# Cannabinoids and Endocannabinoids

# 58

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## Contents

Historical Background ........ 1812
The Endocannabinoid System ........ 1814
Cannabinoid Receptors ........ 1814
2-Arachidonoyl-*sn*-Glycerol (2-AG) ........ 1816
Anandamide ........ 1821
Additional Endogenous Cannabinoid Ligands ........ 1826
Cannabinoid-Based Therapeutics ........ 1826
$CB_1$ Receptor Agonists ........ 1826
$CB_1$ Receptor Antagonists ........ 1828
$CB_2$ Receptor Agonists ........ 1829
Endocannabinoid Deactivation Inhibitors ........ 1829
Outlook ........ 1832
References ........ 1832

**Abstract**

Marijuana is a common name for the plant *Cannabis sativa*, whose intoxicating and medicinal effects have been known for thousand of years. The active principle of marijuana, (−)-*trans*-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC), exerts its pharmacological effects by binding to selective receptors present on the membranes of neurons and other cells. These cannabinoid receptors are normally engaged by a

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family of lipid mediators, called endocannabinoids, which are thought to participate in the regulation of a diversity of brain functions, including pain, mood, appetite and memory.

The endocannabinoid system is comprised of the endocannabinoids, mainly anandamide (arachidonoylethanolamide) and 2-arachidonoylglycerol (2-AG), proteins that control their formation and deactivation, and cell-surface receptors ($CB_1$ and $CB_2$) that transduce their actions. The key components of endocannabinoid signaling are found in the brain and spinal cord, but also in many peripheral organs and tissues.

In this chapter, we outline current views on how endocannabinoid substances are produced, act on cannabinoid receptors, and are deactivated in the brain. In addition, we review recent progress on the development of pharmacological agents that interfere with endocannabinoid deactivation and discuss their potential utility in the treatment of cannabinoid-based therapeutics.

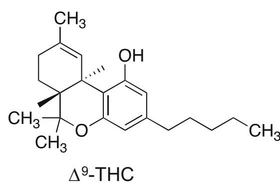
**Keywords**

2-Arachidonoyl-*sn*-glycerol (2-AG) • Bioactive 2-AG metabolites • Enzymatic steps • Formation and deactivation • 2-AG signalosome • Anandamide • Anxiety • Cancer • Cannabinoids • $CB_1$ receptor agonists • $CB_1$ receptor antagonists • $CB_2$ receptor agonists • Chemical structures • Cannabis addiction • Endocannabinoid system • Cannabinoid receptors • Deactivation inhibitors • Fatty acid amide hydrolase (FAAH) • Monoacylglycerol lipase (MGL) • *N*-acyl transferase (NAT) activity • *N*-acylethanolamine acid amidase (NAAA) • Perisynaptic annulus • Phospholipase C (PLC) • Phospholipase D (PLD) • Schizophrenia • Tetrahydrocannabinol

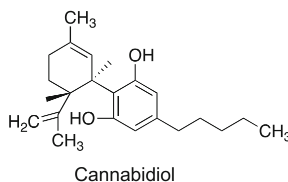
## Historical Background

Marijuana is a common name for the plant *Cannabis sativa*, whose intoxicating and medicinal effects have been known for thousands of years. The chemical constituent responsible for the majority of such effects – a terpene-like molecule called tetrahydrocannabinol – was first identified in the early 1940s by the American chemist Roger Adams, who also devised its first synthesis (Adams 1942). This work was soon confirmed by others (Wollner et al. 1942) and completed in 1964 by Raphael Mechoulam (Gaoni and Mechoulam 1964), who precisely defined the structure of bioactive tetrahydrocannabinol as (−)-*trans*-$\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) (Fig. 1a). In spite of these structural advances, $\Delta^9$-THC remained rather mysterious for the following 20 years. Indeed, its mechanism of action was strongly debated until 1988, when Allyn Howlett discovered that synthetic molecules designed to mimic its effects, such as the compound CP-55,940 developed by Pfizer in the late 1980s (Fig. 1b), bind to unique receptive sites and engage G proteins to inhibit adenylyl cyclase activity in brain tissue (Devane et al. 1988). The subsequent

**A. Phytocannabinoids**

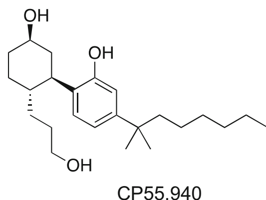



$\Delta^9$-THC

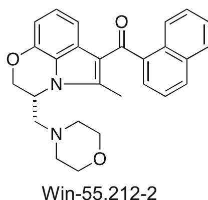



Cannabidiol

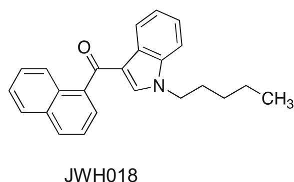
**B. Synthetic Cannabinoids**




CP55,940

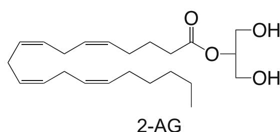



Win-55,212-2

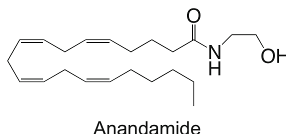



JWH018

**C. Endocannabinoids**




2-AG




Anandamide

**Fig. 1 Chemical structures of representative ligands for cannabinoid receptors**. (**a**) Two terpene-like chemicals present in the *Cannabis* resin. $\Delta^9$-Tetrahydrocannabinol ($\Delta^9$-THC) is responsible for the majority of the psychotropic effects of the drug; it acts by binding to G protein-coupled cannabinoid receptors in the brain and other tissues of the body. Cannabidiol displays a distinct set of pharmacological properties (e.g., antipsychotic and antiepileptic), which do not involve cannabinoid receptor activation. (**b**) Synthetic $CB_1$ receptor agonists. CP-55,940 and Win-55,212-2 (developed by pharmaceutical companies Pfizer and Winthrop, respectively) and JWH018 (originally synthesized by medicinal chemist J.W. Huffman) are cannabinoid receptor agonists. CP-55,940 and Win-55,212-2 are widely used experimentally, while JWH018 is a component of street drugs such as “Spice.” (**c**) The two primary endogenous activators of cannabinoid receptors. 2-AG and anandamide are produced on demand through cleavage of membrane lipid precursors and are involved in various short-range signaling processes including several forms of synaptic plasticity

mapping of these binding sites in the rat central nervous system (CNS) (Herkenham et al. 1990) and the molecular cloning of the first cannabinoid receptor gene (Matsuda et al. 1990), now called $CB_1$, provided definitive evidence that the mammalian brain contains a selective cell-surface receptor that recognizes $\Delta^9$-THC and underpins many of its biological actions. Shortly after the cloning of $CB_1$, a second G protein-coupled receptor, $CB_2$, was identified in lymphocytes and other immune cells (Munro et al. 1993).

In the years that followed, the obligatory role of cannabinoid receptors in mediating the effects of $\Delta^9$-THC and other cannabimimetic ("cannabis-like") substances was unambiguously demonstrated using selective pharmacological tools (receptor agonists and antagonists) as well as genetic mouse models (mutant mice lacking the $CB_1$ and $CB_2$ receptor genes, *cnr1* and *cnr2*) (for reviews, see Kano et al. 2009; Chevaleyre et al. 2006; Freund et al. 2003). Meanwhile, the discovery of cannabinoid (CB) receptors had launched a search for endogenous substances that might normally interact with these proteins. This quest culminated in the isolation of two lipid-derived molecules – anandamide (arachidonoylethanolamide) in 1992 (Devane et al. 1992), and 2-arachidonoylglycerol (2-AG) in 1995 (Sugiura et al. 1995; Mechoulam et al. 1995) – by the laboratories of Mechoulam and Takayuki Sugiura. Many neuroscientists – accustomed to studying conventional water-soluble neurotransmitters – were initially surprised by the lipid nature of these compounds. Skepticism was eventually overcome, however, by the elucidation of the compounds' unique biogenesis (Di Marzo et al. 1994; Stella et al. 1997), the demonstration of their "on demand" formation and release in live brain preparations (Di Marzo et al. 1994; Stella et al. 1997; Giuffrida et al. 1999), and the progressive accumulation of evidence indicating that they function as local regulators of synaptic activity, rather than typical transmitters (for reviews, see Piomelli 2003; Piomelli et al. 2007) (more on this point later in the chapter).

