co-expressed with CB_1 receptors in pyramidal neurons and in a subpopulation of GABAergic interneurons in the hippocampus (Guggenhuber *et al*, 2015), thereby likely determining the ability of eCBs to regulate excitatory and inhibitory

neurotransmission in specific cell subpopulations. However, CRIP1A is also present in cells such as dentate granule cells or other cell types where CB_1 receptors are likely not expressed (Smith *et al*, 2015), indicating that this protein might have additional functions in the brain.

Same Receptor, Different Repertoire of G Proteins?

The identification and discovery of CB_1 receptors finds its roots in the observation that exogenous cannabinoids are able to specifically modulate cAMP levels in cultured cells (Howlett, 1984, 1987; Howlett *et al*, 2002). Thus, the first G protein-dependent pathway described for CB_1 receptor signaling posited an inhibition of adenylyl cyclase (AC) activity through activation of $G_{i/o}$ proteins. Early studies added the AC-dependent or -independent regulation of specific ion channels and, importantly, the impact of CB_1 receptor signaling on other intracellular cascades, such as MAP kinases and others (for extensive review, see Howlett (2005); Nogueras-Ortiz and Yudowski (2016)). The exclusive coupling of CB_1 receptors with $G_{i/o}$ proteins was challenged years ago, when an interesting functional interplay between CB_1 and dopamine D2 receptors was identified, indicating that their functional and/or physical interaction is able to switch CB_1 receptor signaling from $G_{i/o}$ to G_s (Glass and Felder, 1997; Kearn *et al*, 2005). Considering that CB_1 and D2 receptors are co-expressed in a limited subpopulation of brain neurons (in basal ganglia and other brain regions (Hermann *et al*, 2002; Marsicano and Kuner, 2008)), this can be considered as one of the first examples of an emerging property of cannabinoid signaling: the presence of active or inactive D2 receptors determines the outcome of CB_1 receptor stimulation.

Earlier studies demonstrated how neurotransmission could be modulated by CB_1 receptors through the inhibition of calcium channels independently of cAMP, suggesting direct G protein-dependent mechanisms (Mackie and Hille, 1992; Twitchell *et al*, 1997). Moreover, adding a bit more complexity to the picture, recent work indicates that the G protein coupling of CB_1 receptors definitely extends far beyond $G_{i/o}$. For instance, whereas the presynaptic control of neurotransmitter release in neurons is compatible with an inhibitory effect on AC signaling, it was recently shown that blockade of $G_{i/o}$ in the globus pallidus can switch the effect of CB_1 receptors toward activation of G_s and potentiation of neurotransmission (Caballero-Floran *et al*, 2016). In this sense, early studies also showed how CB_1 receptors can couple to G_s, demonstrating the signaling complexity of CB_1 receptors (Glass and Felder, 1997). Another clear example of multiple CB_1-dependent G protein signaling is in astrocytes. In this cell type, activation of CB_1 receptors increases intracellular calcium, which is likely mediated by G_q proteins (Navarrete and Araque, 2008). Together with a previous work (Prather *et al*, 2000), a recent thorough study specifically aimed at identifying the G proteins activated by CB_1 receptors in the mouse cortex using a combination of GTPgamma-binding assays coupled to specific immunoprecipitation with antibodies against different subtypes of G proteins (Diez-Alarcia *et al*, 2016). The results clearly identified specific CB_1 receptor coupling with different subunits of the classic inhibitory $G_{i/o}$, but also with $G\alpha_z$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Prather *et al*, 2000; Diez-Alarcia *et al*, 2016). Interestingly, Diez-Alarcia and colleagues (2016) observed biased signaling patterns depending on the cannabinoid used, suggesting not only that CB_1 receptors can couple to different G proteins but also that different endogenous or exogenous ligands might preferentially direct the signaling toward specific pathways. Given that CB_1 receptors expressed in different cell types in the brain have differential effects (Busquets-Garcia *et al*, 2015), we suggest that this heterogeneity in G protein coupling by CB_1 receptors and the associated biased actions of specific agonists is at least partially due to cell-type-specific expression of the receptor. It would be extremely interesting to test whether the G protein activation patterns might be altered, possibly in a cell-type-dependent manner, under different physiological or pathological conditions. Indeed, as mentioned above, pathophysiological conditions such as stress or excessive neuronal activity markedly alter CB_1 receptor signaling. Future studies will address these new possibilities, potentially making the brain ECS one of the first targets of a new signaling- and/or cell-type-specific pharmacology, which will be essential for interventional therapies against neurological and psychiatric disorders.

Beyond G Protein Signaling

Besides the regulation of G protein signaling, CB_1 receptors also activate β-arrestin 1 and 2, which mediate their internalization (Raehal and Bohn, 2014; Turu and Hunyady, 2010), and trigger other intracellular pathways, such as activation of MAPK (Rozenfeld and Devi, 2008). Interestingly, β-arrestin 1 pathways are likely G protein-independent (Ahn *et al*, 2013). Although the respective roles of G proteins, β-arrestin 1 and β-arrestin 2 need to be further clarified, the activation of CB_1 receptors in the brain results in two major downstream effects, namely the regulation of ion channels and of intracellular kinases, which have been reviewed extensively (Freund *et al*, 2003; Howlett, 2005; Howlett *et al*, 2002; Howlett *et al*, 2004; Lu and Mackie, 2016). Briefly, CB_1 receptors can inhibit N- and P/Q-type Ca^{2+} channels, activate different types of K^+ channels and promote phosphorylation of extracellularly regulated kinases (ERKs). More recently, experiments on retinal ganglion cell revealed that CB_1 receptor activation can lead to the AMP-activated kinase-dependent inhibition of the Na-K-Cl co-transporter (NKCC1) activity, eventually reducing intracellular levels of Cl^- (Miraucourt *et al*, 2016).

The inhibition of AC activity by CB_1 receptor recruitment of $G_{i/o}$ proteins leads to a decrease in the levels of cAMP (Howlett *et al*, 2002) and consequently of the activity of the protein kinase A (PKA). G protein-dependent or -independent mechanisms link cannabinoid actions to the activation

of ERK and FAK kinases (Ahn *et al*, 2013; Derkinderen *et al*, 1996; Derkinderen *et al*, 2003). Recent evidence points to the idea that CB_1 receptors can activate the main target of rapamycin (mTOR) pathway, a key intracellular signaling regulating protein synthesis and synaptic plasticity (Costa-Mattioli and Monteggia, 2013; Laplante and Sabatini, 2012; Puighermanal *et al*, 2009). Notably, recent findings showed that CB_1 receptor activation enhances protein synthesis via the mTOR pathway that control presynaptic local protein synthesis to modulate neurotransmitter release during brain long-term plasticity (Younts *et al*, 2016). Interestingly, the improvement of several behavioral abnormalities observed in a mouse model of fragile X syndrome by blockade of CB_1 receptors is linked to the decreased activation of hippocampal mTOR signaling (Busquets-Garcia *et al*, 2013).

Subcellular Signaling of CB_1 Receptors: Just on Plasma Membranes?

