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The Endocannabinoid Signaling System in the CNS: A Primer

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Abstract

The purpose of this chapter is to provide an introduction to the mechanisms for the regulation of endocannabinoid signaling through CB1 cannabinoid receptors in the central nervous system. The processes involved in the synthesis and degradation of the two most well studied endocannabinoids, 2-arachidonoylglycerol and *N*-arachidonyl ethanolamine are outlined along with information regarding the regulation of the proteins involved. Signaling mechanisms and pharmacology of the CB1 cannabinoid receptor are outlined, as is the paradigm of endocannabinoid/CB1 receptor regulation of neurotransmitter release. The reader is encouraged to appreciate the importance of the endocannabinoid/CB1 receptor signaling system in the regulation of synaptic activity in the brain.

Keywords

endocannabinoid; CB1 cannabinoid receptor; synaptic plasticity; synthesis; degradation

Introduction

Cannabis sativa has been used by humans for thousands of years as a medicinal agent and for its euphoric and relaxing properties. The active principal of *cannabis sativa* was isolated and identified as Δ^9 -tetrahydrocannabinol (THC; Gaoni & Mechoulam, 1964], a landmark discovery that lead to the ultimate uncovering of the endocannabinoid system (ECS) which modulates virtually every brain region and thereby contributes to nearly every function of the CNS.

Using levo-nantradol, a structural analog of THC (Milne, Koe, & Johnson, 1979), Howlett and colleagues provided evidence that THC had biochemical effects consistent with activation of a G protein coupled receptor (GPCR) (Howlett, 1984, 1985; Howlett & Fleming, 1984; Howlett, Qualy, & Khachatrian, 1986). Another THC analog, [3 H]CP55940, was used to demonstrate that a high affinity receptor for THC and structural analogs was present at high density throughout the brain (Devane, Dysarz, Johnson, Melvin, & Howlett, 1988). In a clever set of studies utilizing autoradiography in cerebellar neuron mutant mice, cannabinoid receptor was found to be enriched in axon terminals, which hinted that their activation would modulate neurotransmitter release (Herkenham, Groen, Lynn, De Costa, & Richfield, 1991). Molecular cloning of the cannabinoid receptor followed shortly after, officially introducing the CB1 cannabinoid receptor (CB1R) gene and its protein product to the scientific world (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990). Subsequent

studies have added considerably to our understanding of the ECS; however, none of these would have been possible without these and other seminal observations made between 1965 and 1990.

As the other chapters in this issue describe, there is strong evidence that dysregulation of the ECS contributes to many human maladies, including pain, psychiatric disorders, neurodegenerative diseases, and inflammation. Thus, therapies that alter the ECS could have usefulness as treatments for diseases and disorders that can significantly reduce quality of life. The purpose of this chapter is to introduce the ECS and review what is currently known about its regulation and role in synaptic function.

The endocannabinoids

Definitions

The definition of endocannabinoid used in this chapter is “an endogenous molecule that activates CB1R signaling”. This definition is somewhat arbitrary, since receptors in addition to the CB1R are activated by phytocannabinoids, and the endocannabinoids can bind to other receptors that are not activated by phytocannabinoids.

The first endocannabinoid identified is the very low abundance brain lipid, *N*-arachidonyl ethanolamine (also called anandamide; AEA) (Devane et al., 1992). AEA is one of a family of *N*-acyl ethanolamines (NAEs), first identified by Udenfriend (Bachur, Masek, Melmon, & Udenfriend, 1965; Bachur & Udenfriend, 1966; Colodzin, Bachur, Weissbach, & Udenfriend, 1963) and studied in depth by Schmid and colleagues (Schmid, Schmid, & Natarajan, 1990; Schmid et al., 1995). Shortly after the identification of AEA, two laboratories simultaneously and independently reported that a high abundance 2-monoacyl glycerol, 2-arachidonoyl glycerol (2-AG), also bound and activated CB1R (Mechoulam et al., 1995; Sugiura et al., 1995). Other endogenous lipids that can bind CB1R include O-arachidonyl ethanolamine (virodamine), which is a weak, partial agonist (Porter et al., 2002) and noladin ether, the ether of arachidonic acid (AA) and glycerol (Hanus et al., 2001). There is far less known about the roles of these lipids in the regulation of the ECS than is known about AEA and 2-AG.

In vitro assays demonstrated that the peptide, hemopressin, a nonapeptide derived from the α chain of hemoglobin, binds to the CB1R with high affinity and signals as an inverse agonist (Heimann et al., 2007). More recent studies suggest that hemopressin is not likely the primary signaling molecule but is a cleavage product of RVD-hemopressin (Bomar & Galande, 2013). RVD-hemopressin and several other hemopressin peptides have activity as negative allosteric modulators of the CB1R (Bomar & Galande, 2013).

The focus of this chapter will be on the lipid endocannabinoids, AEA and 2-AG, given the large amount of data supporting their role in brain function and as endocannabinoids.

Mechanisms of AEA biosynthesis

Precursor synthesis—Available evidence indicates that the primary mechanisms for AEA synthesis (and NAE synthesis in general) involve hydrolysis of a minor phospholipid

class, *N*-acyl phosphatidylethanolamines (NAPEs; Figure 1). NAPEs can be synthesized by an *N*-acyl transferase (NAT) that catalyzes transfer and formation of an amide bond between the fatty acyl moiety at the *sn*-1 position of a donor phospholipid and the ethanolamine of phosphatidylethanolamine (PE; Cadas, di Tomaso, & Piomelli, 1997; Schmid, Schmid, & Natarajan, 1996; Sugiura et al., 1996) or plasmenylethanolamine (Hansen, Moesgaard, Hansen, & Petersen, 2000). NAT activity is dependent on high micromolar concentrations of calcium and is present in membranes harvested from brain (Cadas et al., 1997; Cadas, Gaillet, Beltramo, Venance, & Piomelli, 1996; Schmid et al., 1990; Schmid, Schmid, & Natarajan, 1990).

The phospholipase A/acyltransferase (PLA/AT) family of enzymes also carries out the acyl transfer reaction needed to form the NAPEs (Golczak et al., 2012; Shinohara et al., 2011; Uyama et al., 2013). One member of this family, PLA/AT-1, is expressed in brain of human, mouse and rat; and catalyzes the same reaction as NAT, but is calcium independent. Cellular overexpression of PLA/AT-1 results in significantly increased concentrations of NAPE, while its silencing in endogenously expressing cells leads to reduced NAPE (Uyama et al., 2013).

Since acyl groups in the *sn*-1 position are retained in the NAEs, and there is very little AA in this position, these mechanisms would be expected to produce very little *N*-arachidonylPE. the precursor of AEA. Indeed, the concentrations of *N*-arachidonylPE and AEA are very low compared to the saturated substrate and product pairs, such as *N*-palmitoylPE and palmitoylethanolamide (PEA; Ueda, Tsuboi, & Uyama, 2010).

NAPE conversion to NAE: NAPE-PLD—Multiple pathways have been described for the conversion of NAPE into NAEs (Figure 1). The first is a phosphodiesterase of the phospholipase D (PLD) family (NAPE-PLD) that converts NAPEs into phosphatidic acid and NAE (Schmid et al., 1990). NAPE-PLD does not exhibit selectivity for the *N*-acyl moiety, suggesting that it regulates formation of the entire family of NAEs (Wang et al., 2006). The majority of available evidence demonstrates a lack of calcium dependence by NAPE-PLD (Rahman et al., 2014), but little else is known about the mechanisms that regulate its activity. There are a few studies investigating NAPE-PLD mRNA expression. The transcription factor Sp1 regulates basal NAPE-PLD in macrophages, while endotoxin decreases its expression, supporting a link between NAE synthesis and inflammatory state (Zhu et al., 2011). NAPE-PLD expression in the human endometrium changes across the menstrual cycle, suggesting hormonal regulation of expression (Scotchie, Savaris, Martin, & Young, 2015).