## The Endocannabinoid System

The endocannabinoid system is comprised of a set of lipid-derived messengers (the endocannabinoids), proteins that control their formation and deactivation, and cell-surface receptors ($CB_1$ and $CB_2$) that transduce their actions. The key components of endocannabinoid signaling are found in the brain and spinal cord, but also in most peripheral organs and tissues.

### Cannabinoid Receptors

$CB_1$ and $CB_2$ receptors exhibit 48 % amino acid sequence identity and signal through the transducing G proteins, $G_i$ and $G_o$ (for reviews, see Mackie 2006; Freund et al. 2003). The binding of $\Delta^9$-THC and other cannabinoid agonists to these receptors causes inhibition of adenylyl cyclase activity, closing of certain voltage-gated calcium channels, opening of inwardly rectifying potassium channels, and stimulation of various protein kinases (Mackie 2006). In the brain, where $CB_1$ is primarily localized to axon terminals, two important consequences of its activation are the suppression of neuronal excitability (via increase of K+ channel activity) and the reduction of neurotransmitter release (via inhibition of $Ca^{2+}$ channel activity) (Fig. 2).

$CB_1$ receptor expression is greatest in brain structures that are most implicated in the psychoactive effects of *Cannabis*. In humans and other mammals, high

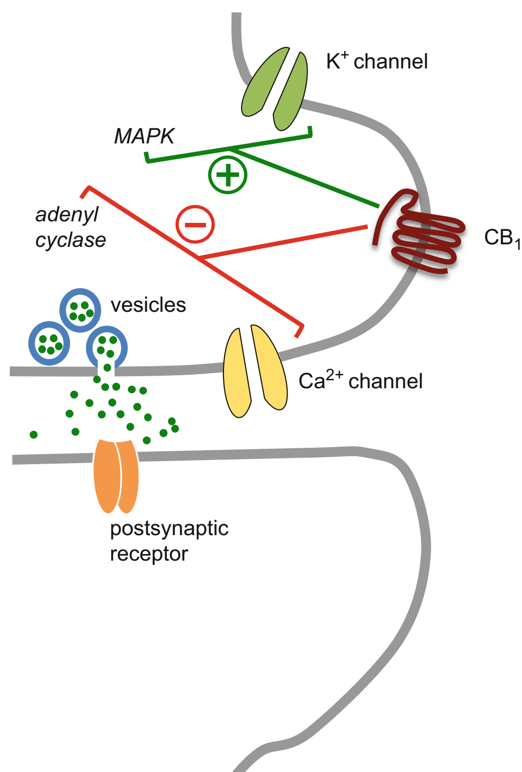



**Fig. 2 $CB_1$ receptor signaling in the brain**. $CB_1$ receptors are present in nerve terminals of excitatory (glutamatergic), inhibitory (GABAergic), and modulatory (e.g., serotonergic) neurons of the brain. By recruiting G proteins, $CB_1$ increases the activity of potassium ($K^+$) channels (reducing neuronal excitability) and decreases activity of calcium ($Ca^{2+}$) channels (inhibiting neurotransmitter release). Additionally, $CB_1$ inhibits adenylyl cyclase (lowering intracellular cyclic AMP levels) and stimulates various protein kinases, including mitogen-activated protein kinases (MAPKs) and focal adhesion kinase, leading to phosphorylation of synaptic proteins

concentrations of $CB_1$ are found in the neocortex, hippocampus, basal ganglia, and cerebellum. Substantial receptor levels are also present in the basolateral amygdala, hypothalamus, brain stem, and spinal cord (Mackie 2006). $CB_1$ is also found outside the brain and spinal cord. Functionally significant amounts of the receptor are found in small intestine, liver, white adipose tissue, pancreas, and skeletal muscle, where its presence likely reflects the pervasive influence exerted by the endocannabinoids on energy balance and peripheral metabolism (for reviews, see DiPatrizio and Piomelli 2012, 2015).

Activation of $CB_1$ receptors by full agonist ligands initiates a process of desensitization that eventually renders subjects exposed to $\Delta^9$-THC and other cannabinoid agonists tolerant to the central and peripheral effects of these drugs (for a review, see González et al. 2005). (Full agonists are compounds that bind to and activate a receptor, producing full efficacy at that receptor. Partial agonists have only partial efficacy compared to a full agonist.) This process can also occur in humans: positron emission tomography studies have shown that chronic marijuana use causes a downregulation of $CB_1$ receptors in cortical regions of the brain, and that abstinence reverses this effect (Hirvonen et al. 2012).

In addition to $CB_1$, the brain also contains a small number of $CB_2$ receptors, which are probably localized to neurons and microglia (Mackie 2006). However, this

receptor subtype is expressed at much higher levels in cells of the peripheral immune system, including macrophages and macrophage-derived cells such as osteoclasts and osteoblasts (Mackie 2006). These cells also express $CB_1$, albeit to a lesser extent than $CB_2$, with both receptor types exerting a broad spectrum of modulatory effects on cytokine release, apoptosis, and cell migration.

## 2-Arachidonoyl-*sn*-Glycerol (2-AG)

There is a general consensus that 2-AG and anandamide are the two primary endocannabinoid substances produced in mammalian tissues (Fig. 1c). Though other endogenous lipid-derived molecules have been found, which activate cannabinoid receptors in vitro, only 2-AG and anandamide have been consistently shown to meet three key conditions that define a neurotransmitter: activity-dependent release from neurons, modulation of synaptic transmission via activation of cell-surface receptors, and rapid deactivation (Piomelli 2003; Piomelli et al. 2007).

### 2-AG Formation and Degradation

At the molecular level, we understand 2-AG much better than we do anandamide. The metabolism of this fatty acyl ester, illustrated in Fig. 3, starts with the cleavage of a membrane phospholipid, phosphatidylinositol-4,5-bisphosphate ($PIP_2$), and ends with the generation of the free fatty acid, arachidonic acid. The first steps in this pathway are sequentially catalyzed by two lipid hydrolases, phospholipase C (PLC) and diacylglycerol lipase (DGL), which are both localized to dendritic spines of excitatory synapses (Stella et al. 1997; Katona et al. 2006). PLC converts $PIP_2$ into 1,2-diacylglycerol (1,2-DAG) (Bennett et al. 1988). This reaction is stimulated by agonist-bound $G_q$ protein-coupled receptors (e.g., type-1 metabotropic glutamate receptors, mGluR5) and in brain tissue is specifically mediated by the β isoform of PLC (PLC-β) (Jung et al. 2005). 1,2-DAG regulates the activity of protein kinase C and other cellular effectors, but also serves as substrate for the α isoform of DGL (DGL-α), which cleaves 1,2-DAG to produce 2-AG (Stella et al. 1997; Bisogno et al. 2003). Of note, 1,2-DAG can also be transformed by diacylglycerol kinase into phosphatidic acid, another intracellular second messenger (for a review, see Shulga et al. 2011).

It is quite common for lipid-derived messengers to be produced through multiple biogenetic routes (Piomelli et al. 2007). Another pathway that may contribute to 2-AG production involves phospholipase $A_1$ ($PLA_1$). This enzyme cleaves membrane phospholipids at the *sn*-1 position, forming arachidonic acid-containing lysophospholipids that are subsequently converted into 2-AG by a lyso-PLC activity (Pete et al. 1994) (Fig. 3).