GPCRs, such as CB_1 receptors, are classically seen as plasma membrane proteins located in the optimal position to convert extracellular signals into intracellular responses (Figure 1). Indeed, a high-school student consulting Wikipedia or the Encyclopedia Britannica for a homework project on GPCRs would find these definitions, respectively: 'GPCRs are proteins that detect molecules outside the cell and activate internal signal transduction pathways and, ultimately, cellular responses' (https://en.wikipedia.org/wiki/G_protein%E2%80%93coupled_receptor), or '[GPCR is a] protein located in the cell membrane that binds extracellular substances and transmits signals from these substances to an intracellular molecule called a G protein' (https://www.britannica.com/science/G-protein-coupled-receptor). The plasma membrane position, optimal to 'detect molecules outside the cell' has been demonstrated by a multitude of ultralocalization and functional studies. Indeed, most of the classical knowledge on GPCR biochemistry and biophysics derives from seminal studies on beta-adrenergic receptors (www.nobelprize.org, 2014), which detect adrenalin, a water-soluble hormone. However, the idea that GPCRs are present only at the plasma membrane has been challenged over the years by elegant studies, showing that different types of GPCRs can be functionally located inside the cell (Irannejad *et al*, 2017; Irannejad *et al*, 2013; Jong *et al*, 2014; Tsvetanova *et al*, 2015; for review: Khan *et al*, 2016; Jalink and Moolenaar, 2010). If this is true for GPCRs targeted by water-soluble ligands, it might be even more likely for lipid receptors. Indeed, the largest class of GPCRs in mammals is represented by odor receptors that are generally volatile compounds that dissolve well in organic solvents but have low solubility in water-based media (Forss, 1972). The CB_1 receptor is primarily a lipid receptor: the recent interesting discovery of a novel class of cannabinoid peptides considered negative allosteric modulators of CB_1 receptor (pepcans (Bauer *et al*, 2012; Hofer *et al*, 2015)) aside, the large majority of plant-derived, synthetic, and endogenous arachidonic acid derivatives eCBs are lipids. Despite the clear differences between water-soluble and -insoluble ligands, we lack a comprehensive understanding of the specific features of GPCRs targeted by lipids. One important difference may be how the ligand reaches the receptor. In this context, there is now evidence that eCBs access the binding pocket of CB_1 receptor via the lipid bilayer, suggesting that ligand entry via a lipid portal is quite likely for this GPCR (Hurst *et al*, 2010; Hurst *et al*, 2013; Reggio, 2010). Consistent with this idea, exogenous cannabinoids appear to rely on lateral diffusion through the membrane lipid bilayer to reach their binding sites on CB_1 and CB_2Rs (Kimura *et al*, 2009). Although further studies are necessary, the recent analyses of the crystal structure of the CB1 receptor protein (Hua *et al*, 2016; Shao *et al*, 2016; Hua *et al*, 2017) is compatible with and provide further information on this peculiar binding path of lipid cannabinoids, which implies that these compounds can easily move within cellular membranes. Indeed, a consolidated vision of lipid cellular organization suggests that eukaryotic cell membranes form a sort of unique entity, called the endomembrane system (Morré and Mollenhauer, 1974), within which lipids can easily move either via active or passive mechanisms (Voelker, 1991). Interestingly, early studies showed that many lipid molecules undergo rapid transport amongst different cellular membranes (Voelker, 1991). In certain situations, the access to intracellular compartments might be even easier for eCBs. Although more research is needed, it is interesting to note here that lipid eCBs are produced and degraded within both the plasma membrane and organelle membranes inside cells (Gulyas *et al*, 2004; Morozov *et al*, 2004). For example, the fatty acid amide hydrolase (FAAH) enzyme, which degrades the eCB anandamide (AEA), is present in intracellular membrane constituents (eg, in mitochondria; Morozov *et al*, 2004). Similarly, monoacylglycerol lipase (MAGL), the degrading enzyme of 2-arachidonoyl-glycerol (2-AG), the other major lipid eCB also seems to be present in subcellular compartments (Blankman *et al*, 2007). In agreement with these localization studies, biochemical assays indicate that 2-AG and AEA are present in intracellular purified brain mitochondria and that the dual inhibitor of FAAH and MAGL JZL195 increases eCBs in isolated mitochondria, thereby modulating mitochondrial respiration (Benard *et al*, 2012) (see also below).

In addition to the eCB degradative enzymes, there is also evidence supporting the presence of intracellular CB_1 receptors. Early anatomical studies revealed that a large proportion of CB_1 receptors in brain cells are intracellular (Freund *et al*, 2003). However, given that GPCRs were believed to be functional only at plasma membranes, the presence of intracellular CB_1 receptors was exclusively interpreted as 'trafficking' proteins, caught in the process to be transported to or recycled from their 'natural' functional location, the plasma membrane (Freund *et al*, 2003). Altogether, these observations revealed that the constituent pieces of the ECS are present inside cells and set the stage for more recent studies demonstrating a