Genetic deletion of NAPE-PLD in mice (NAPE-PLD^{-/-}) results in a significant increase in the concentrations of the NAPEs, evidence that NAPE-PLD-mediated hydrolysis plays an important role in the regulation of NAPE concentrations (Leung, Saghatelian, Simon, & Cravatt, 2006; Tsuboi et al., 2011). Concentrations of saturated NAEs are significantly reduced, but not completely absent, in brains from NAPE-PLD^{-/-} mice (Leung et al., 2006) and brain homogenates of NAPE-PLD^{-/-} mice incubated with *N*-arachidonylPE exhibit only 25% of the conversion to AEA of wild type mice (Liu et al., 2008). However, brain concentrations of AEA and other polyunsaturated NAEs in NAPE-PLD^{-/-} are not

significantly different from wild type mice, which suggests that other pathways are important in the biosynthesis of AEA and that these pathways could be up-regulated in the NAPE-PLD^{-/-} mice.

NAPE-PLD is associated with intracellular membranes (Okamoto, Morishita, Tsuboi, Tonai, & Ueda, 2004). In the ventral pallidum, NAPE-PLD is in presynaptic terminals that are opposed to other axon terminals that express the CB1R, suggesting that AEA could contribute to axo-axonal regulation of CB1R signaling (Pickel, Shobin, Lane, & Mackie, 2012). Studies in the hippocampus also demonstrate presynaptic distribution of NAPE-PLD in CB1R-negative, glutamatergic terminals (Nyilas et al., 2008). NAPE-PLD is found in both axons and dendrites in the hypothalamus (Reguero et al., 2014).

NAPE conversion to NAE: multi-enzyme pathways—Two alternative pathways have been discovered that can convert NAPE to NAE (Figure 1). NAPE can be deacylated to lysoNAPE via phospholipase A₂ family members (Sun et al., 2004) and by alpha-beta hydrolase domain-containing protein 4 (Abhd4; Simon & Cravatt, 2006). Abhd4 is expressed in mouse brain and can also deacylate lysoNAPE, resulting in the formation of glycerophospho-*N*-acylethanolamine (GP-NAE; Figure 1).

GP-NAE is a substrate for glycerophosphodiester phosphodiesterase 1 (GDE1) which catalyzes its hydrolysis to glycerol-3-phosphate and NAE (Figure 1; (Simon & Cravatt, 2008). GDE1 is widely distributed in mammalian tissues, including brain and spinal cord. GDE1^{-/-} mice have been used to examine the role of the Abhd4-GDE1 pathway in the synthesis of NAEs *in vivo* (Simon & Cravatt, 2010). Brains from GDE1^{-/-} mice exhibit no detectable conversion of GP-NAE to NAE; however, brain concentrations of the NAEs, including AEA, are not different from wild type mice indicating that GDE1 is not essential for NAE synthesis *in vivo*. GDE1^{-/-}/NAPE-PLD^{-/-} double knock out mice demonstrate no conversion of NAPE to NAE in brain homogenates; however, cultured neurons from these mice can convert NAPE to NAE (Simon & Cravatt, 2010). These data indicate that there are mechanisms other than NAPE-PLD and Abhd4-GDE1 that can convert NAPE to NAE in an intact cell that are not operative when brain tissue is homogenized.

An additional, multi-step pathway that generates NAE from NAPE which involves phospholipase C (PLC) has also been described (Figure 1). This pathway, elucidated in macrophages, involves PLC-mediated conversion of NAPE to diacylglycerol (DAG) and phospho-NAE, which is subsequently dephosphorylated by several phosphatases, including protein tyrosine phosphatase, non-receptor type 22 (PTPN22) and Src homology 2-containing inositol phosphatase-1 (SHIP-1; Liu et al., 2008; Liu et al., 2006). The PLC/phosphatase pathway was shown to be essential for endotoxin-induced synthesis of AEA in macrophages (Liu et al., 2006).

Inhibitor studies suggest both the Abhd4/GDE-1 and PLC/phosphatase pathways contribute to AEA synthesis in NAPE-PLD^{-/-} mice (Liu et al., 2008). Interestingly, the kinetics of these pathways differ; the PLC/phosphatase pathway is active during the first 1–10 min after the addition of NAPE, and the Abhd4/GDE-1 pathway contributes to AEA accumulation only at later times of NAPE incubation (Liu et al., 2008). Phospho-AEA can be detected in

brain and its concentration is significantly increased in the presence of vanadate, which provides further support for the possibility that the PLC/phosphatase pathway is involved in brain NAE formation (Liu et al., 2008).

AEA synthesis from AA—A synthetic pathway for AEA has been identified in mammalian tissues that does not involve NAPE as a precursor (Figure 1). In this pathway, the NAE hydrolyzing enzyme, fatty acid amide hydrolase (FAAH), acts in “reverse” as an NAE synthase to directly couple AA and ethanolamine to form AEA (Arreaza et al., 1997; Katayama et al., 1997; Kurahashi, Ueda, Suzuki, Suzuki, & Yamamoto, 1997). This mechanism generates AEA during severe hepatic damage (Mukhopadhyay et al., 2011) and in post mortem brain (Patel et al., 2005), conditions that are characterized by large concentrations of ethanolamine, which is necessary to drive the synthase function of the enzyme.

Summary—The synthesis of AEA occurs by a number of possible routes, but it is not clear whether any of the known mechanisms contribute to stimulation of AEA production for the purpose of activating ECS. The answer to this question has not been provided by genetic deletion of possible enzymes, suggesting that the multiple mechanisms can compensate for one another. The development of effective and selective inhibitors of the various synthetic pathways are needed to delineate these processes.

Mechanisms of AEA hydrolysis

Fatty acid amide hydrolase (FAAH)—Early studies identified an enzymatic activity in liver microsomes that hydrolyzed NAEs to free fatty acid and ethanolamine (Schmid, Zuzarte-Augustin, & Schmid, 1985). The activity identified was likely due to the enzyme identified molecularly by Cravatt and colleagues and given the name fatty acid amide hydrolase, or FAAH (Cravatt et al., 1996). In addition to saturated and unsaturated, long and short chain NAEs, FAAH also hydrolyzes oleamide (a primary amine) and *N*-acylethanolamines, which are very significantly increased brain tissue from FAAH^{-/-} mice (Saghatelian & Cravatt, 2005). FAAH can also function as an esterase and hydrolyze 2-AG in vitro (Patricelli & Cravatt, 1999), although genetic deletion of FAAH in mice does not affect brain 2-AG contents (Patel et al., 2005), suggesting that FAAH does not play a prominent role in 2-AG hydrolysis.

FAAH is an integral membrane protein, present primarily on endoplasmic reticulum (Hillard, Wilkison, Edgemond, & Campbell, 1995) and mitochondria (Gulyas et al., 2004). FAAH is localized primarily in large, outflow neurons and is ubiquitously expressed throughout the brain (Tsou et al., 1998). FAAH is active over a wide range of pH values and its activity is unaffected by either the addition or removal of divalent cations (Hillard et al., 1995; Schmid et al., 1985).

FAAH is constitutively active and several studies suggest that its activity is can be regulated by post-translational processes. For example, follicle stimulating hormone treatment increases FAAH activity through a mechanism that requires increased protein kinase A (PKA) activity, which appears to phosphorylate an accessory protein, not FAAH itself (Grimaldi, Rossi, Catanzaro, & Maccarrone, 2009). Activation of receptors for corticotropin

releasing factor receptor 1 (CRF1) by corticotropin releasing hormone (CRH) *in vivo* results in increased FAAH activity *ex vivo* through a mechanism consistent with a post-translational modification (Gray et al., 2015). Given that CRF1 receptors can also couple to activation of PKA (Pollandt et al., 2006), it is possible that similar mechanisms are involved in the actions of both follicle stimulating hormone and CRH.