After diffusing out of the dendritic spine, newly formed 2-AG reaches axon terminals where it encounters both $CB_1$ receptors, which are responsible for its presynaptic actions, and the enzyme monoacylglycerol lipase (MGL), which interrupts such actions (Fig. 4). MGL is a ubiquitous serine hydrolase that converts

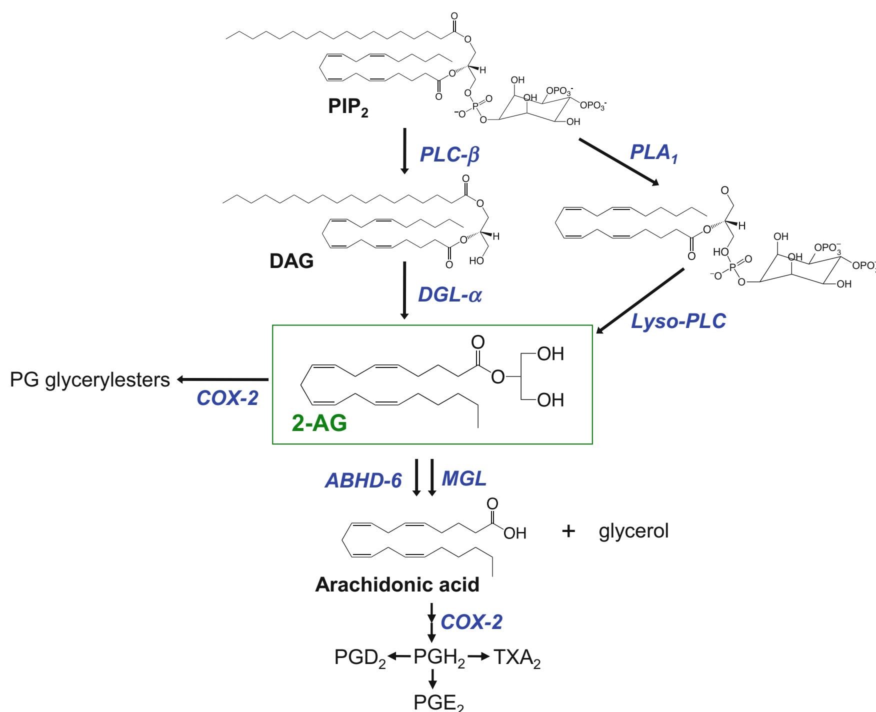



**Fig. 3 Enzymatic steps involved in 2-AG formation and degradation**. In cellular membranes, phospholipase C (*PLC-β*) converts phosphatidylinositol-4,5-bisphosphate (*PIP*$_2$) into 1,2-diacylglycerol (*DAG*). DAG is hydrolyzed by diacylglycerol lipase (*DGL-α*) forming the endocannabinoid 2-AG. Alternatively, 2-AG could be produced through serial hydrolyses of phospholipids by phospholipase $A_1$ (*PLA*$_1$) and lyso-PLC activities. 2-AG is subjected to hydrolytic cleavage catalyzed either by monoacylglycerol lipase (*MGL*) or α/β-hydrolase domain-6 (*ABHD-6*). Additionally, 2-AG can be oxygenated by cyclooxygenase-2 (*Cox*-2) to yield a family of non-endocannabinoid prostaglandin (*PG*) glycerol esters

monoacylglycerols such as 2-AG into fatty acid and glycerol (Fig. 3). It was first molecularly characterized in adipocytes, where it catalyzes the last step in triacylglycerol hydrolysis (lipolysis) (Karlsson et al. 1997), and was later shown to be also responsible for 2-AG deactivation in the brain (Dinh et al. 2002). This conclusion is supported by results obtained with pharmacological inhibitors and genetically modified mice that either lack or over-express MGL (Hohmann et al. 2005; Jung et al. 2012a). In the brain, MGL is primarily found in axon terminals and is almost equally distributed between the cytosol and the inner aspect of the presynaptic cell membrane (Dinh et al. 2002, 2004; Gulyas et al. 2004). This localization suggests that MGL may readily gain access to the pool of 2-AG that interacts with $CB_1$ receptors in axon terminals and, therefore, may efficiently catalyze the hydrolysis of this compound at its main site of action. Studies in a neural cell line have provided evidence that the MGL-mediated degradation of 2-AG

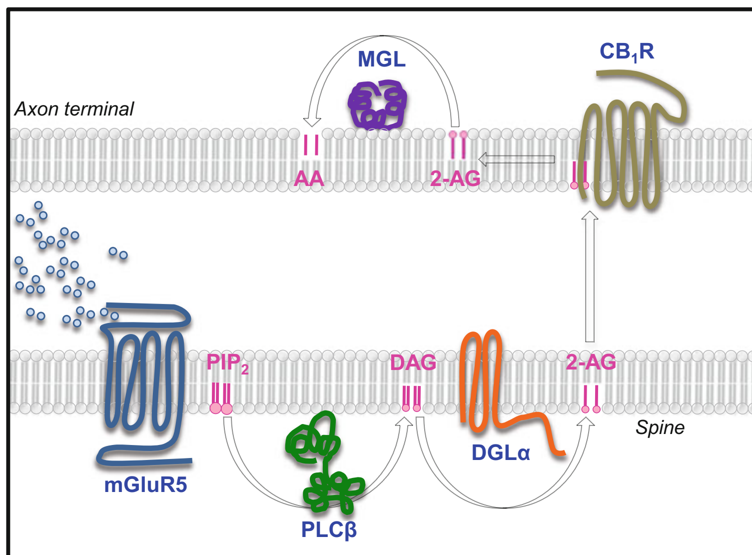



**Fig. 4** **Molecular architecture of 2-AG signaling in the brain**. Cell membrane-associated lipid hydolases phospholipase C-β (*PLC-β*) and diacylglycerol lipase-α (*DGL-α*) are involved in the biosynthesis of 2-AG. On demand activation of receptors such as metabotropic glutamate receptor subtype 5 (*mGluR5*) may trigger the biosynthetic pathway. Monoacylglycerol lipase (*MGL*) hydrolyzes and deactivates 2-AG, terminating its effects

is driven by the subsequent condensation of arachidonic acid with coenzyme A (Beltramo and Piomelli 2000), a pivotal step in phospholipid remodeling catalyzed by acyl-CoA synthetase (for a review, see Farooqui et al. 2000).

MGL accounts for about 85 % of the total 2-AG hydrolase activity present in the rodent brain (Dinh et al. 2004). The remainder have been attributed to two distinct serine lipases, α/β hydrolase domain-containing protein (ABHD)-6 and ABHD-12 (Blankman et al. 2007), which are also able to catalyze the hydrolysis of 2-AG in vitro. The role of these enzymes in terminating the actions of 2-AG remains uncertain. Data supporting such a role have been obtained for ABHD-6, the inhibition of which prolongs the effects of 2-AG at brain synapses (Jung et al. 2012a; Marrs et al. 2010). By contrast, ABHD-12 appears to be primarily involved in the degradation of lysophosphatidylserine (Blankman et al. 2013), a phospholipid that is not directly involved in endocannabinoid signaling.

## The 2-AG Signalosome

Strictly confined to an area of the dendritic spine that is adjacent to the postsynaptic density (PSD) – the so-called perisynaptic annulus – the 2-AG signalosome is a multi-molecular protein complex that joins in a single functional unit, held together by scaffolding Homer proteins, three key players in 2-AG production: mGluR5, PLC-β, and DGL-α (Katona et al. 2006; Jung et al. 2007, 2012b) (Fig. 5). When

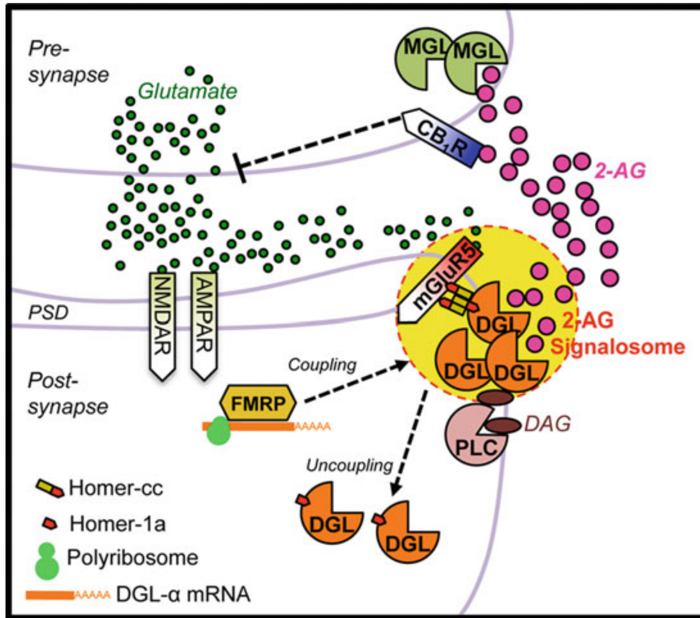



**Fig. 5 The 2-AG signalosome at excitatory brain synapses**. This supramolecular complex, selectively localized to the perisynaptic zone of the dendritic spine, connects in a single functional unit three key proteins involved in 2-AG production – mGluR5 metabotropic glutamate receptors, phospholipase C-β (*PLC*-β), and diacylglycerol-α (*DGL-α*). Evidence suggests that these proteins are held together by the scaffolding proteins Homer-cc and Shank. Fragile X mental retardation protein (*FMRP*) may help target DGL-α to the 2-AG signalosome, possibly by positioning the DGL-α or Homer message(s) in specific subcellular nanodomains (coupling). It is hypothesized that expression of multimerization-incompetent Homer1a isoform could cause a dissociation of the 2-AG signalosome (uncoupling). The proximity of mGluR5 to PLC-β and DGL-α allows for the rapid accumulation of 2-AG, which travels across the synaptic cleft to activate $CB_1$ receptors on axon terminals. The 2-AG that reaches presynaptic terminals may be quickly hydrolyzed by monoacylglycerol lipase (*MGL*), while the 2-AG that fails to reach the terminals may be degraded by α/β hydrolase domain-containing protein 6 (*ABHD-6*). *AMPAR* AMPA receptors, *NMDAR* NMDA receptors, *PSD* postsynaptic density

