The Receptors

Mary E. Abood
Roger G. Sorensen
Nephi Stella *Editors*

endoCANNABINOIDS

Actions at Non-CB1/CB2 Cannabinoid Receptors

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Preface

The concept for this book began as a proposal by one of us (RGS) in response to a request for ideas for symposium topics. A high program priority for the National Institute on Drug Abuse (NIDA), within the National Institutes of Health, US Department of Health and Human Services, is the development of effective medications for the treatment of drug abuse and addiction, and to prevent relapse to drug use [NIDA (2010) *Strategic Plan.* NIH Publication Number 10-6119]. NIDA is constantly looking for new compounds that can interact with novel targets that have the potential of being developed into pharmacotherapies for treating substance use disorders. Research supported by NIDA had been exploring CB1 receptor antagonists for treating addictive disorders and CB2 agonists for treating acute and chronic pain. Yet it was apparent that cannabinergic compounds also had actions independent of CB1 and CB2 receptors. A symposium to explore and discuss these latter, atypical effects of cannabinoids was proposed, which became one of the sessions, *Non-Cannabinoid Receptor-Mediated Actions of Endo-Cannabinoids*, held as part of the 2009 NIDA Mini-Convention, Frontiers in Addiction Research, in October 2009. The goal of this session was to provide an overview of the role of cannabinoids in neuronal function and discuss several non-CB receptor-mediated actions of cannabinoids within the central and peripheral nervous systems.

In this book, this topic of atypical actions of cannabinoids has been expanded from the goals of the symposium to include atypical actions of cannabinoids within the nervous system as well as in other organs and tissues. Within the chapters of this book we have attempted to present a description of the currently known atypical actions of cannabinoids. We also encouraged the contributors to describe current limitations in atypical cannabinoid research and discuss future research needs and directions. Clearly, more research needs to be done. We expect that the future will find additional atypical molecular and cellular responses to cannabinoids, the identification of new receptors and ligands, and confirmation of the physiological role of these responses. It is our expectation that this book will complement other publications and resources that focus primarily on the CB1 and CB2 receptor actions of cannabinoids. We hope that you enjoy reading this volume as much as we enjoyed putting this volume together. Furthermore, we hope that in reading the chapters

contained in this volume, you will be inspired to pursue new avenues and new directions in cannabinoid research or consider the potential of cannabinoid actions in your study of human disease. We want to thank all of the contributors to this volume for their hard work in preparing their chapters and for their patience as we brought this book to its fruition. Without their help, this book would not have been possible.

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Part I
Overview of Non-Cannabinoid Receptors

Chapter 1
Overview of Nonclassical Cannabinoid Receptors

Grzegorz Godlewski and George Kunos

1.1 Introduction

The resin of the female flowering marijuana plant, *Cannabis sativa L.*, has been widely used as medicine and illicit narcotic since ancient times. It has also been the target of extensive investigation in contemporary biomedical research. These efforts have resulted in the elucidation of the chemical structures of most of the bioactive plant constituents including the key psychomimetic principle (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC) (Mechoulam and Gaoni 1965), the identification of high-affinity stereoselective sites in the mammalian brain, the so-called cannabinoid receptors that bind Δ^9-THC, its analogs (Devane et al. 1988) and endogenous cannabinoids (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995), and the elucidation of a complex endogenous cannabinoid system (for reviews, see Di Marzo 2009; Pacher et al. 2006). Although the picture is still not complete, endocannabinoids have emerged as important regulators of many pathophysiological processes. There is a large body of literature covering not only aspects of the chemistry, pharmacology, molecular biology, and function of cannabinoids and their receptors, but also providing clues for the presence of novel molecular targets. This chapter discusses evidence pertaining to such additional targets beyond the two cannabinoid receptors identified in the 1990s, with particular emphasis on G protein-coupled receptors (GPCRs). Since the recognition of new receptors is frequently based upon pharmacological profiling alone, a clear delineation of the properties of the known components of the cannabinoid system is also essential.