The FAAH promoter has been analyzed; its mRNA is transcribed from multiple transcription start sites that lack a TATA box element (Puffenbarger, Kapulina, Howell, & Deutsch, 2001). The promoter region contains several estrogen receptor binding elements (Grimaldi et al., 2012; Waleh, Cravatt, Apte-Deshpande, Terao, & Kilduff, 2002); and estrogen (Grimaldi et al., 2012) and the xenoestrogen, bisphenol A (Vermeer, Gregory, Winter, McCarron, & Berman, 2014) increase FAAH gene transcription. Progesterone also increases FAAH expression in T lymphocytes through the transcription factor, Ikaros (Maccarrone, Bari, Di Rienzo, Finazzi-Agro, & Rossi, 2003). Glucocorticoid receptor binding sites are present in the FAAH promoter and reporter studies demonstrate that glucocorticoid receptors regulate FAAH expression in a negative manner (Waleh et al., 2002). However, glucocorticoid administration in the drinking water results in increased FAAH activity without any effect of mRNA expression in brain (Bowles et al., 2012), suggesting that this process may not occur *in vivo*.

In vitro treatment with endotoxin reduces FAAH expression at a transcriptional level in human peripheral lymphocytes (Maccarrone et al., 2001) and in peripheral blood monocytes of mice (Wolfson et al., 2013). Recent data indicate that treatment of lymphocytes with IL6 results in increased FAAH expression and that a cAMP response element in the FAAH promoter is likely involved in the regulatory mechanism (Gasperi et al., 2014). Leptin receptors activate the FAAH promoter in T lymphocytes through STAT3; perhaps also acting via a cAMP-responsive element (Maccarrone, Di Rienzo, Finazzi-Agro, & Rossi, 2003; Maccarrone, Gasperi, Fezza, Finazzi-Agro, & Rossi, 2004).

A single nucleotide polymorphism (SNP) has been identified in the coding region of the human FAAH gene that results in a missense mutation changing a conserved proline residue to threonine (Sipe, Chiang, Gerber, Beutler, & Cravatt, 2002). The amino acid change does not alter FAAH kinetics, but results in reduced protein amounts, likely as a result of protein instability (Chiang, Gerber, Sipe, & Cravatt, 2004). Recently, a transgenic mouse line was established in which a nucleotide substitution homologous to the rare allele in humans was introduced (Dincheva et al., 2015). The transgenic mice exhibit reduced FAAH protein amounts and higher brain AEA contents.

FAAH^{-/-} mice exhibit 10 fold increases in basal concentrations of AEA and other NAEs in brain tissue (Cravatt et al., 2001), suggesting that FAAH activity is essential for the regulation of NAE concentrations in brain. FAAH^{-/-} mice have been used in a large number of behavioral paradigms and changes are generally ascribed to increased AEA tone. However, the other NAEs are increased to an even greater extent than AEA by FAAH deletion and these lipids have non-CB1R targets, most significantly, the peroxisome proliferation activating receptors (PPARs; Panlilio, Justinova, & Goldberg, 2013). In addition, the *N*-acyl taurine family of lipids is very significantly increased in FAAH^{-/-} mice

(Saghatelian & Cravatt, 2005) can affect TRPV channel function (Saghatelian, McKinney, Bandell, Patapoutian, & Cravatt, 2006).

There are several widely used pharmacological inhibitors of FAAH that are very effective *in vivo*, including URB597 (Tarzia et al., 2003), PF-3845 (Ahn et al., 2009) and JNJ5003 (Hill et al., 2013). One FAAH inhibitor (PF-04457845) is currently in phase 2 clinical trials for the treatment of post-traumatic stress disorder (http://www.pfizer.com/sites/default/files/product-pipeline/February_27_2015_Pipeline_Update_Final2.pdf).

NAE-hydrolyzing acid amidase (NAAA): A peripheral AEA hydrolase—A second amidohydrolase, NAAA, has been identified in peripheral tissues (Ueda, Yamanaka, & Yamamoto, 2001). NAAA is present in lysosomes, active at acidic pH, and prefers PEA as a substrate over AEA. Several inhibitors of NAAA have been developed and shown to increase endogenous concentrations of PEA (Rahman et al., 2014). There are no effects of NAAA inhibitors on AEA concentrations and no overlap or substitution of NAAA for FAAH-mediated hydrolysis of AEA in brain (Rahman et al., 2014).

Mechanisms of 2-AG Biosynthesis

Diacylglycerol lipase—2-AG is present in brain tissue and microdialysates at nanomolar concentrations (Buczynski & Parsons, 2010). A well-supported mechanism for the synthesis of 2-AG involves hydrolysis of the ester bond at the *sn*-1 position of diacylglycerol (DAG) by diacylglycerol lipase (DAGL; Figure 2). Two isoforms of DAGL are expressed in mammals, DAGL α and DAGL β , that share a transmembrane domain of 4 loops, coupled to a catalytic domain and regulatory loop (Bisogno et al., 2003; Reisenberg, Singh, Williams, & Doherty, 2012). DAGL α has an additional, large C terminal tail. Both isoforms are expressed in brain; however, DAGL α is present in high density at perisynaptic regions of dendrites in many brain regions (Katona et al., 2006; Matyas et al., 2008; Uchigashima et al., 2007; Yoshida et al., 2006), which is consistent with a prominent role for this isoform in ECS-mediated regulation of synaptic plasticity (described below).

Mass spectrometry studies demonstrate that both isoforms of DAGL are phosphorylated within the catalytic and regulatory domains; and identify many phosphor-residues in the C terminal tail of DAGL α (Reisenberg et al., 2012). There is some evidence that both PKA and protein kinase C (PKC) can stimulate DAGL activity (Malcher-Lopes et al., 2006; Rosenberger, Farooqui, & Horrocks, 2007; Vellani et al., 2008). DAGL α is phosphorylated on two serines in the C terminal region by calcium/calmodulin dependent kinase II (CamKII); this phosphorylation inhibits DAGL α activity and thereby possibly provides a mechanism by which calcium can produce a feedback inhibition of 2-AG synthesis (Shonesy et al., 2013).

Proteomic analyses demonstrate that both DAGL isoforms can be palmitoylated (Kang et al., 2008; Martin & Cravatt, 2009; Yang, Di Vizio, Kirchner, Steen, & Freeman, 2010). Although both DAGLs are membrane intrinsic proteins, palmitoylation could contribute to membrane localization, particularly to specific membrane regions in which other palmitoylated proteins are localized. However, no studies that specifically explore the role of palmitoylation in localization or activity of DAGL have been published.

The catalytic activities of DAGL α and DAGL β do not differ (Bisogno et al., 2003), so the additional C terminal domain of DAGL α is apparently not important for enzymatic activity. In addition to being a target for phosphorylation, the DAGL α C terminus contains a consensus motif for binding to Homer proteins [Jung et al., 2007], which are a family of adaptor proteins that can tether synaptic proteins, such as metabotropic glutamate receptors (mGluRs) to the post synaptic density (Gao, Tronson, & Radulovic, 2013). Although interaction between DAGL α and Homer proteins was found to be important for DAGL α association with the plasma membrane in a cell line, this interaction was not required for 2-AG synthesis (Jung et al., 2007). On the other hand, several studies have demonstrated that Homer is required for ECS-mediated changes in synaptic transmission (Fourgeaud et al., 2004; Roloff, Anderson, Martemyanov, & Thayer, 2010; Won, Puhl, & Ikeda, 2009), suggesting that DAGL α location within the cell is regulated by Homer proteins and that this process is required for efficient activation of 2-AG-mediated signaling.