glutamate released by excitatory terminals binds to mGluR5, the physical proximity of this protein to PLC-β and DGL-α enables 2-AG to be generated in large amounts within this focal area. Newly formed 2-AG leaves the postsynaptic membrane to activate $CB_1$ receptors on adjacent nerve terminals, causing in turn a reduction in calcium channel activity and glutamate release. The fraction of 2-AG that reaches the terminals and activates $CB_1$ may be then rapidly hydrolyzed by MGL, while the 2-AG that remains associated with the spine might be eliminated by ABHD-6, which is localized postsynaptically (Marrs et al. 2010; Jung et al. 2012b) (Fig. 5). The 2-AG signalosome was identified at excitatory synapses of the ventral striatum and pre-frontal cortex (Jung et al. 2012a) but is likely to be present in other regions of the mammalian CNS (Mátyás et al. 2008 and Nyilas et al. 2009). The identification of

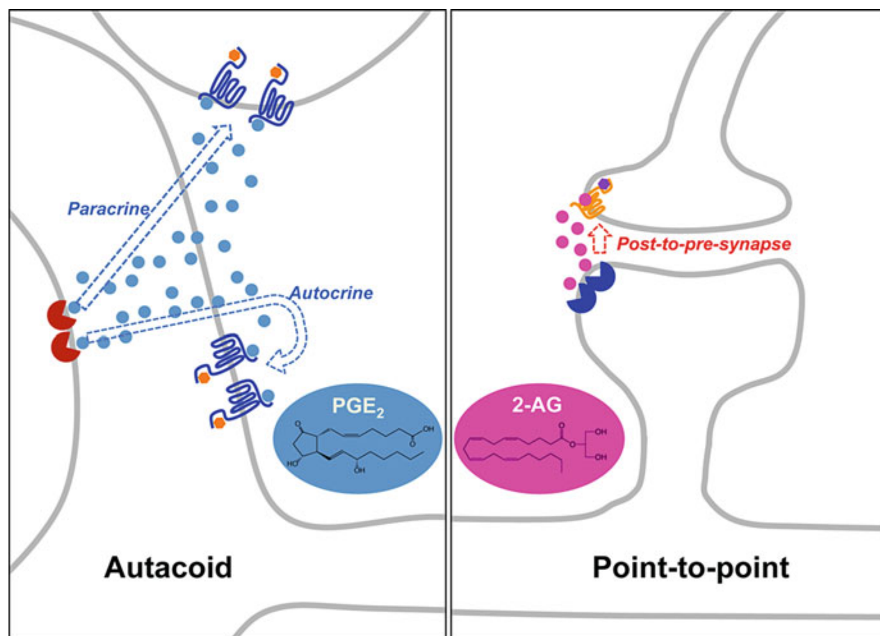



**Fig. 6 2-AG as a point-to-point regulator of synaptic activity**. 2-AG signaling at excitatory synapses of the brain provides a first example of point-to-point-type lipid signaling. In contrast to classical autacoid-type lipid signaling (e.g., via diffusible prostaglandins) (**a**), point-to-point signaling requires that the proteins essential for the formation and deactivation of 2-AG are spatially arranged to make signaling both rapid and efficient (**b**)

the 2-AG signalosome (Jung et al. 2012a) provides an unusual example of point-to-point lipid signaling. In contrast to classical autacoid-type signaling (e.g., by diffusible eicosanoids), this signaling modality requires that the enzymes needed to produce 2-AG be arranged in such a way as to make 2-AG-mediated retrograde transmission rapid and efficient (Fig. 6).

The structural arrangement outlined above has been documented at excitatory synapses, which contain relatively low levels of $CB_1$ receptors (Marsicano and Lutz 1999). Inhibitory synapses formed by cholecystokinin-containing GABAergic interneurons – where retrograde signaling has been also demonstrated (for reviews, see Castillo et al. 2012 and Katona and Freund 2012) and $CB_1$ is present in large numbers (Katona et al. 1999) – are likely to control endocannabinoid signaling through different mechanisms, which remain unknown.

## Bioactive 2-AG Metabolites

The sequential action of PLC-β, DGL-α, and MGL contributes to the release of free (non-esterified) arachidonic acid from membrane phospholipids (Allen et al. 1992; Bell et al. 1979) (Fig. 3). Like other polyunsaturated fatty acids, free arachidonate is either immediately reinserted into membrane phospholipids (part of a process known

as "phospholipid remodeling") or utilized for the production of the eicosanoids, a large family of cyclooxygenase metabolites that include prostaglandins, thromboxanes, and leukotrienes (Piomelli et al. 2007). These are important bioactive lipids that control the neural response to psychological stress, body temperature, and energy homeostasis, among other processes (for reviews, see Piomelli et al. 2007; Harizi et al. 2008). 2-AG itself can be oxygenated by cyclooxygenase-2 (Cox-2) to yield a family of prostaglandin glyceryl esters, which do not bind to cannabinoid receptors yet display interesting biological activities (Kozak et al. 2000). It appears that neural cells can steer 2-AG toward alternative fates of metabolic activation (e.g., formation of prostaglandin glyceryl esters) or deactivation (e.g., hydrolysis followed by arachidonic acid reesterification into phospholipids or Cox-2-dependent oxidation). The selection between these paths is likely to depend on the cells' signaling needs, but how such selection is made and enforced is entirely unknown.

## Anandamide

Figure 7 illustrates the main molecular pathways involved in the formation and degradation of anandamide. In contrast with 2-AG, the reactions leading to the production of this endocannabinoid are relatively unprecedented in lipid biochemistry, and anandamide and other members of its chemical family – the amides of ethanolamine with long-chain fatty acids (known as *N*-acylethanolamines or fatty acid ethanolamides) – were initially dismissed as being terminal products of *post mortem* tissue degradation rather than physiologically meaningful signaling molecules (Schmid et al. 1995). The functional significance – and indeed the very existence (Kempe et al. 1996) – of anandamide remained controversial until the mechanisms underlying the production and deactivation of this compound were outlined using primary cultures of rat brain neurons (Cadas et al. 1996, 1997; Di Marzo et al. 1994) and its activity-dependent release in the CNS of freely moving rats was demonstrated by using a combination of in vivo microdialysis and gas chromatography/mass spectrometry (Giuffrida et al. 1999).

### Anandamide Formation

Three interconnected enzyme pathways have been implicated in anandamide production (Fig. 7). The "canonical route," shown in the center of the figure, was elaborated in 1994–1997 (Cadas et al. 1996, 1997; Di Marzo et al. 1994). According to this model, anandamide is released by hydrolysis of the phospholipid precursor, *N*-arachidonoyl-phosphatidylethanolamine (*N*-arachidonoyl-PE), which is catalyzed by a phospholipase D (PLD) that preferentially recognizes *N*-acyl-substituted PE species (collectively called NAPEs) over other more common phospholipids (for a review, see Ueda et al. 2013). A unique PLD that selectively hydrolyzes NAPEs, including *N*-arachidonoyl-PE, was molecularly cloned (Ueda et al. 2013) and its structure was recently resolved by X-ray crystallography (Magotti et al. 2015). Nevertheless, its physiological role in anandamide formation remains to be fully elucidated (for a review, see Piomelli 2014).