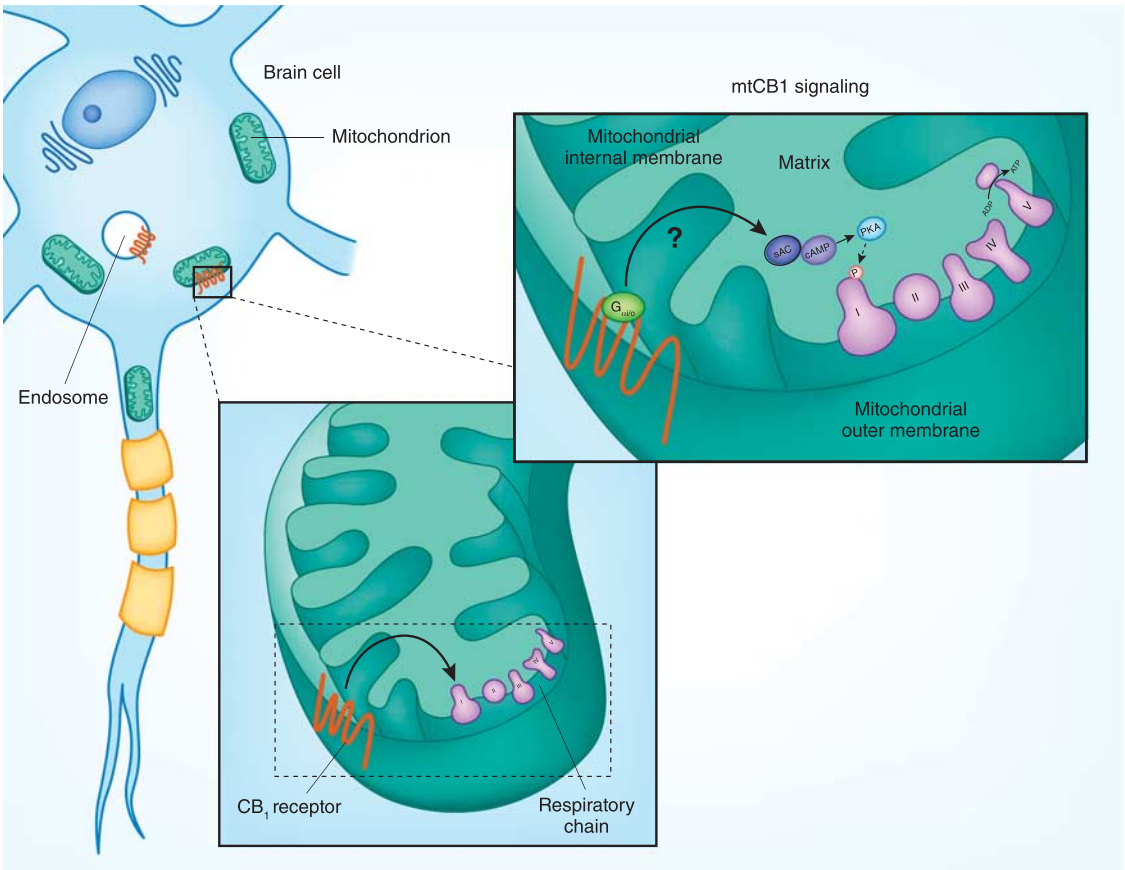

Figure 2. Schematic view of the $mtCB_1$-dependent signaling pathway. CB_1 receptors are present in brain mitochondria likely in the external membrane where they regulate the respiratory chain and ultimately the mitochondrial functions (eg, ATP production). On the right, we represented the signaling pathway downstream $mtCB_1$ receptors. It has been described that $mtCB_1$ receptors mediate its effects involving intra-mitochondrial $G_{i/o}$ protein signaling, mitochondrial cAMP synthesis that is catalyzed by a soluble form of adenylyl cyclase (sAC), and the decrease of intra-mitochondrial PKA activity that also reduced phosphorylation of specific subunits of complex I (eg, NDUFS2). All these events can impair the respiratory chain decreasing mitochondrial respiration, likely affecting other mitochondria functions. For additional information refer to the main text.

functional role for intracellular CB_1 receptor signaling in brain functions.

One of the first pieces of evidence for the presence of intracellular cannabinoid signaling came from the observation that cannabinoids can activate CB_1 receptors localized in late endosomal/lysosomal compartments (Thibault *et al*, 2013), where they can trigger G protein-dependent signaling (Rozenfeld and Devi, 2008). Accordingly, a recent study showed how a β-arrestin-mediated signaling from CB_1 receptors could modulate the endocytic trafficking (Delgado-Peraza *et al*, 2016). Perhaps the most complete evidence so far for intracellular functional cannabinoid signaling relies on the presence of CB_1 receptors on mitochondrial membranes. Already in the Seventies of last century, different studies reported effects of cannabinoids on mitochondria, including decrease of complex I or V activities and changes in mitochondrial ultrastructure (Bartova and Birmingham, 1976; Bino *et al*, 1972; Chari-Bitron and Bino, 1971; Mahoney and Harris, 1972; Schurr and Livne, 1975). These effects remained unexplained and, with the identification of CB_1 receptors as typical plasma membrane GPCRs, they were ascribed to unspecific alterations of mitochondrial membrane properties by lipid molecules (Bartova and Birmingham, 1976; Howlett *et al*, 2002) or to indirect CB_1 receptor-dependent signaling (Campbell, 2001).

However, in 2012, electron microscopic immunogold experiments accompanied by controlled functional assays revealed that a small but significant proportion of hippocampal CB_1 receptors are localized at mitochondrial membranes (called mtCB1), where they mediate reduction of O_2 consumption by exogenous and endogenous cannabinoids (Benard *et al*, 2012). Interestingly, similar mitochondrial localization of CB_1 receptors has been also shown in peripheral tissues, such as sperm cells (Aquila *et al*, 2010) and muscles, where the proportion of $mtCB_1$ receptors appears to be higher than in the brain (Mendizabal-Zubiaga *et al*, 2016). The signaling of $mtCB_1$ receptors in the brain started to be deciphered. Pharmacological and genetic experiments showed that the effects of cannabinoids on mitochondrial respiration, cAMP levels, and PKA activity are blocked by pertussis toxin, suggesting an involvement of intra-mitochondrial $G_{i/o}$ protein signaling. Mitochondrial cAMP synthesis is catalyzed by a soluble form of AC (sAC), and manipulation of sAC activity also blocked the effects of

cannabinoids on brain mitochondria. Moreover, a physical interaction between G proteins and sAC was identified in brain mitochondria, which was increased upon cannabinoid treatments, suggesting that $mtCB_1$ signals through a G protein and sAC-dependent intra-mitochondrial pathway. Following the consequent decrease of PKA activity, specific proteins of the OXPHOS chain (in particular of complex I) appear to be less phosphorylated possibly explaining the final effect on O_2 consumption. Importantly, as mentioned below, genetic approaches showed that decreased intra-mitochondrial PKA activity and reduced phosphorylation of a specific subunit of complex I (NDUFS2) are necessary for specific effects of cannabinoids *in vitro* and *in vivo* (Hebert-Chatelain *et al*, 2016) (Figure 2).

The $mtCB_1$-dependent intra-mitochondrial signaling cascade is far from being completely understood and presents surprising elements. In particular, it is notable that sAC is thought to be a G protein-independent form of AC (Buck *et al*, 1999; Chen *et al*, 2000). Indeed, sAC lacks key structural features that allow membrane-bound AC enzymes to interact with G protein (Steegborn, 2014). Therefore, it is not clear yet how $mtCB_1$ receptors could trigger the reduction of O_2 consumption by brain mitochondria via an interaction between G proteins and sAC. It is possible that cell-type- or organelle-specific regulation of sAC activity exists, perhaps mediated by the formation of intra-mitochondrial signaling complexes. In other words, brain mitochondria may express unique complexes that induce the indirect interaction between G proteins and sAC. Consistent with this idea, recent reports demonstrate that sAC is involved in an endocytosis-dependent cAMP response suggesting that the link between GPCRs and sAC depends on a larger scaffolding complex and not just on the activation of GPCRs at the plasma membrane (Inda *et al*, 2016). Clearly, further studies are required to clarify this and other issues linked to the discovery of $mtCB_1$ receptors. These should include efforts directed at identifying both the specific effects of cannabinoids on mitochondrial functions and the potential impact these GPCR–mitochondrial interactions have on ECS function. Indeed, by regulating innumerous cellular processes beyond ATP production, mitochondria exert a plethora of functions that are particularly crucial for one of the most energy-avid organs of the body, such as the brain (Mattson *et al*, 2008).

Endogenous Allosteric Modulation of CB_1 Receptors

CB_1 receptors are endowed with important physiological and endogenous regulatory mechanisms able to enhance or limit their activity in the brain and in the body (Piazza *et al*, 2017). For instance, the endogenous anti-inflammatory lipid lipoxin A4 has been shown to be an allosteric enhancer of CB_1 receptor signaling in the brain. In particular, the presence of lipoxin A4 is able to increase the affinity of AEA at CB_1 receptors, thereby potentiating the signaling and behavioral effects of this eCB. This mechanism likely has an important physiological role in the modulation of CB_1 receptor signaling (Pamplona *et al*, 2012) and might have important behavioral consequences (see below).