Several DAGL inhibitors have been identified and used to implicate 2-AG synthesis in cellular and physiological function. Tetrahydrolipstatin (THL; orlistat) is a high affinity inhibitor of DAGL at concentrations that are without effect on monoacylglycerol lipase (MAGL) or NAPE-PLD (Bisogno et al., 2006; Lee, Kraemer, & Severson, 1995) and is a potent inhibitor of 2-AG *in vitro* (Hashimotodani, Ohno-Shosaku, Maejima, Fukami, & Kano, 2008; Won et al., 2009). However, THL inhibits a broad range of gastric and pancreatic lipases and is irreversible (Guercioli, 1997), which limits its use *in vivo*. RHC 80267 is also a DAGL inhibitor, but it interferes with the CB1R-mediated effects of exogenously administered 2-AG, suggesting direct effects on CB1R signaling (Hashimotodani et al., 2008). OMDM-188, a THL analog (Ortar et al., 2008), is also a selective and potent inhibitor of DAGL *in vitro* (Hashimotodani et al., 2013; Min et al., 2010). LEI105 is a reversible inhibitor of both DAGL α and DAGL β ; is without effect on the known 2-AG catabolic enzymes or FAAH; and reduces 2-AG but not AEA concentrations in Neuro2A cells (Baggelaar et al., 2015). If OMDM-188 and LEI105 have *in vivo* efficacy and selectivity, they will be important additions to the ECS pharmacological toolbox.

Mechanisms of DAG synthesis—The DAG substrate for DAGL can be generated in multiple ways (Figure 2). The PLC family of enzymes, particularly PLC β and PLC γ isoforms, act on phosphatidylinositol 4,5 biphosphate (PIP $_2$) to produce DAG and inositol-trisphosphate (IP $_3$). This pathway links the regulation of 2-AG synthesis to GPCRs, since the G α_q family of G proteins activate PLC β . The group I mGluRs are G α_q coupled and their activation in brain slices has been shown to increase 2-AG concentrations (Jung et al., 2005) through a mechanism that requires DAGL α (Jung et al., 2007). Other G α_q -activating GPCRs that have been shown to elevate 2-AG include muscarinic (Straiker & Mackie, 2007; Uchigashima et al., 2007); orexin 1 (Ho et al., 2011); angiotensin (Turu et al., 2007); alpha 1 adrenergic (Turu et al., 2009); vasopressin (Turu et al., 2009); bradykinin (Turu et al., 2009); and neurotensin (Kortleven, Bruneau, & Trudeau, 2012) receptors. Studies in the cerebellum demonstrate a close spatial relationship between PLC β 1, mGluRs and DAGL α in dendritic spines (Fukaya et al., 2008), supporting the synthetic pathway outlined and specifically placing these components together in the perisynaptic region.

Like PLC β , PLC γ isoforms also act on PIP₂ and generate DAG, but are downstream of tyrosine kinase linked, growth factor receptors, suggesting that 2-AG synthesis can also be regulated by that receptor class. In support of this notion, brain derived neurotrophic factor induces 2-AG synthesis through a trkB receptor/PLC γ -requiring mechanism (Zhao & Levine, 2014). Thus 2-AG synthesis can potentially be mediated by a wide variety of receptor types, including GPCRs and growth factor receptors. The proximity of DAGL determines whether 2-AG will be produced from DAG when PLC activating receptors are engaged.

There is evidence in N18TG2 cells that 2-AG synthesis can be evoked by calcium without a requirement for PLC (Bisogno, Melck, De Petrocellis, & Di Marzo, 1999). A combination of inhibitor studies and analysis of intermediates indicates that calcium evokes production of DAG via a two enzyme pathway in this cellular model: phospholipase D resulting in production of phosphatidic acid (PA), followed by removal of the phosphate group of PA via PA phosphatase (Figure 2).

Mechanisms of 2-AG Catabolism

Monoacylglycerol lipase—2-AG is catabolized by hydrolysis of the ester bond between the AA backbone and glycerol through the actions of several enzymes (Figure 2). MAGL is responsible for more than 85% of 2-AG hydrolysis by brain homogenates (Blankman, Simon, & Cravatt, 2007) and is considered the dominant mechanism for the inactivation of 2-AG in its role as CB1R agonist in neurons (Murataeva, Straiker, & Mackie, 2014). MAGL is a serine hydrolase that hydrolyzes both 1(3)- and 2 -monoacylglycerols with little ability to hydrolyze triacylglycerols or diacylglycerols (Tornqvist & Beifrage, 1976). MAGL is ubiquitously distributed throughout the body, including brain. Interestingly, mouse and rat brain MAGL (but not that of other tissues) appear as a doublet on Western blot (one at the predicted molecular weight of 33 and a second that migrates at approximately 35 kDa; Dinh et al., 2002; Karlsson et al., 2001). The reason for this is not known; it is possible that the proteins have the same amino acid sequence but differ in post translational modification. Alternatively, previous studies have shown that multiple mRNA species are present for mouse MAGL that could give rise to proteins of varying amino acid length (Karlsson et al., 2001). How this would occur in a tissue-specific manner is not clear.

In situ hybridization has been used to assess the distribution of MAGL mRNA expression in the brain, showing good agreement of brain regions expressing the CB1R and MAGL (Dinh et al., 2002). MAGL is found in presynaptic terminals (Gulyas et al., 2004; Horvath et al., 2014; Suarez et al., 2008) and enzymatic activity is enriched in synaptosomal preparations (Farooqui & Horrocks, 1997; Vyvoda & Rowe, 1973). A recent study provides evidence that MAGL is expressed in astrocytes as well as neurons; and astrocytic MAGL contributes to overall regulation of brain 2-AG content (Viader et al., 2015).

Although MAGL does not contain canonical transmembrane domains, subcellular fractionation studies find MAGL enzymatic activity in particulate (i.e. membrane) as well as cytosolic fractions (Bisogno et al., 1997; Di Marzo et al., 1999; Goparaju, Ueda, Taniguchi, & Yamamoto, 1999; Sakurada & Noma, 1981). Although there are no detectable differences in catalytic activity between cytosolic and membrane associated MAGL (Goparaju et al.,

1999), recent studies using nanodisk models of phospholipid bilayers demonstrate that interactions of MAGL with the bilayer hold MAGL in a conformation that facilitates substrate access to the catalytic region (Nasr et al., 2013). This, together with the likelihood that 2-AG will partition to membranes rather than cytosol, provides evidence that MAGL associated with the plasma membrane is more important for termination of 2-AG action at the CB1R than cytosolic MAGL. There are several studies demonstrating compartment-selective changes in MAGL protein or activity. Cytosolic MAGL activity was found to be reduced by 50% in adipocytes from fasted rats while particulate MAGL activity was not changed (Sakurada & Noma, 1981). Importantly for its function in regulating brain ECS, chronic stress selectively reduced MAGL protein detected in membrane but not cytosolic fractions of the basolateral amygdala (Sumislawski, Ramikie, & Patel, 2011).

Several studies demonstrate decreased MAGL mRNA expression in tissues that are inflamed (Engeli et al., 2014; Lappas, 2014; Mai et al., 2015). Sustained elevation of neuregulin-1 increases MAGL expression in hippocampal slices, likely through activation of the ErbB4 receptor (Du, Kwon, & Kim, 2013). The transcription factor and tumor suppressor Prdm5 is a repressor of MAGL expression and its loss acts synergistically with WNT pathway activation to increase MAGL expression and to increase adenoma formation (Galli et al., 2014). Genetic deletion of PLC β 1 results in reduced MAGL expression, suggesting that its expression is regulated by 2-AG concentrations (Filis, Kind, & Spears, 2013). MAGL protein is degraded in developing cholinergic neurons through a process that involves nerve growth factor upregulation of the E3 ubiquitin ligase, BRCA1 (Keimpema et al., 2013).

Although there are no studies to date examining the role of posttranslational modifications in the regulation of MAGL activity, one study showing rapid changes in MAGL activity during ischemia is consistent with phosphorylation or other short term regulatory mechanisms (Strosznajder, Singh, & Horrocks, 1984).