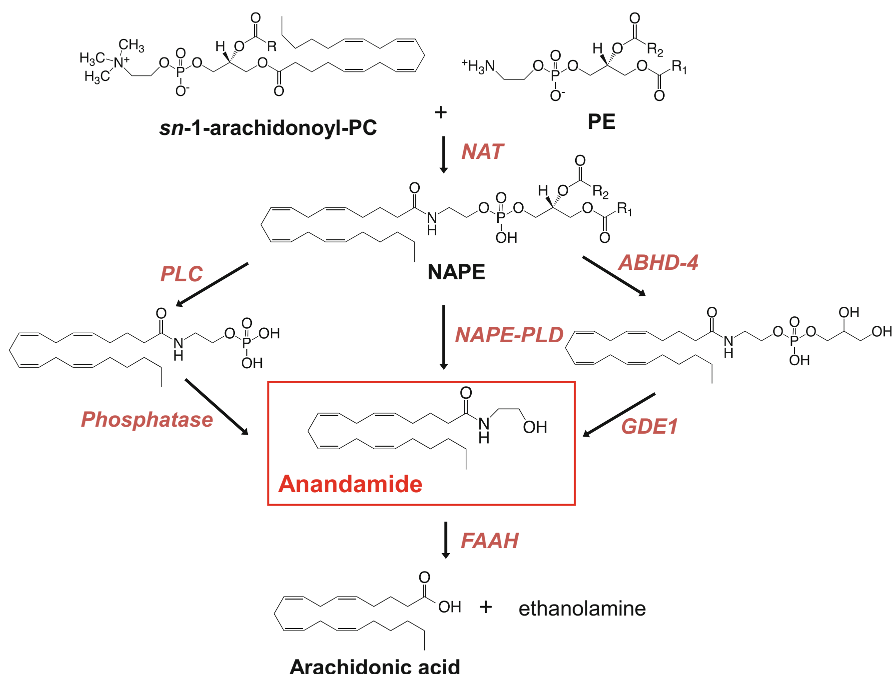



**Fig. 7 Enzymatic steps involved in anandamide formation and degradation**. The canonical route of anandamide biosynthesis is shown in the *center*. According to this model, anandamide is released by hydrolysis of the phospholipid precursor, *N*-arachidonoyl-phosphatidylethanolamine (*N*-arachidonoyl-PE), catalyzed by a phospholipase D (*PLD*). *N*-arachidonoyl-PE is produced through a two-step reaction in which arachidonic acid (*AA*) is transferred from the *sn*-2 position of a phospholipid to the *sn*-1 position of lyso-phosphatidylcholine (*PC*), producing diarachidonoyl-PC. The *sn*-1 arachidonoyl chain of diarachidonoyl-PC is then transferred to the free amino group of PE, generating *N*-arachidonoyl-PE. Two additional routes of anandamide biosynthesis have been proposed. *Left*: an as-yet-uncharacterized phospholipase C (*PLC*) converts *N*-arachidonoyl-PE into phospho-anandamide, which is then dephosphorylated by a phosphatase forming anandamide. *Right*: *N*-arachidonoyl-PE is hydrolyzed by α/β hydrolase domain-containing protein 4 (*ABHD-4*), forming glycerophospho-anandamide, which generates anandamide after losing the glycerophosphate group. Anandamide is degraded intracellularly by the serine amidase, fatty acid amide hydrolase (*FAAH*)

The levels of the anandamide precursor, *N*-arachidonoyl-PE, are vanishingly low in resting neurons but quickly increase when the neurons are exposed to stimuli that elevate intracellular calcium concentrations (Cadas et al. 1996, 1997; Di Marzo et al. 1994; Stella and Piomelli 2001). For *N*-arachidonoyl-PE to be produced, a sequence of two distinct enzyme-mediated reactions must occur. First, arachidonic acid must be transferred from the *sn*-2 position of various phospholipids, where it normally resides, to the *sn*-1 position of lysophosphatidylcholine, producing a low-abundance phosphatidylcholine species that incorporates arachidonic acid at both *sn*-1 and *sn*-2 positions (Fig. 7). The newly generated diarachidonoyl-

phosphatidylcholine gives quickly away its *sn*-1 acyl chain to the free amino group of phosphatidylethanolamine, generating *N*-arachidonoyl-PE. This reaction is catalyzed by an *N*-acyl transferase (NAT) activity that has not been molecularly characterized yet (Fig. 7). The sequential formation of diarachidonoyl-phosphatidylcholine and *N*-arachidonoyl-PE is strictly calcium-dependent and represents the rate-limiting step in the production of anandamide in intact neurons (Cadas et al. 1997). Despite their functional importance, the enzymes (or enzyme) responsible for the concerted production of *N*-arachidonoyl-PE are still unknown (Ueda et al. 2013).

Two detours from the canonical biosynthesis of anandamide have been proposed, both of which utilize *N*-arachidonoyl-PE as a starting point and substitute NAPE-PLD with one or more lipid hydrolases (Fig. 7). Macrophages exposed to the bacterial toxin, lipopolysaccharide (LPS), emit a burst of lipid mediators that include anandamide (Liu et al. 2006) and other bioactive derivatives of arachidonic acid (Dennis et al. 2010). This response is unlikely to be mediated by NAPE-PLD – the expression of which is, in fact, strongly suppressed by LPS (Zhu et al. 2011) – but rather appears to require the consecutive action of two as-yet-uncharacterized enzymes: a PLC activity that converts *N*-arachidonoyl-PE into phospho-anandamide and a phosphatase activity that cleaves the latter into anandamide and free phosphate (Liu et al. 2006) (Fig. 7). In addition to this PLC-initiated mechanism, *N*-arachidonoyl-PE may be also subjected to a double deacylation at its *sn*-1 and *sn*-2 positions, catalyzed by the enzyme ABHD-4, to produce glycerophospho-anandamide (Simon and Cravatt 2006). This intermediate may be transformed into anandamide by cleavage of its phosphodiester bond, catalyzed by the phosphodiesterase GDE1, and release of glycerol phosphate (Simon and Cravatt 2008).

The existence of multiple routes of anandamide production might reflect the diversity of physiological stimuli that are able to mobilize this endocannabinoid – which include, in neural cells, membrane depolarization, intracellular calcium transients, and dopamine $D_2$ receptor activation (Di Marzo et al. 1994; Giuffrida et al. 1999; Liu et al. 2006; Lourenço et al. 2011; Steffens et al. 2003; and Stella and Piomelli 2001). However, it is important to point out that each of the three pathways described above attributes a central place to the enzyme system that catalyzes the formation of diarachidonoyl-PC and *N*-arachidonoyl-PE. Since this is the only system that directs NAPE hydrolysis toward the selective production of anandamide, its molecular characterization will be crucial to fully understand this important branch of the endocannabinoid signaling system.

### Anandamide Deactivation

After release into the extracellular space, anandamide acts as a partial agonist at $CB_1$ receptors and is subsequently deactivated by cellular uptake and intracellular hydrolysis (Fig. 8). The molecular mechanisms utilized by neural cells to degrade anandamide are reasonably well understood. Anandamide is a preferred endogenous substrate for the intracellular serine amidase, fatty acid amide hydrolase (FAAH), a member of the amidase signature family of enzymes that catalyzes the cleavage of various long-chain fatty acyl amides (Cravatt et al. 1996; Desarnaud et al. 1995; Hillard et al. 1995; Ueda et al. 1995a). Other lipid hydrolases, such as