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1.2 Cannabinoid System

1.2.1 Cannabinoid Receptors

The International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification and its Subcommittee on Cannabinoid Receptors have originally coined the term “cannabinoid receptor” based on the interaction of these receptors with Δ^9-THC and its analogs. It is now clear that these receptors also recognize endogenous lipid ligands structurally unrelated to Δ^9-THC (Pertwee et al. 2010). To date, the identity of at least two distinct cannabinoid receptors, each belonging to the GPCR superfamily, has been established by molecular cloning. The CB_1 receptor (or CNR1) was originally cloned from rat cerebral cortex as an orphan GPCR receptor, termed SKR6 (Matsuda et al. 1990). Its identity as a cannabinoid receptor was subsequently revealed by the overlap between the brain distribution of its mRNA and the distribution of specific binding sites for radiolabeled cannabinoid ligands (Herkenham et al. 1990). The human homolog of CB_1 receptors was identified shortly thereafter (Gerard et al. 1991). Splice variants of the human CB_1 receptor mRNAs that encode putative proteins with modified amino-terminals have also been described (Ryberg et al. 2005; Shire et al. 1995). When cDNAs derived from these mRNAs were expressed in cultured cells, the resulting CB_1 receptors exhibited distinct signaling properties (Straiker et al. 2012) and sensitivity to endocannabinoids (Ryberg et al. 2005). Polymorphisms in the CNR1 gene have suggested a link between CB_1 receptors and schizophrenia (Leroy et al. 2001; Tiwari et al. 2010) or Parkinson’s disease (Barrero et al. 2005). The CB_1 receptor is highly conserved across mammalian species with the amino acid homology ranging from 81 % between human and rat to 93 % between rat and mouse (Abood 2005; Griffin et al. 2000; Lutz 2002; Munro et al. 1993). The CB_1 receptors are the most abundant GPCR in the mammalian central nervous system (CNS) (Herkenham et al. 1991). They are present at particularly high levels in cerebellum, hippocampus, and basal ganglia where they mediate inhibition of the release of various excitatory and inhibitory neurotransmitters from neuronal terminals to affect cognition, memory, motor, and metabolic functions (for reviews, see Howlett et al. 2002; Szabo and Schlicker 2005). Activation of CB_1 receptors in the brain by Δ^9-THC and synthetic cannabinoids has been shown to mediate a classic tetrad of behavioral effects in mice, including catalepsy, hypothermia, analgesia, and hypomotility. These effects can be counteracted by CB_1 receptor antagonists and are absent in CB_1 receptor-deficient mice (Ledent et al. 1999; Zimmer et al. 1999). Lower, yet functionally relevant levels of CB_1 receptors are also present in the peripheral nervous system as well as somatic cells of most tissues including adipose tissue, liver, heart, vascular smooth muscle and endothelium, kidneys, and testis, where they control metabolic, cardiovascular, reproductive, and other pathophysiological functions (Gerard et al. 1991; Herkenham et al. 1990; Ishac et al. 1996; Liu et al. 2000: for reviews, see Pacher et al. 2006; Pertwee et al. 2010). The role of CB_1 receptors in the above processes has been further confirmed through

the use of genetically altered mice that lack CB_1 receptors (Ledent et al. 1999; Zimmer et al. 1999).

The CB_2 receptor (or CNR2) was first cloned from rat spleen (Munro et al. 1993), and subsequently confirmed to have distinct actions from the CB_1 receptor through the creation of CB_2 receptor-deficient mice (Buckley et al. 2000). The human CB_2 receptor shows 44 % amino acid sequence homology with the CB_1 receptor, which is increased to 68 % among the transmembrane regions (Munro et al. 1993). CB_2 receptors are expressed predominantly, although not exclusively, in immune and hematopoietic cells (Munro et al. 1993). More recently, functional CB_2 receptors have been identified both in neurons and glial cells of the CNS (Onaivi et al. 2006; Xi et al. 2011) where they may be involved in mechanisms underlying addictive behaviors (Onaivi et al. 2008; Xi et al. 2011), as well as in the liver where they have been linked to the control of lipid metabolism and fibrosis (Teixeira-Clerc et al. 2006). When activated, CB_2 receptors modulate immune cell migration and cytokine release both in the brain and in peripheral tissues (for reviews, see Howlett et al. 2004; Pertwee et al. 2010). Polymorphisms in the CNR2 gene have also been identified and link CB_2 receptors with postmenopausal osteoporosis (Bab et al. 2011; Norrod and Puffenbarger 2007).