Several inhibitors of MAGL have been developed. URB602 is a relatively weak inhibitor of recombinant MAGL *in vitro* (King et al., 2007). URB602 increases 2-AG concentrations when injected into the periaqueductal gray (Hohmann et al., 2005) and significantly increases depolarization-induced increases in 2-AG in microdialysates of rat nucleus accumbens (Wiskerke et al., 2012). JZL184 is a more potent inhibitor of mouse MAGL (Long, Li, et al., 2009), but is not as an effective inhibitor of rat MAGL (Long, Nomura, & Cravatt, 2009; Pan et al., 2009). Microdialysis studies in nucleus accumbens of rat and mice confirm that JZL184 is effective at elevation of 2-AG in mice but not rats (Wiskerke et al., 2012). Although these findings could suggest that JZL184 is not a good indirect agonist of CB1R signaling in rat, it has been shown to increase CB1R-mediated behavioral effects in rats without affecting tissue 2-AG concentrations (eg. Woodhams et al., 2012). Since both URB602 and JZL184 show evidence of being efficacious *in vivo* in spite of relatively poor inhibition of MAGL *in vitro*, it is likely that even modest reductions of MAGL activity can significantly enhance 2-AG actions. There are several recent reports of other MAGL inhibitors that have not been used as widely as JZL184 and URB602 (Ignatowska-Jankowska et al., 2014; Kapanda et al., 2012; Tuccinardi et al., 2014).

Although acute inhibition of MAGL activity potentiates CB1R signaling (Pan et al., 2009), genetic deletion (Chanda et al., 2010) and chronic pharmacological inhibition (Schlosburg et al., 2010) of MAGL both result in functional reductions in CB1R signaling rather than activation. Interestingly, the MAGL^{-/-} mice have increased tonic CB1R activity (Pan et al., 2011), but exhibit region-specific desensitization of CB1R agonist-induced activation of G proteins (Navia-Paldanius et al., 2015). Given that both genetic deletion and chronic inhibition of MAGL produce very large increases in brain 2-AG contents, these data are consistent with agonist (i.e. 2-AG)-induced desensitization of CB1R signaling. The profound effects of reduced MAGL activity on 2-AG suggest that 2-AG homeostasis is regulated more by its degradation than synthesis. In support of this hypothesis, recent data demonstrate that 2-AG is generated continuously at hippocampal GABA synapses and that MAGL activity is critical for opposing this steady stream of 2-AG to maintain the CB1R in a low ligand state (Lee et al., 2015).

Other enzymes that hydrolyze 2-AG in the brain—Abhd6 and Abhd12 were identified as potential 2-AG hydrolases using a functional proteomic approach (Blankman et al., 2007). Subsequent studies have demonstrated that Abhd6 is expressed by neurons in postsynaptic compartments and its activity can regulate 2-AG-mediated activation of CB1R (Marrs et al., 2010). Given the postsynaptic distribution, Abhd6 has been proposed to regulate 2-AG concentrations at the site of synthesis (Savinainen, Saario, & Laitinen, 2012), which complements the role of MAGL to regulate 2-AG concentrations in the axon terminal. As was argued above, this function of Abhd6 could play a very important role in basal CB1R activity by controlling the amount of 2-AG that survives to exit the postsynaptic terminal.

Studies in brain homogenates suggest that Abhd12 accounts for approximately 9% of brain 2-AG hydrolysis (Blankman et al., 2007). Abhd12 is mutated in the human neurodegenerative disorder, PHARC (Blankman, Long, Trauger, Siuzdak, & Cravatt, 2013). There is little information regarding the role of Abhd12 in brain 2-AG homeostasis; however, its mRNA is enriched in microglia, suggesting it may be important in the termination of 2-AG-mediated CB2 receptor activation (Fiskerstrand et al., 2010).

Contribution of 2-AG to AA concentrations—Early studies in platelets demonstrated that free AA was increased by the metabolism of DAG and 2-AG (Bell, Kennerly, Stanford, & Majerus, 1979; Prescott & Majerus, 1983). MAGL^{-/-} mice exhibit significantly lower brain tissue concentrations of AA in addition to elevated 2-AG (Schlosburg et al., 2010). This finding, together with data that acute inhibition of MAGL abolishes endotoxin-induced increases in brain AA and prostaglandin E₂ (Nomura et al., 2011), strongly indicate that 2-AG is a biologically significant precursor for AA and that MAGL is a critical enzyme in the provision of free AA for further metabolism. A recent study using cell specific MAGL deletion suggests that astrocyte MAGL is mainly responsible for converting 2-AG to inflammatory prostaglandins in brain (Viader et al., 2015). Similar roles for 2-AG and MAGL have been demonstrated in hepatic injury (Cao et al., 2013) and for the synthesis of vasodilatory arachidonates in coronary arteries (Gauthier et al., 2005). Studies carried out

using vascular tissue suggest that the AEA/FAAH pair can serve a parallel function in the synthesis of vasoactive AA metabolites (Pratt, Hillard, Edgemond, & Campbell, 1998).

These and other studies demonstrate integration and synergism between the endocannabinoid and AA signaling systems. This conclusion is further supported by the findings discussed immediately below that AEA and 2-AG can also serve as substrates for enzymes that metabolize AA.

Other inactivation mechanisms for AEA and 2-AG

Uptake, accumulation and sequestration—The addition of labeled AEA to the outside of cells results in its cellular association in a manner that is consistent with accumulation and in some cases, intracellular sequestration (Hillard & Jarrahian, 2003). However, details of the mechanisms involved in this “uptake” process remain unclear. FAAH-mediated catabolism of AEA can maintain the concentration gradient and thereby enhance AEA uptake into the cells by either passive or facilitated diffusion and many inhibitors of AEA uptake also inhibit FAAH-mediated catabolism of AEA (Deutsch et al., 2001; Glaser et al., 2003; Kaczocha, Hermann, Glaser, Bojesen, & Deutsch, 2006). However, AEA is also accumulated by cells that express very low or no FAAH (Hillard, Edgemond, Jarrahian, & Campbell, 1997; Hillard & Jarrahian, 2005; Nicolussi, Chicca, et al., 2014), suggesting that other processes can contribute to accumulation. Indeed, other intracellular proteins have been identified that bind AEA and could thereby serve as sequestration sites. These include fatty acid binding proteins (FABPs; Kaczocha, Glaser, & Deutsch, 2009); heat shock proteins (Oddi et al., 2009) and sterol carrier protein 2 (Liedhegner, Vogt, Sem, Cunningham, & Hillard, 2014). In addition, AEA associates with membrane lipid rafts (McFarland et al., 2004; McFarland, Terebova, & Barker, 2006), which could also serve as a sequestration site following uptake. Importantly, it is likely that AEA uptake occurs via different processes in different cells (Hillard & Jarrahian, 2005).

There are several experiments that indirectly support a protein transporter that can translocate AEA across the plasma membrane in a bidirectional manner (Chicca, Marazzi, Nicolussi, & Gertsch, 2012; Hillard et al., 1997; Ligresti et al., 2004; Ronesi, Gerdeman, & Lovinger, 2004). These data suggest that an endocannabinoid transporter could participate in both inactivation (as a first step in a cellular sequestration process) and in the release of AEA. FLAT, a variant of FAAH that is without catalytic activity, has been suggested as a putative AEA transport protein (Fu et al., 2012). However, other studies dispute these findings (Leung, Elmes, Glaser, Deutsch, & Kaczocha, 2013). Although fewer experiments have been done, inhibitor studies suggest that 2-AG could also be subjected to similar regulation by uptake and/or sequestration (Bisogno et al., 2001; Nicolussi & Gertsch, 2015).