Even more intriguing, recent studies showed that the neurosteroid pregnenolone is an allosteric signal-specific inhibitor of CB_1 receptors, able to protect the brain from excessive cannabinoid intoxication (Vallee *et al*, 2014 but see also Krohmer *et al*, 2017; Khajehali *et al*, 2015). Pregnenolone has been long considered as the *per se* biologically inactive precursor of other steroids. Vallee *et al* (2014) showed that high doses of THC or other exogenous cannabinoids impressively increase the levels of pregnenolone in different brain regions. In turn, pregnenolone is able to decrease certain effects of cannabinoids (see below) by binding an identified allosteric site in the sequence of the CB_1 receptor. Very importantly, pregnenolone does not inhibit, like an orthosteric antagonist, all signaling pathways triggered by CB_1 receptor activation. Whereas the decrease of cAMP by cannabinoids is not altered by pregnenolone, the neurosteroid fully inhibits the CB_1-dependent modulation of the ERK pathway and of mitochondrial functions (Vallee *et al*, 2014). This unique mode of action suggests that pregnenolone-derived drugs (more stable and better absorbed than pregnenolone itself) can be used to treat conditions characterized by excessive activation of CB_1 receptors (Piazza *et al*, 2012; Vallee *et al*, 2014). Indeed, after completing preclinical studies showing very interesting properties, clinical trials are running at the moment of writing, aimed at testing the efficacy of a pregnenolone derivative (AEF0117) on cannabis addiction.

Whereas the origin of lipoxin A4 in the brain is not fully elucidated, pregnenolone is produced, like all steroids, by cell mitochondria. Considering that high levels of eCB s have been implicated in the activation of $mtCB_1$ receptors (Benard *et al*, 2012), it is tempting to speculate that a subcellular mechanism might underline the negative feedback actions of pregnenolone. In this sense, activation of CB_1 receptors (possibly directly at mitochondrial membranes) might stimulate the production of pregnenolone to partially inhibit CB_1 signaling in the same subcellular compartment, same cell, and/or in neighboring ones. If confirmed by experimental evidence, this would be a very interesting and sophisticated example of autocrine/paracrine self-control of a receptor system in the brain. In this context, it is also important to mention the recent identification of pepcans, a family of endogenous peptides proposed to exert allosteric regulation of CB1 receptors (Bauer *et al,* 2012).

SYNAPTIC FUNCTIONS OF THE ECS: EXTRACTING SPECIFICITY FROM UBIQUITY

It is clear that the machinery required for the production of eCBs is located at synapses. This means that eCBs release occurs at, or very close to, synaptic sites. For example, at hippocampal glutamate synapses, the 2-AG synthesizing enzyme, DGL-α, is highly accumulated in nanodomains in

the perisynapse region (Katona *et al*, 2006). Similar nanodomain localization has been described in some regions of the cortex and amygdala between CCK-positive basket cells and their targets (Omiya *et al*, 2015). Intriguingly, the basket cells express the vesicular glutamate transporter (vGluT3) at some, but not all their synapses. Thus, postsynaptic elements opposing vGluT3 express DGL-α (Omiya *et al*, 2015 but see also Yoshida *et al*, 2011), suggesting a link between vGluT3 functions and eCB production, which implies selective regulation of eCB signaling at different synapses of the same neuron.

What remains unclear, however, is whether each synaptic site is regulated independently of another site. One of the key features of synapses is that they offer relatively private and privileged communication between one presynaptic element and its postsynaptic partner. eCBs, however, are likely liberated from all sites of production in response to a postsynaptic depolarization. In this sense, as eCBs can be released following GPCR activation (eg, mGLUR5, muscarinic receptors), these molecules can be considered as a coincidence detector of depolarization and GPCR activation (Kano *et al*, 2009). Of course, there will be some decay of the depolarization that will mean release at distant sites is less likely to occur, but this spatial gradient approach to neuronal signaling is curious. By contrast, once mobilized, the spread of the eCBs is very tightly controlled as shown by the demonstration that liberation of eCBs from one pyramidal neuron does not have any effect on CB_1 receptors at synapses on a neighboring neuron (Younts *et al*, 2013). However, in this study, the theta burst stimulation used was not able to activate CB_1 receptors on Schaffer collaterals to produce long-term depression (LTD). As mentioned above, this can be caused by the highly specialized basket cell synapses onto CA1 pyramidal neurons (Omiya *et al*, 2015; Yoshida *et al*, 2011).

Indeed, all cells examined so far are capable of producing eCBs that, in turn, can exert biological effects. Yet, the highly specific localization of CB_1 receptors, their differential signaling effects, the strong dependence of these effects on neuronal circuit activity, and the strong temporal regulation of eCB mobilization indicate that this ostensibly unspecific mode of action is, in fact, highly regulated.

At the cellular, or synaptic level, eCBs have been conceptualized as 'circuit breakers' (Katona and Freund, 2008,2012). This view is derived from a vast literature showing that once liberated from the postsynaptic cell, eCBs act on presynaptic CB_1 receptors to decrease the probability of release. Although this review is focused on CB_1 receptors, some works identified TRPV1 channels as an additional player in this concept of circuit breaker suggesting a more complex scenario (Puente *et al*, 2011; Chávez *et al*, 2010). Yet, linking this circuit breaker function to the output of a neural network, let alone behavior, has remained elusive. Are there pieces of the eCB puzzle that remain hidden? (likely). Are we over-simplifying, to make convenient links between what we currently know at the microscopic level, to what we know at the macroscopic level? (perhaps). How do we begin to bridge the divide to make a more explicit link between synaptic/cellular signaling and behavior? In an attempt to solve this complex puzzle, and before entering into the behavioral aspects, we will discuss different synaptic concepts including 'on demand' and 'tonic' activity of the ECS, specific mechanisms of eCB mobilization, the synaptic role of CB_1 receptors in astrocyte, or the role for postsynaptic CB_1 receptors.