Both CB_1 and CB_2 receptors signal through $G_{i/o}$ proteins to inhibit adenylyl cyclase and regulate ion channels, including G protein-coupled inwardly rectifying potassium channels (GIRK) (McAllister et al. 1999) or N-type voltage-gated calcium channel ($Ca_{v2.2}$) (Wilson et al. 2001; Agler et al. 2003) (Table 1.1). There is also evidence that CB_1 receptors can signal through G_s proteins (Chen et al. 2010; Glass and Felder 1997). Cannabinoid receptors regulate, in a G-protein-independent manner, the activity of a variety of intracellular kinases, e.g., mitogen activated protein kinases and extracellular signal-regulated kinases (MAPK/ERK pathway), cJun N-terminal kinases (JNKs), and protein kinase B (AKT) (for reviews, see Di Marzo 2009; Howlett 2005: for review of signal transduction pathways, see Howlett 2005).

1.2.2 Cannabinoid Ligands

The cloning of cannabinoid receptors in mammalian tissues has triggered a search for endogenously occurring counterparts of plant-derived cannabinoids. The first such "endocannabinoid" identified was a lipid amide isolated from porcine brain, *N*-arachidonoyl ethanolamide (anandamide or AEA) (Devane et al. 1992). Subsequent studies have revealed that AEA is generated in vivo from membrane phospholipid precursors via transacylation that yields *N*-arachidonoyl phosphatidylethanolamide (NAPE) (Di Marzo et al. 1994), which is then hydrolyzed via multiple parallel pathways to yield AEA (Cravatt et al. 1996; Liu et al. 2008; Placzek et al. 2008). An alternative biosynthetic pathway involving the condensation of arachidonic acid and ethanolamine (Devane and Axelrod 1994) may also operate under certain in vivo conditions, such as in the post-hepatectomy, regenerating liver (Mukhopadhyay et al. 2011), and possibly under postmortem conditions in the brain