There are several inhibitors of the cellular accumulation of the endocannabinoids, although the lack of complete understanding of the processes involved has made it difficult to ascribe mechanisms to the inhibitors. Some inhibitors of AEA uptake, including AM404, VDM11, AM1172 and LY2183240, also inhibit or are substrates for FAAH (Alexander & Cravatt, 2006; Fowler, Tiger, Ligresti, Lopez-Rodriguez, & Di Marzo, 2004; Glaser et al., 2003; Vandevoorde & Fowler, 2005) and can compete with endocannabinoids for binding to intracellular proteins (Kaczocha, Vivieca, Sun, Glaser, & Deutsch, 2012; Liedhegner et al.,

2014). Recent reports have identified the natural product, guineensine, as a potent inhibitor of AEA uptake in several cell types (Nicolussi, Viveros-Paredes, et al., 2014) and a series of *N*-alkylcarbamates as extremely potent inhibitors of both AEA uptake and FAAH activity (Nicolussi, Chicca, et al., 2014). Characteristics of the inhibitory effects of guineensine and the *N*-alkylcarbamates support the notion that FAAH activity contributes to AEA uptake, but it is not sufficient to explain all forms of uptake in all cell types. Development and study of inhibitors with novel structures will undoubtedly move our understanding of the mechanisms involved in AEA and 2-AG uptake further.

In vivo treatment with uptake inhibitors AM404 and UCM707 increase brain tissue concentrations of AEA and, to a lesser extent, 2-AG (de Lago et al., 2005; Di et al., 2005); however, AM404 only affects 2-AG, and UCM707 is ineffective at elevating either AEA or 2-AG in brain extracellular space, measured using microdialysis (Wiskerke et al., 2012). Since effective inhibitors of FAAH and MAGL have very significant effects on microdialysate AEA and 2-AG, respectively, these findings suggest that the processes of endocannabinoid uptake and accumulation may contribute only a small amount to their overall clearance. On the other hand, small changes in clearance, particularly of 2-AG, could result in a significant increase in CB1R activation and might even be a very useful approach because it should avoid inducing receptor desensitization.

Oxygenation of the arachidonate backbone—The acyl chain of AA can be modified by cyclooxygenases, lipoxygenases and cytochrome P450s, resulting in the production of prostaglandins, leukotrienes, and epoxyeicosatrienoic acids, respectively. Subtypes of each of these enzyme classes can also utilize AEA and 2-AG as substrates, resulting in the formation of ethanolamide and glycerol ester analogs of the arachidonates (see Urquhart, Nicolaou, & Woodward, 2015 for an excellent recent review). The result of these processes are a large number of lipid mediators, some of which have been shown to have their own targets. On the other hand, metabolism of AEA and 2-AG along any of these pathways is likely to reduce affinity for CB1R, thus they are inactivation mechanisms for the CB1R signaling roles of these lipids.

In particular, 2-AG is an excellent substrate for COX-2, having K_m and k_{cat} values that are very similar to those of AA (Rouzer & Marnett, 2011). Based upon structure function studies showing that 2-AG and AA bind differentially to COX-2, substrate-specific inhibitors of COX-2 that selectively reduce 2-AG metabolism while preserving prostaglandin formation have been designed (Hermanson, Gamble-George, Marnett, & Patel, 2014). The in vivo effects of these inhibitors support the hypothesis that COX-2 mediated metabolism of 2-AG contributes in a significant manner to the regulation of CB1R signaling (Hermanson et al., 2013).

Endocannabinoid Receptors

Introduction

The focus of this review is on the CB1R. However, other receptors have been identified that can bind the endocannabinoids AEA and 2-AG and it is highly likely that these receptors contribute to the biological effects of the lipids. Both AEA and 2-AG bind to CB2

cannabinoid receptors (CB2R; Gonsiorek et al., 2000), which are GPCRs (Munro, Thomas, & Abu-Shaar, 1993). Although 2-AG has the characteristics of a full CB2R agonist, AEA does not induce CB2R-mediated GDP/GTP exchange (Gonsiorek et al., 2000; Hillard et al., 1999) and is likely a weak partial agonist of the CB2R. CB2R are expressed in circulating immune cells (Bouaboula et al., 1993), spleen (Galiege et al., 1995) and tissue resident macrophage populations, including microglial cells (Carlisle, Marciano-Cabral, Staab, Ludwick, & Cabral, 2002). CB2R are also expressed by some neuronal populations, although the expression levels are far lower than CB1R (Van Sickle et al., 2005; Zhang et al., 2014).

AEA is an agonist of the vanilloid type 1 receptor, also called TRPV1 (Kim et al., 2007; Ross et al., 2001; Saghatelian et al., 2006). TRPV1 is a nonselective cation channel expressed widely in the CNS. Intracellular AEA induces opening of the channel and this function contributes to many of the non-CB1 mediated effects of AEA. AEA and other NAEs are agonists of PPARs, particularly PPAR α (Bouaboula et al., 2005; Fu et al., 2003; Lo Verme et al., 2005). Recent data suggests that PPAR α mediated changes could contribute to the effects of FAAH inhibition (Panlilio et al., 2013).

CB1 cannabinoid receptors

CB1R are heterogeneously expressed throughout the CNS (Hu & Mackie, 2015). CB1R are present at extremely high density in the cingulate gyrus, frontal cortex, hippocampus, cerebellum and the basal ganglia. Moderate receptor densities are found in the basal forebrain, amygdala, nucleus accumbens, periaqueductal grey and hypothalamus; and low density is seen in the midbrain, pons and medulla. Relatively little receptor is found in primary motor cortex or thalamus. In the forebrain, CB1R mRNA is expressed at very high density in a restricted number of neurons (Marsicano & Lutz, 1999). These CB1R-expressing neurons project widely, resulting in a dense network of CB1R positive processes. Double-labeling studies demonstrate that these highly expressing cells are GABAergic interneurons that also express the neuropeptide cholecystokinin (CCK; Katona et al., 1999). Other neurons express the CB1R at lower densities; these neurons are more heterogeneous and consist of both non-CCK, GABAergic interneurons and glutamatergic terminals (Hu & Mackie, 2015).

The CB1R is also expressed by non-neuronal cells in the CNS, including astrocytes (Navarrete & Araque, 2008; Salio, Doly, Fischer, Franzoni, & Conrath, 2002), oligodendrocytes (Molina-Holgado et al., 2002) and by endothelial (Golech et al., 2004) and smooth muscle cells (Gebremedhin, Lange, Campbell, Hillard, & Harder, 1999) of the cerebral circulation.

CB1R signaling—Activation of the CB1R results in inhibition of adenylyl cyclase activity in most tissues and cells via activation of G α i-mediated signaling (Howlett, 1985; Howlett & Fleming, 1984). As is the case for most GPCRs that couple to G α i, CB1R also engage G α o-mediated signaling (Glass & Northup, 1999) which results in inhibition of the opening of voltage-dependent calcium channels (VDCCs) through the release of associated $\beta\gamma$ subunits (Caulfield & Brown, 1992; Mackie & Hille, 1992). This mechanism is likely the

primary process by which CB1R regulate short-term changes in neurotransmitter release (described below). Signaling through G α i/o also results in activation of inward rectifying potassium channels (Henry & Chavkin, 1995). There is some evidence that CB1R agonists can exhibit bias toward activation of specific G protein α subtypes (Turu & Hunyady, 2010), suggesting that they can selectively engage inhibition of calcium influx versus adenylyl cyclase, for example. If G α i/o proteins are unavailable, CB1R will couple to G α s and thereby enhance adenylyl cyclase activity (Glass & Felder, 1997).

CB1R activation is coupled to activation of p42/p44 and p38 mitogen activated kinases and Jun N-terminal kinase through a variety of signaling mechanisms, including G proteins (Turu & Hunyady, 2010) and β -arrestin (Ahn, Mahmoud, & Kendall, 2012). CB1R activation has also been linked to the activation of PLC (Lograno & Romano, 2004) and Akt signaling in some cells (Gomez et al., 2011); through these pathways CB1R activation can influence intracellular calcium concentrations, protein kinase activities and other signaling cascades that regulate cell growth and differentiation.