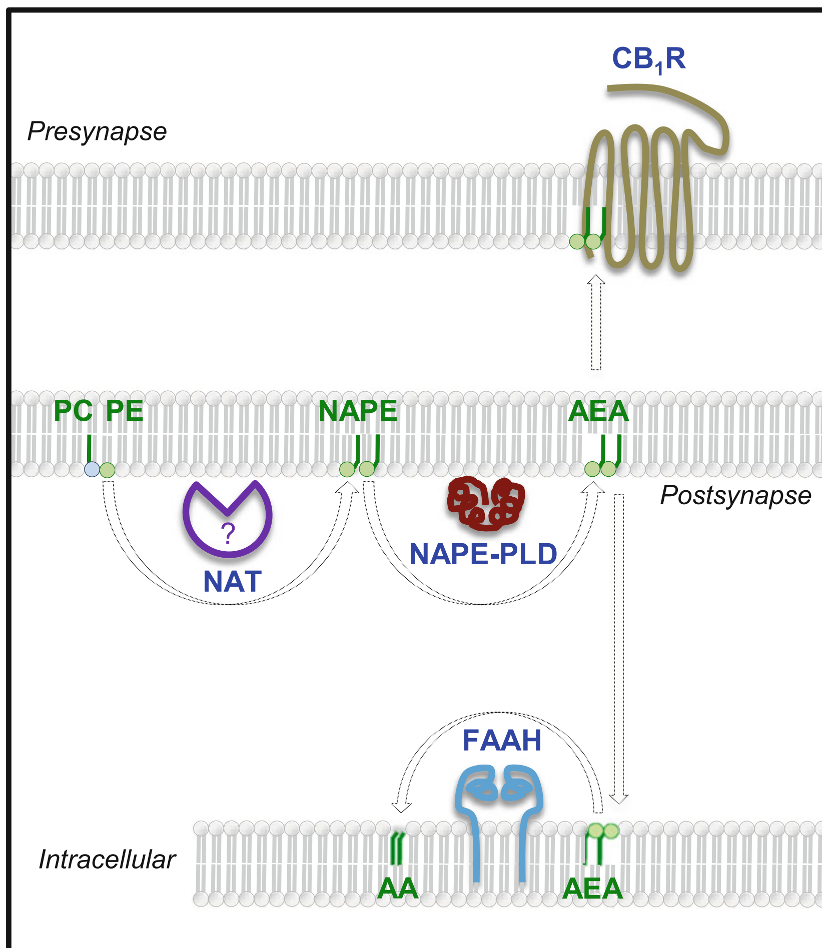



**Fig. 8 Molecular players in the endocannabinoid system: anandamide**. *N*-acyl-phosphatidyl-ethanolamine-selective phospholipase D (*NAPE-PLD*) and an unknown *N*-acyl transferase (*NAT*) may be involved in the biosynthesis of anandamide. Fatty acid amide hydrolase (*FAAH*) hydrolyzes and deactivate the compound

*N*-acylethanolamine acid amidase (NAAA) (for a review, see Ueda et al. 2010) and acid ceramidase (AC) (Li et al. 1998; for a review, see Park and Schuchman 2006), are also able to hydrolyze lipid amide bonds but show little or no affinity for anandamide and are unlikely to play an important role in its deactivation. Consistent with this view, interventions that interrupt FAAH activity cause a profound enhancement in anandamide-mediated signaling at $CB_1$ receptors (Cravatt et al. 2001 and Kathuria et al. 2003), whereas blockade of NAAA or AC exerts no such effect (Realini et al. 2013 and Sasso et al. 2013).

FAAH is expressed at high levels throughout the CNS. In situ hybridization studies in the rat have shown that FAAH transcription is highest in the neocortex and hippocampus; intermediate in the cerebellum, thalamus, olfactory bulb, and striatum; and lowest in hypothalamus, brain stem, and pituitary gland (Thomas et al. 1997). Immunohistochemical experiments largely confirmed this distribution, showing that principal neurons in the cerebral cortex, hippocampus, cerebellum, and olfactory bulb have the strongest expression of FAAH protein (Egertová et al. 1998). Many FAAH-positive neurons in the brain are found in proximity of nerve terminals that contain $CB_1$ receptors, supporting a role for FAAH in anandamide deactivation (Egertová et al. 2003; for a review, see McKinney and Cravatt 2005). There are, however, several regions of the brain where no such correlation can be demonstrated. This discrepancy may reflect the participation of FAAH in the catabolism of non-cannabinoid fatty-acid ethanolamides, such as palmitoylethanolamide (PEA) and oleoylethanolamide (OEA), which are endogenous ligands for the nuclear receptor PPAR-α (peroxisome proliferator-activated receptor type-α) and the G protein-coupled receptor GPR119 (Fu et al. 2003; for reviews, see LoVerme et al. 2005; DiPatrizio and Piomelli 2015). Supporting this idea, pharmacological inhibition of FAAH activity or genetic disruption of the *faah* gene results in marked increases in the levels of these FAEs, along with anandamide's (Cravatt et al. 2001).

FAAH is housed on intracellular organelle membranes of dendritic spines (Gulyas et al. 2004). This localization raises the question of how anandamide might be able to cover the distance between its site of action ($CB_1$ receptors on axon terminals) and its site of degradation (organelles in postsynaptic spines). There is evidence that this process is mediated through a selective carrier system present in both neurons and glial cells. This system exhibits three identifying features of a carrier-mediated transport process: first, saturation kinetics – plots of the initial rate of anandamide accumulation against extracellular anandamide concentrations yield apparent Michaelis constants that are similar to those measured for other known neurotransmitter uptake systems (Beltramo et al. 1997); second, substrate specificity – rat brain neurons and other cells in culture internalize anandamide but not closely related analogs (Beltramo et al. 1997); third, selective inhibition – a variety of natural and synthetic compounds, including chiral analogs of anandamide, block [$^3$H] anandamide transport in a competitive and stereospecific manner (Piomelli et al. 1999). Moreover, anandamide transport is distinct from FAAH, since FAAH inhibitors do not interrupt its activity (Beltramo et al. 1997). Several proteins that bind anandamide and might facilitate its intracellular movements have been identified, including a splicing variant of FAAH-1, named FAAH-like anandamide transporter (Fu et al. 2011). However, the exact role played by these proteins in the termination of anandamide signaling remains disputed.

Anandamide can be also metabolized by lipoxygenases and cyclooxygenases to oxygen-containing products that display significant biological activities at as-yet-unidentified non-cannabinoid targets (Ueda et al. 1995b; Starowicz et al. 2013; for a review, see Woodward et al. 2008). An important unanswered question is whether these reactions represent an alternative path for anandamide deactivation (Kim and

Alger 2004), provide a mechanism for the generation of distinct classes of lipid mediators (Starowicz et al. 2013; Woodward et al. 2008), or both.

### Additional Endogenous Cannabinoid Ligands

In addition to 2-AG and anandamide, other naturally occurring lipid molecules have been shown to bind to and activate cannabinoid receptors. These include noladin ether, an ether-linked analog of 2-AG (Hanus et al. 2001); virodhamine, the ester of arachidonic acid with ethanolamine (Porter et al. 2002); and *N*-arachidonoyl-dopamine, an endogenous vanilloid agonist that also exhibits affinity for cannabinoid receptors in vitro (Bisogno et al. 2000). However, the physiological significance of these substances, if any, remains unknown and their pathways of formation and degradation have not been fully characterized.

## Cannabinoid-Based Therapeutics

European and American pharmacopeias of the late nineteenth and early twentieth century listed *Cannabis* as an analgesic, hypnotic, and anticonvulsant (Iversen 2000; Russo 2007). The lawful use of *Cannabis* as a medicine came to a stop in the 1920s and 1930s, following the introduction in various countries, including the United States, of severe legal restrictions on its sale and use. This scenario has dramatically changed in recent years. As of November 2015, 23 states of the Union, the District of Columbia, and Guam have allowed the medical use of marijuana, quickly leading to a resurgence of its application to a variety of ailments, including glaucoma, loss of appetite, nausea, chronic pain, and muscle spasticity (Aggarwal et al. 2009). Most of these indications lack adequate support from controlled clinical studies (Wei and Piomelli 2015), though many find a rational basis in the biology of the endocannabinoid system (Fig. 9). Importantly, however, the primary use of *Cannabis* remains a recreational one. According to the National Institute on Drug Abuse (NIDA), marijuana is the most commonly used illicit drug in the United States (information available at http://www.nida.gov). Indeed, the drug's ability to cause dependence and addiction (Wei and Piomelli 2015) continues to play a major role in setting the agenda for cannabinoid-based therapeutics.