Table 1.1 Pharmacological profile of non-cannabinoid receptors

Receptor properties	Cannabinoid		Putative endothelial	Putative neuronal			Orphan			
	CB_1	CB_2		Cerebellar	Hippocampal	Glial	GPR55	GPR119	GPR18	GPR92
Agonists	AEA, 2-AG, Δ^9-THC, R-(+)-WIN55212-2, CP55940, noladin ether	JWH133, JWH015	AEA, AbnCBD, O-1602, ARA-S, VSN16, virodhamine, ARA-Gly	AEA, R-(+)-WIN55212-2	R-(+)-WIN 55212-2, CP55940	AEA, R-(+)-WIN55212-2 AbnCBD	AbnCBD, O-1602, AM251, rimonabant, LPI, 2-ARA-Glyc, N-ARA-S, JWH015	N-oleoyl dopamine, OEA, PEA, AEA, lysophospholipids, 2-oleoyl-gycerol, oleoyl-LPI, palmitoyl-LPI, stearoyl-PI	ARA-Gly, AbnCBD, O1602, PAL-Gly	ARA-Gly, FPP, LPA
Antagonists	Rimonabant, taranabant, AM251, AM6545	SR144528	Rimonabant, O-1918, CBD	Not reported	Rimonabant, capsazepine	O1918	O1918, CBD	Not reported	O1918, CBD	Not reported
Effective concentration	Pico-/nanomolar	Pico-/nanomolar	Micromolar	Micromolar	Micromolar	Nano-/micromolar	Nano-/micromolar	Nano-/micromolar	Nano-/micromolar	Nano-/micromolar
G-protein	$G_{i/o}$, G_s	$G_{i/o}$	$G_{i/o}$	Not reported	$G_{i/o}$	$G_{i/o}$	$G_{\alpha 13}$, $G_{\alpha 12}$, $G_{\alpha q}$	G_s	$G_{i/o}$	$G_{q/11}$, G_s
Downstream signaling	AC inhibition, ↓cAMP, MAPK/ERK, AKT, JNKs, GIRK, $Ca_{v2.2}$	AC inhibition, ↓cAMP, MAPK/ERK, AKT,	AC inhibition, ↓cAMP, MAPK/ERK, AKT, GC, ↑cGMP, PKG, BK_{Ca}	Not reported	Not reported	AC inhibition, ↓cAMP	Small GTPases (RhoA, csc42, rqc1), ↑Ca_i^{2+}, PLC	AC stimulation, ↑cAMP, K_{ATP} and Ca_v channels	AC inhibition, ↓cAMP,↑Ca_i^{2+}	AC stimulation, ↑cAMP, PKA,↑Ca_i^{2+}
References	Di Marzo (2009), Howlett (2005), and Pertwee et al. (2010)		Begg et al. (2005), Mo et al. (2004), Offertáler et al. (2003), and Pertwee et al. (2010)	Di Marzo (2009), Howlett (2005), and Pertwee et al. (2010)	Di Marzo (2009), Howlett (2005), and Pertwee et al. (2010)	Di Marzo (2009), Howlett (2005), and Pertwee et al. (2010)	Di Marzo (2009), Godlewski et al. (2009b), Howlett (2005), and Pertwee et al. (2010)	Godlewski et al. (2009b) and Pertwee et al. (2010)	Pertwee et al. (2010)	Pertwee et al. (2010)

(Patel et al. 2005). AEA is degraded in vivo by fatty acid amide hydrolase (Cravatt et al. 1996). A second endocannabinoid, isolated 3 years later from the gut (Mechoulam et al. 1995) and the brain (Sugiura et al. 1995) was a glycerol ester, 2-arachidonoylglycerol (2-AG), which is preferentially metabolized by monoacylglycerol lipase (Dinh et al. 2002), with additional involvement of αβ-hydrolase domain-containing 6 and 12 (Straiker et al. 2009). Both endocannabinoids were found to mobilize on demand, in response to stimuli that elevate intracellular calcium levels and mimic the biological effects of Δ^9-THC at cannabinoid receptors (Liu et al. 2008). Other identified endogenous cannabinoid ligands include amides, i.e., *N*-arachidonoyl dopamine (Sugiura et al. 1995), esters, i.e., virodhamine (Porter et al. 2002) and *N*-dihomo-γ-linolenoylethanolamine (Van Der Stelt et al. 2000), and ethers, i.e., noladin ether (Hanus et al. 2001).

Identification of biological processes regulated by the endocannabinoid system was facilitated by the development of potent, subtype-selective synthetic cannabinoid receptor ligands. These include nonselective cannabinoid receptor agonists, e.g., HU210, CP55940, R-(+)-WIN55212-2; selective CB_2 receptor agonist, JWH015; global CB_1 receptor antagonists/inverse agonists, i.e., rimonabant, AM251, taranabant; peripheral CB_1 antagonist, AM6545; and global CB_2 receptor antagonists/inverse agonists, i.e., SR144528 (Table 1.1) (for reviews, see Pacher et al. 2006; Pertwee et al. 2010).