There is evidence that the CB1R exhibits considerable constitutive activity, meaning that it can activate signaling cascades in the absence of agonist binding (Lee et al., 2015; Nie & Lewis, 2001; Savinainen, Saario, Niemi, Jarvinen, & Laitinen, 2003).

CB1R pharmacology—The endocannabinoids 2-AG and AEA are arachidonates that bind to the CB1R with affinities in the mid to high nanomolar range. 2-AG acts as a full agonist at the CB1R, at least with respect to G protein activation, while AEA is a partial agonist (Hillard, 2000). Ethanolamides of several other long chain, unsaturated fatty acids are also agonists of the CB1R, including the ethanolamide of docosahexaenoic acid (Hillard & Campbell, 1997). Two synthetic arachidonates, *N*-arachidonyl-2-chloroethylamide and arachidonylcyclopropylamide that have 500–1000 times greater affinity for the CB1R than CB2R have been developed (Hillard et al., 1999) and are the only CB1R-selective ligands in wide use. Many other synthetic agonists for the CB1R have been synthesized and characterized. These include derivatives of THC, such as levo-nantradol, CP55940 and HU210; and the aminoalkylindole, WIN55212–2 (Pertwee, 2008).

The identification of a CB1R antagonist occurred much later than the synthesis of the agonists. The first antagonist discovered was SR141716A, later named rimonabant (Rinaldi-Carmona et al., 1994). Rimonabant and a structural analog, AM251, are both inverse agonists of the CB1R (Bouaboula et al., 1997; Savinainen et al., 2003), so will reduce both constitutive and endocannabinoid-activated CB1R signaling. Several neutral antagonists have been designed that can be used to differentiate these possible mechanisms (Kirilly, Gonda, & Bagdy, 2012).

Recent studies have identified multiple allosteric ligands that interact with the CB1R. ORG27569 was originally reported to function as a negative allosteric modulator of the CB1R (Price et al., 2005). Subsequent studies found that this compound is a biased allosteric modulator, inhibiting CB1R agonist activation of G protein signaling (Baillie et al., 2013) while increasing high affinity orthosteric agonist binding sites, β -arrestin 1 recruitment, and ERK activation (Ahn et al., 2012). ORG27569 attenuates CB1R-mediated signaling in

hippocampal neurons in culture (Straiker, Mitjavila, Yin, Gibson, & Mackie, 2015); however, is not effective *in vivo*, perhaps because of a poor pharmacokinetic profile (Gamage et al., 2014).

PSNCBAM-1 is a negative allosteric modulator of CB1R-mediated activation of G protein signaling (Horswill et al., 2007). This compound exhibits *in vivo* efficacy to reduce body weight and feeding in a rat model, findings that are consistent with reduced CB1R activation. Further *in vitro* studies have demonstrated that PSNCBAM-1 reduces the effects of CP55940 to modulate synaptic CB1R signaling, but not the effects of WIN55212-2 (Wang, Horswill, Whalley, & Stephens, 2011). PSNCBAM-1 inhibits endocannabinoid-mediated activation of CB1R in cultured hippocampal neurons, suggesting that it negatively modulates 2-AG activation (Straiker et al., 2015).

Two endogenous compounds have been reported to function as CB1R negative allosteric modulators: a family of hemopressin-related peptides, particularly pepcan 12 (Bauer et al., 2012) which are widely expressed in brain (Hofer et al., 2015); and the steroid, pregnenolone (Vallee et al., 2014). While pepcan 12 is effective at reducing endogenous CB1R signaling in cultured hippocampal neurons, pregnenolone is not (Straiker et al., 2015). However, pregnenolone might be more effective at reducing the signaling of THC, and therefore could reduce its adverse effects (Vallee et al., 2014).

The phytocannabinoid, cannabidiol (CBD) was reported to act as a negative allosteric modulator for CB1R-mediated beta-arrestin 2 recruitment and ERK signaling (Laprairie, Bagher, Kelly, & Denovan-Wright, 2015). This is an interesting observation, given that CBD can dampen many of the effects of THC, including its anxiogenic effects.

Two positive allosteric modulators of the CB1R have been described. The first is the naturally occurring arachidonate, lipoxin A4 (Pamplona et al., 2012) and the second is a synthetic, ZCZ011 (Ignatowska-Jankowska et al., 2015). ZCZ011 potentiates binding of [³H]CP55,940 to the CB1R and increases AEA-stimulated [³⁵S]GTPγS binding in mouse brain membranes and β-arrestin recruitment and ERK phosphorylation in CB1R expressing cells. Lipoxin A4 also increases AEA signaling as well as its affinity for the CB1R.

CB1R and retrograde regulation of synaptic activity

The basic paradigms

Short term depression of synaptic transmission—Three simultaneous reports in 2001 described endocannabinoid/CB1R signaling as a mediator of retrograde inhibition of neurotransmitter release (Kreitzer & Regehr, 2001; Ohno-Shosaku, Maejima, & Kano, 2001; Wilson & Nicoll, 2001). Many studies in multiple brain regions carried out since demonstrate that modulation of synaptic plasticity is a major mechanism by which CB1R signaling affects the brain (Freund, Katona, & Piomelli, 2003; Kano, 2014; Patel & Hillard, 2009). In this mechanism, endocannabinoids are mobilized from the postsynaptic neuron in response to triggers, such as depolarization and activation of NMDA receptors, which increase calcium, and/or receptors that couple to an increase in PLC. These triggers result in the synthesis of 2-AG which diffuses from the postsynaptic neuron and binds to CB1R on

the presynaptic terminal. The result of presynaptic CB1R activation is inhibition of neurotransmitter release; in the case of short term modulation, the CB1R signaling mechanisms include inhibition of the opening of VDCCs and perhaps, also increased activation of potassium channels, resulting in hyperpolarization. In the case where depolarization of the post synaptic neuron is the trigger and CB1R activation inhibits GABA release, the plasticity is called depolarization-induced suppression of inhibition (DSI). Evidence supporting the role of the CB1R in DSI include data that DSI is both mimicked and occluded by a CB1R agonist; and that CB1R agonist and DSI increase the paired-pulse ratio and decrease spontaneous, calcium-dependent, miniature IPSPs, both indicative of a reduction in presynaptic vesicular release probability.

Endocannabinoid/CB1R signaling also produces suppression of glutamate release by a parallel mechanism. Specifically, depolarization of CA1 pyramidal neurons results in transient suppression of excitatory postsynaptic potential amplitude that is dependent upon CB1R activation (Ohno-Shosaku et al., 2002). DSE is also dependent upon presynaptic CB1R availability and is the result of activation of CB1R on glutamatergic terminals (Ohno-Shosaku et al., 2002; Ruehle et al., 2013).

DSI is not affected by botulinum toxin applied to the postsynaptic neuron, indicating that the retrograde messenger does not utilize a synaptic release mechanism. This finding is consistent with an “on-demand” synthesis of the endocannabinoids (Freund et al., 2003). Further studies have demonstrated that acute inhibition of DAGL suppresses (Hashimotodani et al., 2013) while acute inhibition of MAGL prolongs (Pan et al., 2009) hippocampal DSI. DSI/DSE are completely absent in slices from hippocampus, cerebellum, striatum and prefrontal cortex mice in which DAGL α has been genetically deleted (Gao et al., 2010; Tanimura et al., 2010; Yoshino et al., 2011). These findings, together with a lack of effect of FAAH inhibition on DSI (Pan et al., 2009), indicate that 2-AG is the endocannabinoid subserving this type of synaptic plasticity.