### $CB_1$ Receptor Agonists

Three currently available medications target cannabinoid receptors (for a review, see Pertwee 2012). $\Delta^9$-THC itself is marketed under the trade name of *Marinol*® and is used in the clinic to increase appetite in anorexic HIV patients and combat nausea caused by cancer chemotherapeutics. A synthetic analog of $\Delta^9$-THC, *nabilone* (*Cesamet*®), is prescribed in Europe for similar indications. The third approved

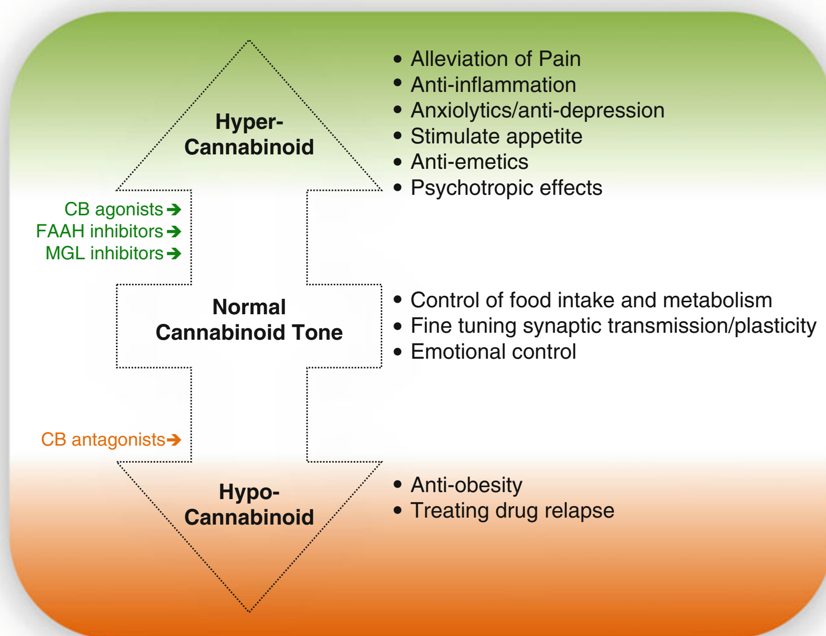



**Fig. 9** **Possible therapeutic modulation of the endocannabinoid system**. The roles played by the endocannabinoid system in the control of a variety of physiological processes and the availability of drugs modulating this system provide multiple opportunities for drug discovery. Pharmacological modulation of the endocannabinoid system can be achieved by direct agonists and antagonists for cannabinoid receptors, and by inhibitors targeting enzymes responsible for endocannabinoid inactivation

cannabinoid-based medication is a *Cannabis* extract primarily composed of $\Delta^9$-THC and cannabidiol (Fig. 1a). Commercialized in Canada and several European countries under the name of *nabiximols* (*Sativex*®), this extract is utilized as a sublingual spray for the relief of pain and muscle spasticity due to multiple sclerosis and as an adjunctive analgesic treatment in cancer patients (Pertwee 2012).

In addition to $\Delta^9$-THC and nabilone, various highly potent cannabinoid agonists have been described in the literature (Fig. 1b) (for reviews, see Iversen 2001; Pertwee 2012). The therapeutic utility of these ligands is limited by their $CB_1$-mediated psychotropic side effects, which also provide the rationale for the illicit use of some of them as an alternative to recreational marijuana (for a review, see Wells and Ott 2011). Products such as *Spice*, *K2*, and *Eclipse* are a blend of various herbs and spices, which have been laced with one of these synthetic cannabinoids. It is generally assumed that their effects are similar to those of $\Delta^9$-THC, but the preclinical and clinical data supporting this assumption are still very limited.

## $CB_1$ Receptor Antagonists

Rimonabant (*Acomplia*®, previously known as SR141716A) was the first $CB_1$ antagonist to be developed and remains one of the most extensively studied members of this class of drugs (Mackie 2006) (Fig. 10). It selectively binds to $CB_1$ receptors with nanomolar affinity and, in most experimental settings, it behaves as an inverse agonist (Mackie 2006). ("Inverse agonists" elicit pharmacological responses that are opposite to those exerted by agonists. "Neutral antagonists" (or simply "antagonists") block the access of agonists to their receptors without producing a response.) An important effect of rimonabant and other $CB_1$ antagonists is to counteract the regulatory control exerted by endocannabinoid substances on central and peripheral mechanisms of energy conservation (DiPatrizio and Piomelli 2012). In obese animals and humans, this effect translates into significant improvements in lipid profiles, central obesity, and insulin resistance, along with a sustained albeit moderate weight loss (for a review, see Engeli 2012). The therapeutic potential of $CB_1$ antagonists is limited, however, by the fact that the endocannabinoids can also strongly influence the activity of neural circuits involved in the regulation of stress responses, affect, mood, and motivation (for a review, see Clapper et al. 2009). Indeed, treatment with $CB_1$ antagonists produces in humans a series of psychiatric adverse events (including anxiety, depression, and suicidal thoughts), which have led to stop their clinical development as anti-obesity medications (Engeli 2012). An alternative approach that is currently under investigation is the use of peripherally restricted antagonists to target $CB_1$ receptors in the adipose organ, pancreas, and liver. In preclinical models, these receptors have been shown to play important roles in the anti-obesity effects of $CB_1$ blockade (Tam et al. 2010).

Considering the complex changes in brain circuits and pleomorphic mechanisms underlying drug abuse, it is not surprising that no unifying theme has been identified for the function of endocannabinoid signaling in addiction. Nevertheless, it is notable that preclinical and human clinical studies have pointed to a pivotal role for the endocannabinoid system in nicotine addiction. For example, $CB_1$ antagonists prevent nicotine-induced conditioned place preference and nicotine-induced

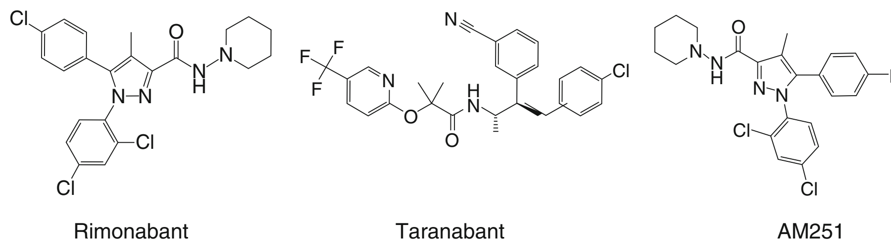



**Fig. 10 Chemical structures of $CB_1$ receptor antagonists**. Rimonabant (SR141716) was the first $CB_1$ antagonist to be reported. It was approved for use as an anti-obesity drug in Europe and other countries under the product name "*Acomplia*®" but was subsequently withdrawn due to side effects. Also shown are the structures of two additional $CB_1$ antagonists, taranabant and AM251

dopamine release in the nucleus accumbens (Castane et al. 2002; Cohen et al. 2002). These actions suggest that $CB_1$ receptor blockade may decrease the strength of specific environmental cues associated with nicotine intake. However, there is no clinical evidence supporting the use of $CB_1$ receptor antagonism in smoking cessation (for a review, see Cahill et al. 2013). It is notable that the endocannabinoid system is also implicated in the reinforcing effects of alcohol, heroin, and cocaine. For example, $CB_1$ activation enhances alcohol consumption while $CB_1$ blockade has the opposite effect. Genetic deletion of the $CB_1$ receptor reduces alcohol-induced conditioned place preference (Thanos et al. 2005). Likewise, administration of $CB_1$ antagonists or genetic deletion of $CB_1$ receptors reduced cocaine-seeking behavior of mice (Soria et al. 2005; Orio et al. 2009; for review, see Maldonado et al. 2006).

## $CB_2$ Receptor Agonists

A particularly attractive feature of selective $CB_2$ receptor agonists as therapeutics is that they are seemingly devoid of central side effects. Nevertheless, a number of preclinical studies have shown that these agents are highly effective in animal models of chronic neuropathic pain, peripheral inflammation, and sensitization (Ibrahim et al. 2003; Hohmann et al. 2004). They also appear to be useful in combating cocaine addiction (Xi et al. 2011), through a mechanism that remains unclear, and in counteracting bone loss, which is suggestive of a potential application in osteoporosis. Supporting this theory, a single nucleotide polymorphism (SNP) in the $CB_2$ receptor gene strongly correlates with osteoporosis in a cohort of women (Karsak et al. 2005).

## Endocannabinoid Deactivation Inhibitors

The psychiatric adverse events associated with the use of $\Delta^9$-THC and its synthetic mimics are an obstacle to broader clinical development. As a potential alternative, the endocannabinoid system offers several opportunities to circumvent such events and possibly achieve adequate therapeutic efficacy. In particular, FAAH inhibitors have reached clinical testing and are currently being considered for the treatment of anxiety, *Cannabis* addiction, and pain. There are also intriguing hints that they might be useful in schizophrenia. Moreover, MGL inhibitors have shown promising activities in animal models of pain and cancer.