Circuit Breakers and Synaptic Discriminators

eCBs exert the majority of their known actions by directly targeting CB_1 receptors located on presynaptic nerve terminals. Many outstanding research papers and reviews have been written describing how either depolarization of the postsynaptic neuron or activation of GPCRs can liberate eCBs. Information about this can be found in several excellent reviews (Castillo *et al*, 2012; Freund *et al*, 2003; Kano *et al*, 2009; Katona and Freund, 2012; Piomelli, 2003; Araque *et al*, 2017) and will not be covered in detail here. Briefly, these molecules, either 2-AG or anandamide, are produced at postsynaptic level, traverse the synaptic cleft, and bind to presynaptic CB_1 receptors. These GPCRs act either directly on vesicular release machinery or at Ca^{2+} channels to decrease the probability of neurotransmitter release (Pr). The simple take-away message is that eCBs weaken synaptic connections, effectively functioning as very efficient, synaptic 'circuit breakers' (Katona and Freund, 2008). The circuit breaker concept emerges from early findings that deletion of CB_1 receptors exclusively from excitatory neurons causes hyperexcitability and predisposes the brain to epileptic-type seizures (Marsicano *et al*, 2003; Monory *et al*, 2006). As a breaker in an electrical circuit protects the circuit from a power surge, 2-AG acting at presynaptic CB_1 receptors on glutamate terminals appears to protect, through a negative feedback, from excessive excitatory neurotransmission. Moreover, several observations indicated that there may be additional nuances that should be considered. First, as eCBs, like 2-AG, are mobilized from multiple synapses during depolarization of the postsynaptic cell, they will impact numerous inputs (provided that these inputs express functional CB_1 receptors). This means that in some brain regions, both excitatory and inhibitory transmission will be affected (Wamsteeker *et al*, 2010b). If the neuronal output is simply an algebraic sum of excitatory and inhibitory synaptic inputs, then it is difficult to envision how this scenario would alter network activity. Clearly, there are additional elements that must be considered. In some systems, CB_1 receptor expression is biased toward GABA synapses, rather than glutamate synapses (Katona and Freund, 2012), suggesting that specificity of eCB signaling lies in the differential expression of CB_1 receptors on nerve terminals. Specificity could also result from highly regionalized mechanisms that control the spread of eCBs from one synapse to another (Younts *et al*, 2013), but how exactly this is regulated is not clear. An alternate view is that eCBs, by decreasing Pr, create a scenario whereby inputs originating from neurons that spike at particular rates are favored (Foldy *et al*, 2006). For

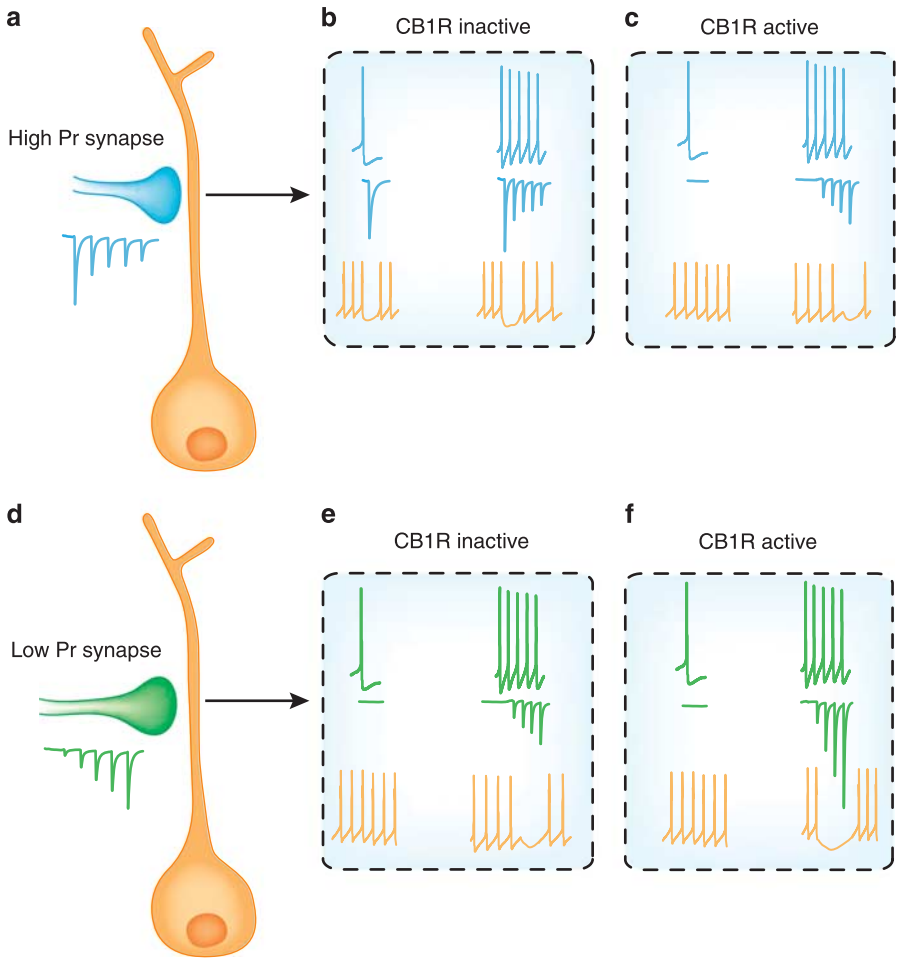

Figure 3. CB_1-mediated effects, release probability, and presynaptic activity on postsynaptic firing. (a) Schematic depiction of a GABA synapse (blue) that has a high initial release probability. With rapid, repeated activation of the presynaptic neuron, GABA release decreases. (b) Blue traces show the spike patterns in the presynaptic neuron and the putative synaptic response immediately below in a condition when CB_1 receptors are not recruited. In orange, the activity of postsynaptic neuron and the effect of the inhibitory event. Note that a single presynaptic action potential is sufficient to elicit a pause in firing of the postsynaptic neuron. A burst of presynaptic action potentials elicits a pause that is marginally longer, but rapid synaptic depression allows postsynaptic firing to resume quickly. (c) When CB_1 receptors are recruited, a single action potential evokes no release and consequently, postsynaptic firing is unaffected. A burst of presynaptic action potentials, however, results in synaptic facilitation and a prolonged pause in postsynaptic firing. (d) Schematic depiction of a GABA synapse (green) that has a low initial release probability. With rapid, repeated activation of the presynaptic neuron, GABA release increases. (e) Green traces show the spike patterns in the presynaptic neuron and the putative synaptic response immediately below in a condition when CB_1 receptors are not recruited. In orange, the activity of postsynaptic neuron and the effect of the inhibitory event. Note that a single presynaptic action potential has no effect on firing of the postsynaptic neuron. A burst of presynaptic action potentials results in synaptic currents that facilitate and cause a delayed pause in firing of the postsynaptic neuron. (f) When CB_1 receptors are recruited, a single action potential still evokes no release and again, postsynaptic firing is unaffected. A burst of presynaptic action potentials, however, results in very profound synaptic facilitation and a prolonged pause in postsynaptic firing.

example, decreasing Pr does weaken synapses when assessed as the response to a single presynaptic action potential. The decrease in Pr, however, also means that during a train of action potentials, synapses can facilitate more readily, resulting in an increase, rather than a decrease in synaptic strength. This has been demonstrated in hypothalamus where blockade of CB_1 receptors increases Pr at GABA synapses, but weakens inhibition to the postsynaptic neuron when GABA inputs are recruited at a high frequency (Oliet *et al*, 2007). This suggests that CB_1 receptors at some synapses may have some basal or constitutive signaling, even in the absence of ligand (Lee *et al*, 2015). Alternatively, local depolarization via kainate receptors (Lourenco *et al*, 2010) could be a critical regulator of this tonic CB_1 receptor activity. In addition, as eCBs decrease the release of quantal events (in the absence of action potentials in the nerve terminal), they may further act to filter specific inputs by increasing the ratio of signal to noise. This suggests that in addition to their role as circuit breakers, eCBs are synaptic discriminators that promote selective communication between neurons at higher frequencies (Figure 3).