The primary triggers for the synthesis of endocannabinoid to produce short term plasticity are increased post synaptic calcium and activation of receptors that increase PLC activity (Kano, 2014). Receptor-driven, endocannabinoid mediated plasticity was first demonstrated for the group I mGluRs (Maejima, Hashimoto, Yoshida, Aiba, & Kano, 2001), which couple to G α_q proteins and therefore increase PLC β activity and generate DAG as described above. Subsequent studies demonstrated that many receptors that activate PLC can also evoke endocannabinoid-dependent short term suppression of neurotransmitter release. Endocannabinoid signaling can be induced by strong G α_q linked receptor activation alone, or by a combination of sub-threshold G α_q activation with a simultaneous increase in postsynaptic calcium (Kim, Isokawa, Ledent, & Alger, 2002). It is not completely clear how increased postsynaptic calcium concentrations results in an increase in 2-AG; a two enzyme pathway for DAG synthesis involving PLD and PAP is calcium sensitive and could serve this function (Bisogno et al., 1999). There is evidence that different DAGL α pools could be responsible for 2-AG generation from DAG synthesized in response to calcium versus PLC activation (Zhang, Wang, Bisogno, Di Marzo, & Alger, 2011).

Long-term depression of transmission (LTD)—The ECS has also been implicated in more persistent forms of synaptic plasticity. Stimulation protocols which induce LTP of excitatory synapses onto CA1 pyramidal neurons also produce long-term depression of GABAergic inputs to the same neurons; a phenomenon called iLTD (Chevalleyre & Castillo, 2003). High frequency stimulation (HFS) of inputs to CA1 pyramidal neurons depresses GABAergic transmission onto these neurons for almost 1 hour, an effect that is presynaptic in nature, and requires activation of mGluR and CB1R (Chevalleyre & Castillo, 2003). Induction of iLTD is blocked by PLC and DAGL inhibition, supporting a role for 2-AG in the initiation of the plasticity (Edwards, Kim, & Alger, 2006). HFS of cortical afferents to the striatum induce LTD of glutamatergic transmission, an effect absent in CB1R^{-/-} mice and mice treated with CB1R antagonist (Gerdeman, Ronesi, & Lovinger, 2002). Interestingly, CB1R antagonist application 10 minutes after HFS does not block LTD, suggesting that the ECS is required for the induction of LTD, but not its long-term maintenance (Ronesi et al., 2004). LTD at this synapse is dependent upon increases in post-synaptic intracellular calcium (Gerdeman et al., 2002).

The triggers for endocannabinoid-mediated LTD are the same as those for short term synaptic suppression; i.e. increased post-synaptic calcium and increased PLC activity. What differentiates short and long term changes in neurotransmitter release are the presynaptic signaling mechanisms evoked by CB1R activation. For ECS-mediated LTD, inhibition of PKA is critical (Castillo, Younts, Chavez, & Hashimoto, 2012) and synergistic changes in the presynaptic terminal are likely required, such as increased calcium concentrations or co-activation of other GPCRs (Kano, 2014).

There is evidence that AEA can also produce changes in synaptic activity at a more limited number of synapses and through TRPV1 signaling. For example, AEA-mediated post-synaptic TRPV1 activation produces LTD in the hippocampus (Chavez, Chiu, & Castillo, 2010), nucleus accumbens (Grueter, Brasnjo, & Malenka, 2010) and extended amygdala (Puente et al., 2011). These effects do not involve CB1R signaling.

Summary

The ECS is a beautiful and fascinating neuromodulatory system that allows for moment to moment, synapse-specific regulation of neurotransmission. The basic building blocks of the proteins involved in endocannabinoid synthesis and degradation together with the receptor targets are utilized throughout the brain, but in very diverse ways depending upon their relative locations and in relationship to other ongoing signaling events. The ECS modulates the functions of the major excitatory and inhibitory neurotransmitters, serving as a break on their release (described above). CB1R can also inhibit the release of biogenic amines (Haring, Guggenhuber, & Lutz, 2012) and neuropeptides (Hirasawa et al., 2004), thereby modulating the modulators! The ECS is likely the mechanism by which steroid hormones such as glucocorticoids (Di, Malcher-Lopes, Halmos, & Tasker, 2003) and estrogen (Huang & Woolley, 2012) alter synaptic plasticity. Recent evidence that a cycle of 2-AG → AA is tightly regulated by MAGL activity (discussed above) adds another layer of complexity to the role of 2-AG homeostasis to brain function. It is not surprising that increasing evidence indicates that dysregulation of ECS contributes to many forms of brain dysfunction,

including psychopathology, developmental problems and plays a role in neurodegenerative diseases as well.

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Abbreviations

AA	arachidonic acid
Abhd	Alpha beta hydrolase domain protein
AEA	<i>N</i> -arachidonylethanolamine
2-AG	2-arachidonoylglycerol
CB1R	cannabinoid receptor subtype 1
CBD	cannabidiol
CCK	cholecystokinin
DAG	diacylglycerol
DAGL	diacylglycerol lipase
DSE	depolarization-induced suppression of excitation
DSI	depolarization-induced suppression of inhibition
EA	ethanolamine
ECS	endocannabinoid system
FAAH	fatty acid amide hydrolase
GDE1	glycerophosphodiesterase 1
GP-AEA	glycerophospho- <i>N</i> -arachidonylethanolamine
GP-NAE	glycerophospho- <i>N</i> -acylethanolamine
GPCR	G protein coupled receptor
HFS	high frequency stimulation
IP₃	inositol triphosphate
LTD	long-term depression
MAGL	monoacylglycerol lipase
mGluR	metabotropic glutamate receptor

NAAA	<i>N</i> -acylethanolamine-hydrolyzing acid amidase
NAE	<i>N</i> -acylethanolamine
NAPE	<i>N</i> -acyl phosphatidylethanolamine
NAPE-PLD	<i>N</i> -acyl phosphatidylethanolamine-specific phospholipase D
NAT	<i>N</i> -acyl transferase; PC: phosphatidylcholine
PA	phosphatidic acid
PAP	phosphatidic acid phosphatase
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PIP₂	phosphatidylinositol 4,5 bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLA/AT	phospholipase A/acyl transferase
PLC	phospholipase C
PLD	phospholipase D
PPAR	peroxisome proliferation activating receptor
PTPN22	protein tyrosine phosphatase, non-receptor type 22
SHIP1	Src homology 2-containing inositol phosphatase-1
THC	⁹ -tetrahydrocannabinol
THL	tetrahydrolipstatin
VDCC	voltage- dependent calcium channel

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Figure 1.

AEA biosynthesis and degradation pathways. The pathways described in the text are shown here specifically for the endocannabinoid, AEA. Abbreviations: AA: arachidonic acid; Abhd4: Alpha beta hydrolase domain protein 4; AEA: *N*-arachidonyl ethanolamine; EA: ethanolamine; FAAH: fatty acid amide hydrolase; GDE1: glycerophosphodiesterase 1; GP-AEA: glycerophospho-*N*-arachidonyl ethanolamine; NAAA: *N*-acyl ethanolamine-hydrolyzing acid amidase; NAPE-PLD: *N*-acyl phosphatidylethanolamine-specific phospholipase D; NAT: *N*-acyl transferase; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PLA/AT: phospholipase A/acyltransferase; PLC: phospholipase C; PTPN22: protein tyrosine phosphatase, non-receptor type 22; SHIP1: Src homology 2-containing inositol phosphatase-1. Scheme is a modification of figure 2 from [Rahman, Tsuboi, Uyama, & Ueda, 2014).

Figure 2.

Biosynthesis and catabolism of 2-AG. Abbreviations: AA: arachidonic acid; abhd6: alpha-beta hydrolase domain containing protein 6; abhd12: alpha-beta hydrolase domain containing protein 12; 2-AG: 2-arachidonoylglycerol; DAG: diacylglycerol; DAGL: diacylglycerol lipase; IP₃: inositol triphosphate; MAGL: monoacylglycerol lipase; PA: phosphatidic acid; PAP: phosphatidic acid phosphatase; PC: phosphatidylcholine; PIP₂: phosphatidylinositol 4,5 bisphosphate; PLC: phospholipase C; PLD: phospholipase D.