1.3 Nonclassical Cannabinoid Receptors

There has been a steady stream of evidence indicating that the biological effects of certain cannabinoids are not mediated by classical CB_1 or CB_2 receptors. Some effects may be linked to the antioxidant or lipophilic chemical properties of cannabinoid ligands (Hampson et al. 1998). Other responses, however, which display structural/steric selectivity and sensitivity to G protein toxins or to other molecular manipulations, provide rationale to consider the existence of additional receptors. These new receptors, different from CB_1 and CB_2 receptors, have often been named non-CB_1/CB_2 or CB_3 receptors, or named after the tissue they were originally described in, i.e., endothelial or hippocampal cannabinoid receptors. They are collectively classified here as "nonclassical cannabinoid receptors." The group comprises a number of targets, which include as-yet-unidentified/putative receptor(s), and established GPCRs as well as ion channels and nuclear receptors, which will be discussed in the following chapters. Recently, orphan GPCRs, namely GPR18, GPR55, and GPR119, have also emerged as potential nonclassical cannabinoid receptor candidates, which are reportedly being activated by various endogenous, plant-derived, and synthetic cannabinoids.

The term "cannabinoid" has frequently been used to describe all ligands that are structural analogs of Δ^9-THC or its endogenous counterparts regardless of their binding affinity to cannabinoid receptors (Pertwee et al. 2010). Thus, by this definition, the group also comprises a number of non-psychoactive and psychoactive

compounds, which do not necessarily interact with cannabinoid CB_1 and CB_2 receptors, but may interact with nonclassical cannabinoid GPCRs. These include:

- Non-psychoactive compounds found in *C. sativa L*, such as cannabidiol (CBD) and its synthetic analogs, i.e., abnormal cannabidiol (AbnCBD), O1918, O1602.
- Non-psychoactive acylethanolamides, analogs of AEA, which are devoid of affinity to CB_1 and CB_2 receptors, i.e., oleylethanolamide (OEA) and palmitoylethanolamide (PEA).
- Non-psychoactive lipoamino acids, e.g., *N*-arachidonoyl L-serine (ARA-S), *N*-palmitoyl L-serine (PAL-S), and *N*-arachidonoyl glycine (ARA-Gly).
- Psychoactive cannabinoid receptor ligands, which may interact with additional targets, i.e., AEA, 2-AG, noladin ether, Δ^9-THC, CP55940, R-(+)-WIN55212-2, AM251, HU210, and rimonabant.

1.3.1 Putative Nonclassical Cannabinoid Receptors

1.3.1.1 Endothelial Receptor

Historically, the first convincing evidence favoring the existence of novel, nonclassical cannabinoid receptors came from studies into the vasodilatory effects of cannabinoids. These early findings showed that AEA and AbnCBD, but not Δ^9-THC, R-(+)-WIN55212-2, or HU210, elicited long-lasting vasodilation of rat isolated perfused mesenteric arterial preparations in a manner sensitive to rimonabant and CBD (Járai et al. 1999; Wagner et al. 1999) and that the effect was dependent on intact vascular endothelium and was lost following endothelial denudation (Chaytor et al. 1999; Ho and Hiley 2003b, 2004; Járai et al. 1999; Mukhopadhyay et al. 2002; O’Sullivan et al. 2004). Moreover, the vasodilatory activity of AEA and AbnCBD still persisted in mice lacking CB_1 receptors and in double CB_1/CB_2 receptor knockout mice (Járai et al. 1999). Consequently, a new endothelial cannabinoid receptor, distinct from CB_1 and CB_2 receptors, was proposed to exist and mediate vascular effects of AEA and AbnCBD (Járai et al. 1999; Wagner et al. 1999). Further confirmation of distinctive features of the putative endothelial non-cannabinoid receptor was that AbnCBD did not bind to rat CB_1 receptors in cerebellar membrane preparations or to human CB_2 receptors expressed in Chinese hamster ovary cells (Offertáler et al. 2003), nor did it induce analgesia, hypomotility, hypothermia, or catalepsy (Járai et al. 1999). The putative endothelial receptor was also found to be distinct from the transient receptor potential vanilloid 1 (TRPV1), for which AEA has been documented to be an agonist (Zygmunt et al. 1999), due to the inability of the TRPV1 antagonist capsazepine to alter the effect of AEA (Ho and Hiley 2003b; Járai et al. 1999; Offertáler et al. 2003). Within the vasculature, this novel site appears to be limited to resistance branches of the mesenteric artery (O’Sullivan et al. 2004) and may also operate in the coronary (Ford et al. 2002) or pulmonary (Kozłowska et al. 2007; Su and Vo 2007) circulations. Additional evidence indicates

the presence of AbnCBD-sensitive receptors in microglia where they mediate cannabinoid effects on cell migration (Walter et al. 2003) (see below).