The scenario outlined above paints the presynaptic neuron as a passive element that is influenced by eCBs if CB_1 receptors are present. A number of reports, however, suggest that the presynaptic neuron can dynamically regulate the expression/function of CB_1 receptors. For example, repeated

activation of GABA neurons or synapses in the hippocampus increases the efficacy of CB_1 receptors on those terminals (Chen *et al*, 2007; Chen *et al*, 2003; Foldy *et al*, 2006; Heifets *et al*, 2008; Zhu and Lovinger, 2007), resulting in more robust DSI. Even the inhibition by CB_1 receptors of GABA release can be overcome by increasing the firing rate of these cells (Foldy *et al*, 2006). The mechanistic link between repeated activation and changes in functional signaling of CB_1 receptors remains unclear. One possibility is that like other GPCRs at presynaptic terminals (Kuzmiski *et al*, 2009; Pelkey *et al*, 2005), there may be activity-dependent insertion of CB_1 receptors that relies on Ca^{2+}-dependent fusion of vesicles containing the receptors. Alternatively, repeated activity may recruit intracellular signaling pathways that either, 'switch on' or 'switch off' CB_1 receptors. For example, in the hippocampus, activity-induced recruitment of calcineurin is crucial for amplifying eCB-mediated LTD at GABAergic synapses (Castillo *et al*, 2012). Moreover, as decreases in cell surface receptor mobility appear to be an important component of desensitization of CB_1 receptors (Mikasova *et al*, 2008), it is conceivable that repeated activity reverses this process, thereby increasing the pool of receptors available to bind the ligand. Finally, in the CA1, presynaptic kainate receptors appear to have a key role in enhancing CB_1 receptor function. Here it is proposed that local depolarization, driven by cation influx through the KAR, is sufficient to enhance CB_1 receptor-dependent signaling (Lourenco *et al*, 2010). These observations indicate that entraining synapses with patterns of activity may be an effective way to up- or downregulate CB_1 receptor function. This idea has been extended beyond the experimental brain slice preparation with the demonstration that electroconvulsive seizures and specific experiences that increase neuronal activity can rapidly increase the functional expression of CB_1 receptors (Wamsteeker Cusulin *et al*, 2014; Wamsteeker *et al*, 2010b).

Beyond Retrograde Signaling

Although eCBs are known primarily as retrograde signals, their capacity to influence brain function is not limited to actions on nerve terminals. Some years ago, it was described how eCBs released from the dendrites of depolarized cerebellar neurons could inhibit nearby cells suggesting that eCBs, through the interneuron arborization, can regulate synaptic inputs far beyond its own limits of diffusion (Kreitzer *et al*, 2002). Moreover, there is now clear evidence that the eCBs also act on postsynaptic neurons as well as neighboring astrocytes (as noted above) (Figure 1). The first observations that eCBs can affect neuronal activity through mechanisms that do not rely on changes in synaptic function were described over a decade ago in the cortex (Bacci *et al*, 2004). This autocrine feedback through which eCBs elicit a slow hyperpolarization that inhibits neuronal activity provides a powerful way to regulate neuronal activity. Even though this concept of direct inhibition provides an elegant mechanism for linking cellular activity and behavior, there have been few descriptions of similar mechanisms in other brain regions. Whether this does, indeed, reflect a region-specific phenomenon or merely highlights the need for more careful investigation in other brain regions remains unknown. A recent report demonstrates that eCBs act in an autocrine fashion in midbrain dopamine neurons to increase neuronal activity through a non-CB_1 receptor-mediated mechanism (Gantz and Bean, 2017). Here the target of eCBs is not CB_1 receptors, but A-type potassium channels. Accordingly, previous works have shown how eCBs could affect the functioning of other channels in a CB_1 receptor-independent manner such as TRPV1, GABA-A, nicotinic, or glycinergic channels (Zygmunt *et al*, 1999; Sigel *et al*, 2011; Oz *et al*, 2003; Xiong *et al*, 2012). Specifically, 2-AG appears to act directly on the rapidly activating and inactivating K channels to increase neuronal activity. These disparate observations indicate that the actions of eCBs are region- and cell-type-specific, and highlight the importance of conducting additional experiments extending the focus of investigators beyond the nerve terminal. Consistent with this idea are recent observations demonstrating that eCBs, through actions on postsynaptic CB_1 receptors, drive a non-canonical signaling mechanism that recruits c-Jun-N-terminal kinases (JNKs) and nitric oxide (Maroso *et al*, 2016). In this recent study, the authors described how the activation of a specific pool of postsynaptic CB1 receptors increases a hyperpolarization-activated K^+ current (Ih) that is present in the dendrites of a subset of hippocampal pyramidal neurons in the superficial layers. Ih has a key role in integrating coincident synaptic signals in the dendrites and the authors show that this decrease in the integration of excitatory synaptic signals inhibits the induction of long-term potentiation and the formation of spatial memory.

An important issue concerning postsynaptic effects of eCB actions is the precise subcellular localization of the target receptors. For the moment, there is lack of anatomical evidence of postsynaptic CB_1 receptors located at plasma membrane (Freund *et al*, 2003) (Figure 1) although it cannot be discarded that low levels of CB_1 receptors below level of detection are present at this precise location. However, intracellular CB_1 receptors, and in particular $mtCB_1$, are clearly present both at presynaptic terminals and at somatodendritic compartments of glutamatergic and GABAergic hippocampal neurons (Benard *et al*, 2012) (Figure 1). It is, therefore, tempting to speculate that postsynaptic effects of eCB s might act intracellularly. Future studies will address this intriguing hypothesis.

As described above, recent data indicate that astrocytes are important additional players that express CB_1 receptors and may affect neuronal/synaptic function. As explained earlier, although CB_1 receptors are generally thought to be 'inhibitory' due to their coupling to G_i proteins, this does not apply to all cell types and conditions. In astrocytes, CB_1 receptors increase intracellular Ca^{2+}, possibly through G_q coupling (Navarrete and Araque, 2008). As a consequence, the present idea is that eCB-dependent activation of CB_1 receptors in astrocytes can liberate gliotransmitters (Navarrete and Araque, 2008) that in turn act at neighbor

or distant neurons (Han *et al*, 2012; Martin *et al*, 2015; Navarrete and Araque, 2008, 2010; Min and Nevian, 2012). This creates a scenario in which a postsynaptic pyramidal neuron not only decreases release probability (Pr) at afferent neuronal synapses but it also signals to astrocytes to release gliotransmitters and activate other neurons (Araque *et al*, 2014; Gomez-Gonzalo *et al*, 2015; Navarrete and Araque, 2010; Araque *et al*, 2017).

In summary, synaptic effects of eCB signaling are emerging as more complex events as compared to what believed just few years ago, further extending the multi-faceted ways through which this 'ubiquitous' system can modulate and determine very specific brain processes. How these novel mechanisms contribute to behavioral outcomes of ECS activity will be the subject of studies in the next decades. At the moment, the community is just starting to grasp the surface of this link, producing new mechanistic concepts and hypotheses, as we will touch upon in the next part of this article.