**Anxiety**. The hypothesis that anandamide is an important regulator of stress-coping behaviors was first suggested by animal experiments, which showed that the FAAH inhibitor URB597 decreases isolation-induced ultrasonic vocalizations in rat pups and increases the time spent by adult rats in the open arms of an elevated maze (Kathuria et al. 2003). Subsequent studies found that URB597 also enhances active stress-coping behaviors in mouse and rat models of acute and chronic stress (Gobbi et al. 2005; Bortolato et al. 2007). Other FAAH inhibitors were later shown to exert

anxiolytic-like effects that were similar to those of URB597 (Bluett et al. 2014). Such effects are prevented by administration of a $CB_1$ antagonist, an indication that they are due to enhanced anandamide-mediated transmission at $CB_1$ receptors. Further implicating anandamide in response to stressful stimuli, a study in healthy volunteers showed that the circulating levels of anandamide were elevated after exposing the subjects to a psychosocial stress test (Dlugos et al. 2012). In this context, it is important to point out that URB597 has no rewarding effects in rodents (Gobbi et al. 2005) and is not self-administered by squirrel monkeys (Justinova et al. 2008), marking a clear mechanistic distinction with $\Delta^9$-THC and suggesting that this FAAH inhibitor might be used in the clinic without overt risk of abuse.

**Cannabis addiction**. The pharmacological profile of FAAH inhibitors predicts that they should alleviate many of the symptoms experienced by abstinent marijuana addicts – including anxiety, depression, and deterioration of sleep quality (for a review, see Clapper et al. 2009). Animal studies support this prediction (Schlosburg et al. 2009). A randomized double blind clinical trial aimed at determining the safety and efficacy of the compound PF-04457845, a FAAH inhibitor that is structurally different from URB597, is currently ongoing (https://clinicaltrials.gov). The outcome of this study will likely influence future research directions in other areas of addiction medicine, such as tobacco (Justinova et al. 2015) and cocaine use disorder (Adamczyk et al. 2009; for reviews, see Panlilio et al. 2013; Piomelli 2014).

**Schizophrenia**. Autoradiography and positron emission tomography studies have shown that $CB_1$ receptor densities are elevated in cortical and subcortical areas of human subjects with schizophrenia (Wong et al. 2010). These results are sometimes interpreted as suggesting that excessive endocannabinoid transmission might be a causative factor in psychosis (Andreasson et al. 1987), but data from other studies offer an opposing perspective. First, a simplistic "endocannabinoid hypothesis of schizophrenia" is negated by the fact that the $CB_1$ antagonist rimonabant did not significantly improve disease symptoms in a placebo-controlled clinical trial of subjects with schizophrenia (Meltzer et al. 2004) or in a subsequent double-blind placebo-controlled trial aimed at assessing the impact of the drug on cognitive function in schizophrenic subjects (Boggs et al. 2012). Second, a study in non-medicated first-episode persons with psychosis showed that cerebrospinal levels of anandamide correlate *inversely* with positive and negative symptoms of schizophrenia (Leweke et al. 2007). Third, in a double-blind randomized clinical trial of cannabidiol in acute psychosis, treatment with the drug was accompanied by a substantial increase in circulating anandamide levels, which was significantly associated with clinical improvement (Leweke et al. 2012). Finally, in patients in the prodromal states of schizophrenia, lower cerebrospinal levels of anandamide were linked with higher risk of an earlier transition to psychosis (Koethe et al. 2009). These results are consistent with animal studies suggesting that anandamide signaling in the basal ganglia of rats and mice may be part of a negative feedback loop that offsets the effects of excessive dopaminergic activity (for a review, see van der Stelt and Di Marzo 2003). Thus, a substantial number of findings support the conclusion that anandamide may act as a homeostatic controller of dopamine neurotransmission and a protective signal in schizophrenia. A corollary of this hypothesis is that FAAH

inhibitors may be beneficial in psychosis and, possibly, in other mental disorders in which hyperactive dopamine transmission might be implicated (e.g., Tourette's syndrome).

**Pain**. Pain perception can be effectively controlled by neurotransmitters that operate within the CNS. This modulation has been well characterized in the dorsal horn of the spinal cord, where impulses carried by nociceptive (pain-sensing) fibers are processed before they are transmitted to the brain. In addition to these central mechanisms, intrinsic control of pain transmission can also occur at terminals of afferent nerve fibers outside the CNS. One prominent example of peripheral regulation is provided by the endogenous opioids, which are released from activated immune cells during inflammation and inhibit pain initiation by interacting with opioid receptors localized on sensory nerve endings (for a review, see Stein and Zöllner 2009). Endocannabinoid mediators such as anandamide might serve an analogous function to that of the opioids, because pharmacological activation of peripheral $CB_1$ and $CB_2$ cannabinoid receptors inhibits pain-related behaviors (Calignano et al. 1998; Dziadulewicz et al. 2007; for a review, see Anand et al. 2009) while genetic disruption of $CB_1$ receptor expression in primary nociceptive neurons exacerbates such behaviors (Agarwal et al. 2007). These and other findings have led to suggest that the peripheral endocannabinoid system may act as a filter for incoming pain signals (for review, see Piomelli and Sasso 2014). Supporting this theory, the peripherally restricted FAAH inhibitor, URB937 – which inhibits FAAH in peripheral tissues but is actively extruded from the brain and spinal cord (Clapper et al. 2010; Moreno-Sanz et al. 2011, 2012, 2013, 2014) – was shown to reduce nociceptive responses in rodent models of acute and persistent pain through a mechanism that requires elevation of peripheral anandamide levels and consequent activation of $CB_1$ receptors (Clapper et al. 2010). Subsequent studies have confirmed and extended those findings, documenting the profound analgesic properties of URB937 in animal models of nociceptive, inflammatory pain and neurogenic pain (Sasso et al. 2012; Greco et al. 2015) and suggesting that peripheral FAAH inhibition may offer a new approach to the therapy of acute pain states.

Like FAAH inhibitors, inhibitors of the 2-AG-deactivating enzyme MGL display antinociceptive effects in a number of animal models of acute, visceral, inflammatory, neuropathic, and/or bone cancer pain (Busquets-Garcia et al. 2011; Sciolino et al. 2011). In addition, they may also be useful in conjunction with nonsteroidal anti-inflammatory drugs (NSAIDs), since the selective MGL inhibitor JZL184 provides protection against gastric hemorrhage produced by diclofenac in mice (Kinsey et al. 2011). This protection depended on $CB_1$ receptors and persisted when animals were pretreated with the MGL inhibitor for 5 days prior to diclofenac treatment (Kinsey et al. 2013).

**Cancer**. Ever since the first demonstration that $\Delta^9$-THC and other phytocannabinoids reduced the rate of growth of lung tumour xenografts (Munson et al. 1975), the potential of cannabinoids as anti-cancer agents has been actively explored (for reviews, see Velasco et al. 2004; Flygare and Sander 2008; Sarfaraz et al. 2008; Freimuth et al. 2010; Fowler et al. 2010; Díaz-Laviada 2011; Malfitano et al. 2011). There is evidence that MGL blockade might be effective against breast,

ovarian, skin, and prostate cancer. For example, the potent MGL inhibitor JZL184 may reduce prostate cancer (PC3) cell migration, invasion, and survival in vitro. Administration of JZL184 or genetic knockdown of MGL reduced tumor sizes in xenograft models of ovarian, melanoma, and colorectal cancers (Nomura et al. 2010; Ye et al. 2011). The potential of MGL inhibitors, however, may be related to their ability to reduce the formation of long-chain fatty acids from their corresponding glycerol esters.

## Outlook

Research on the endocannabinoid system has greatly expanded our understanding on these unique signaling molecules and the roles they play in health and disease. Information gleamed from studies on *Cannabis sativa* has illuminated how $\Delta^9$-THC and other exogenous cannabinoids hijack the endocannabinoid signaling system, leading to serious side effects, but at the same time providing promising opportunities for therapeutic intervention. While important questions remain, it is nevertheless clear that the therapeutic potential of endocannabinoid modulation calls for further scientific and clinical investigation.

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