NEW VIEWS ON BEHAVIORAL FUNCTIONS OF THE ECS

The ECS impacts a plethora of behavioral functions. However, the picture of the underlying mechanisms is still incomplete. Besides the well-known inhibition of synaptic activity, new studies have revealed novel and surprising modes through which the ECS modulate animal behavior.

Different Cells, Opposite Functions

In addition to regulating molecular and synaptic functions in unexpected ways, the specific and differential localization of CB_1 receptors in different cell types also has surprising behavioral consequences. Thus, CB_1 receptors activated either by exogenous ligand or by eCBs in one particular cell type or another can have opposing effects on behavior. Indeed, if we dig into the eCB literature, we can find examples of how CB_1 receptors located in different cellular subtypes such as GABAergic, glutamatergic, serotonergic, noradrenergic and other neurons, or astroglial cells can control different behavioral responses ranging from memory processes to food intake to stress. In this review, we will mention just a few interesting examples.

The specific genetic deletion of CB_1 receptors from different cell types has been particularly important in helping us linking CB_1 receptor expression in distinct cell types and different behavioral responses (see Busquets-Garcia *et al*, 2015 for specific examples). However, it is important to note that the possible role of CB1 receptors in regulating behavioral responses is highly state-dependent. Probably, the best-studied case is how CB_1 receptors are key determinants of the biological balance between the excitatory glutamatergic and the inhibitory GABAergic neurons. Thus, CB_1 receptor activation can lead to biphasic effects in food intake (Bellocchio *et al*, 2010; Hao *et al*, 2000) and anxiety (Rey *et al*, 2012), as well as novelty (Lafenetre *et al*, 2009) or fear fear responses (Metna-Laurent *et al*, 2012). Interestingly, acute pharmacological approaches in mutant and wild-type control mice indicate that CB_1 receptor activation by low doses of the ligand impact glutamatergic transmission increasing food intake, producing anxiolytic-like effects, or favoring active fear responses, whereas higher doses affect GABAergic transmission decreasing food intake, increasing anxiety-like fear responses, or promoting passive fear responses, respectively (Bellocchio *et al*, 2010; Metna-Laurent *et al*, 2012; Rey *et al*, 2012).

The mechanisms underlying this differential recruitment of CB_1 receptors on GABAergic or glutamatergic neurons remain unclear, and the full understanding of these biphasic effects of cannabinoid drugs and how the cell-type-specific expression of CB_1 receptors mediates these effects is a big challenge for the future. As mentioned above, one potential explanation for these bimodal effects of cannabinoids is the possibility of cell-type-specific differences in the coupling of downstream intracellular signaling pathways (Steindel *et al*, 2013). In addition to this, the recently discovered biphasic effects of cannabinoids on the regulation of glucose intake by different brain regions (Miederer *et al*, 2017) may also rely on the differential expression of CB_1 receptors in different cell types or subcellular compartments (see above).

However, the general picture is not as simplistic as understanding the effects of CB_1 receptors on GABAergic or glutamatergic neurons. In addition to complex interactions between receptor signaling, changes in neurotransmitter release, and neuronal firing (discussed above), newly generated mutant mice and pharmacological studies have demonstrated the importance of CB_1 receptors on the modulation of the dopaminergic (Bloomfield *et al*, 2016), noradrenergic (Busquets-Garcia *et al*, 2016), cholinergic (Soria-Gomez *et al*, 2015), or serotonergic (Dubreucq *et al*, 2012; Haring *et al*, 2015) systems, and their participation on the modulation of behavioral responses. Although it seems a utopic objective, the field must dedicate significant future work to combine these interesting findings in an effort to determine whether there is a more uniform set of rules that determine how CB_1 receptors modulate behavior in a state-dependent manner.

Astroglial CB1 Receptors: Few of Them, but How Important?

In the brain, CB_1 receptor expression is not limited to neurons (Figure 1). In the last decade, it has been widely demonstrated that astrocytes can also express functional CB_1 receptors (Bosier *et al*, 2013; Han *et al*, 2012; Navarrete and Araque, 2008, 2010). Although likely not all the astrocytes express CB_1 receptors and the protein expression is difficult to detect by conventional light or fluorescent immunohistochemistry or by *in situ* hybridization approaches, RNASeq studies done in the cortex showed that about 20% of total CB_1 receptor mRNA is present in astrocytes (https://web.stanford.edu/group/barres_lab/cgi-bin/igv_cgi_2.py?lname=CNR1).

Moreover, it is well known that the expression levels of CB_1 receptors on given cell types do not necessarily predict their functional relevance (Marsicano and Kuner, 2008) (ie, quantity is not quality). This fact becomes even more important when recent studies clearly demonstrated how the relatively small pool of astroglial CB_1 receptors could control very complex processes, such as metabolic, electrophysiological, and behavioral responses (Bosier *et al*, 2013; Han *et al*, 2012; Navarrete and Araque, 2008, 2010; Min and Nevian, 2012).

However, the direct *in vivo* evidence for the role of astroglial CB1 receptors on behavior is relatively limited. The first observations came from the generation of mutant mice bearing a specific deletion of the *CB_1* receptor gene in astrocytes (Han *et al*, 2012). These mice failed to exhibit the impairment of short-term working memory that is normally evident in mice given exogenous cannabinoids. This suggested that the activation of astroglial CB_1 receptors is required for the working memory impairment induced by cannabinoids (Han *et al*, 2012). However, as the study of astroglial CB_1 receptors is still nascent, the endogenous roles of these receptors on astrocytes and how precisely this impacts behavior need further investigation (Metna-Laurent and Marsicano, 2015; Oliveira da Cruz *et al*, 2016). Recent data in the striatum suggest that astroglial CB_1 receptors might have a specific role in circuit selection processes (Martin *et al*, 2015). Thus, by determining the selective activity of particular circuits and likely contributing to the formation of selected functional neuronal 'domains', astroglial CB_1 receptors can likely contribute to a fine modulation of behavioral responses. Moreover, CB_1 receptors in astrocytes might also contribute to the modulation of memory processes via the regulation of adult neurogenesis (Sultan *et al*, 2015). Finally, another important aspect where astroglial CB_1 receptors could have an important role is in the neuron–astrocyte metabolic coupling that has been shown to be also important for behavioral responses (Halassa and Haydon, 2010; Suzuki *et al*, 2011). Thus, the research community still has considerable work to do to dissect the specific roles of this relatively small but apparently important pool of CB_1 receptors in modulating behavior both in physiological and pathological conditions.

Memory Impact of Mitochondrial CB1 Receptors

As discussed in the first part of this review, it is often early work that informs new ideas. In the 1970s, a number of reports demonstrated that cannabinoid drugs interfered with mitochondrial functions (Bartova and Birmingham, 1976; Bino *et al*, 1972; Chari-Bitron and Bino, 1971; Mahoney and Harris, 1972; Schurr and Livne, 1975), but this evidence was largely forgotten. There is now compelling evidence that CB_1 receptors are present in mitochondrial membranes of different tissues (eg, brain, spermatozoa, and skeletal muscles) (Aquila *et al*, 2010; Benard *et al*, 2012; Hebert-Chatelain *et al*, 2014; Koch *et al*, 2015; Mendizabal-Zubiaga *et al*, 2016) (Figure 3). Thus, the discovery of CB_1 receptors on brain mitochondrial membranes paved the way to a novel field of research, dealing with the direct bioenergetic functions of GPCR signaling and their impact on behavior.

Recently, the first study showing the behavioral relevance of $mtCB_1$ receptors was published (Hebert-Chatelain *et al*, 2016). This work indicates that acute cannabinoid-induced memory impairment in mice requires activation of hippocampal $mtCB_1$ receptors. Genetic exclusion of CB_1 receptors from hippocampal mitochondria prevents the cannabinoid-induced reduction of mitochondrial mobility, synaptic transmission, and memory formation (Hebert-Chatelain *et al*, 2016). Interestingly, hippocampal inhibition of $mtCB_1$ signaling abolishes bioenergetic and amnesic effects of cannabinoids. Thus, the G protein-coupled $mtCB_1$ receptors regulate memory processes via modulation of mitochondrial energy metabolism. Although these data reveal that acute bioenergetic processes are primary acute regulators of cognitive functions (Harkany and Horvath, 2017), more work is required to define the short- and long-term consequences of the decreased mitochondrial respiration and if similar mechanisms alter other behavioral responses. Recent data demonstrating that $mtCB_1$ receptors are also likely involved on the regulation of food intake (Koch *et al*, 2015) suggest that there may be a growing role for these receptors in controlling various behavioral functions.

There is a robust literature focused on the impact of mitochondrial function or dysfunction in the brain relating to long-term pathological conditions (Cheng *et al*, 2010; Mattson *et al*, 2008; Picard, 2015; Raefsky and Mattson, 2017). On the other hand, very little is known about the direct impact of physiological regulation of mitochondrial activity on ongoing brain functions and behavior. Simply considering the fact that mitochondria-dependent processes such as controlling the levels of ATP (Rangaraju *et al*, 2014), the tight modulation of Ca^{2+} (Brini *et al*, 2014), or the generation of reactive oxygen species (Accardi *et al*, 2014) are absolutely necessary for normal synaptic transmission, it is very likely that slight adjustments of these mitochondrial functions through $mtCB_1$ receptors could have deep and rapid impact on brain functioning and behavior. With the discovery of how this subcellular pool of CB_1 receptors can modulate memory functions, a new path has already started linking cannabinoid signaling, mitochondria, and behavior. However, future studies will provide more information on how these organelles and their modulation by CB_1 receptors are important for physiological and pathological behavioral responses.

Endogenous Regulation of the ECS

The ECS appears to trigger and regulate a very complex network of organ-, tissue-, and cell-specific signaling pathways, explaining the wide impact of CB_1 receptor activation on many different behaviors. Just as a politician that with all the power but no control can lead an entire country into marked situations, CB_1 receptor activity needs to be tightly controlled by brake mechanisms that can ultimately modify

behavior (Piazza *et al*, 2017). As described in the first section of this review, these mechanisms include CB_1 receptor-interacting proteins involved in the development of epileptiform seizures (Guggenhuber *et al*, 2015) and processes involving tolerance/resistance to the behavioral effects of cannabinoid drugs (Yao and Mackie, 2009). Moreover, different mechanisms have been shown to control selected signaling aspects of CB_1 receptors that can clearly lead to behavioral consequences (Vallee *et al*, 2014; Busquets-Garcia *et al*, 2017; Pamplona *et al*, 2012)

New mechanisms aimed at protecting and endogenously modulate the activity of the ECS will be very likely described in the future. Each cell-type or subcellular localization of CB_1 receptors might have a specific regulatory mechanism. The discovery of all these mechanisms, as in the case of pregnenolone (Vallee *et al*, 2014), will provide new tools to modulate the plethora of behavioral effects induced by cannabinoid drugs and, potentially, to develop new therapeutic tools against conditions characterized by excessive or reduced ECS activity.

Postsynaptic Effects

The exclusive presynaptic localization of neuronal CB_1 receptors has been challenged based on new findings. Although the presynaptic localization and functions have been widely described (Castillo *et al*, 2012; Freund *et al*, 2003; Kano *et al*, 2009; Piomelli, 2003), there are studies demonstrating that cortical CB_1 receptors can be also present and functional at the postsynaptic somatodendritic compartments of neurons where they can modulate self-inhibition processes (Bacci *et al*, 2004; Marinelli *et al*, 2009). Indeed, the first evidence suggesting the somatic presence of cannabinoid receptors came from the earlier 2000s when putative postsynaptic actions of cannabinoids in hippocampal neurons were shown (Schweitzer, 2000; Zhuang *et al*, 2005). Importantly, as we described in the second part of this review, recent work showed how the somatodendritic pool of CB_1 receptors controls a specific postsynaptic signaling cascade, which is required for the cognitive impairment induced by cannabinoids (Maroso *et al*, 2016) (Figure 1).

These new results are especially important for the behavioral impact of CB_1 receptor activation in specific cell types (Busquets-Garcia *et al*, 2015; Mackie, 2005). In this sense, the CB_1 receptor functions in the postsynaptic compartment have been implicated in the cannabinoid-induced effects of spatial memory (Maroso *et al*, 2016). A quick search of the literature for 'memory deficits and cannabinoids', reveals a plethora of elegant studies demonstrating the possible involvement of CB_1 receptors present in the presynaptic or postsynaptic compartment, in the plasma, or in mitochondrial membranes, or the involvement of different signaling pathways activated by these receptors (eg, MAPK-dependent pathways, mTOR pathway, and JNK) (Busquets-Garcia *et al*, 2015; Puighermanal *et al*, 2009; Vargish and McBain, 2016). Thus, future studies investigating the possible involvement of $mtCB_1$ in the 'postsynaptic' effects of cannabinoids, the possible link between mTOR, MAPK, or other signaling cascades and postsynaptic signaling pathways dependent of postsynaptic CB_1 receptors, or the deep study on how different memory types engaged different CB_1-dependent mechanisms will be necessary for the field.

GENERAL CONCLUSION

The ECS is amongst the most interesting and exciting discoveries of the last decades in the CNS. This discovery generated waves of 'lo and behold' about the new perspectives that it raised in the attempt to understand basic principles of brain functions. As it often and luckily happens in science, the ECS field has continuously evolved with new findings that push the concepts beyond the 'textbook' views. New data continue to challenge previous dogmas, providing refreshing revisions that boost interest in the large field touched by the ECS. The ambition of this short review was to simply highlight how new observations regarding eCB signaling in the brain still continue to open new perspectives on the complexity of brain function. With these premises, we believe that new surprises await us in the future, with new rigorous data opposing currently consolidated views. Given the pace and breadth of discoveries in the evolving eCB field, our hypothesis (and hope) is that this newly consolidated information will likely render this brief review obsolete in the next few years.

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