In subsequent studies, which utilized the phenylephrine/methoxamine-precontracted resistance segments of rat mesenteric arteries, AEA, AbnCBD, and its analog O1602 caused vasodilation sensitive to rimonabant (Offertáler et al. 2003). Quite unexpectedly and contrary to whole mesenteric arterial preparations, CBD behaved like AbnCBD and relaxed isolated arterial segments, prompting the search for a true antagonist and the design of synthetic CBD derivatives. As a result, the compound O1918 was developed, which lacked detectable affinity for CB_1 and CB_2 receptors, yet still inhibited the vasorelaxant response to AEA, AbnCBD, O1602, and CBD in a concentration-dependent manner (Offertáler et al. 2003; Ho and Hiley 2003a, b). In fact, O1918 also appeared to be effective in vivo and attenuated the AbnCBD-induced hypotension in anesthetized mice at doses that did not attenuate the hypotension induced by the CB_1/CB_2 receptor agonist HU210 (Offertáler et al. 2003).

The potential involvement of a GPCR in the vasorelaxant effect of AEA and AbnCBD was implicated by the sensitivity of arteries to pertussis toxin (Mukhopadhyay et al. 2002; Offertáler et al. 2003). This sensitivity persisted only in intact vessels and disappeared after denudation of endothelium (Begg et al. 2003; Mukhopadhyay et al. 2002; Offertáler et al. 2003), suggesting the involvement of endothelial $G_{i/o}$-coupled receptor in the above effects. Several other reports also confirmed the same phenomenon; thus, mesenteric arteries were relaxed by putative endogenous receptor agonists, i.e., ARA-S (Milman et al. 2006), ARA-Gly (Parmar and Ho 2010), oleamide (Hoi and Hiley 2006), virodhamine (Ho and Hiley 2004; Kozłowska et al. 2008), and the novel water-soluble agonist 3-(5-dimethylcarbamoyl-pent-1-enyl)-*N*-(2-hydroxy-1-methyl-ethyl) benzamide (VSN16) (Hoi et al. 2007) in a manner sensitive to the blockade by pertussis toxin, O1918, rimonabant, and by endothelial denudation. This receptor may also account for the delayed hypotension induced by AEA in vivo (Zakrzeska et al. 2010). With respect to OEA and PEA, only an entourage effect on vasorelaxation to AEA was suggested to occur through TRPV1 receptors (Ho et al. 2008) in a manner dependent on cyclooxygenase activity (Wheal et al. 2010). Effects of AEA and AbnCBD were also observed in some other vessels, i.e., rat coronary artery (Ford et al. 2002), rabbit aorta (Mukhopadhyay et al. 2002), rat aorta (Herradon et al. 2007), and human pulmonary artery (Kozłowska et al. 2008). One notable exception was the study showing that the vasodilatory effect of AbnCBD in the rat mesenteric artery was unaffected by pertussis toxin and seemed to signal mainly through inhibition of voltage-gated L-type calcium channels (Ho and Hiley 2003a). The characterization of the endothelial receptor is still hampered by the poor selectivity and limited availability of potent ligands, with only one neutral antagonist, O1918, available to date. We have recently developed its structural analog, 1,3,5-trimethoxy-5-methyl-2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]benzene (O6064), which also potently blocks the vasorelaxation of AbnCBD in isolated rat small mesenteric arteries (EC_{50} value for AbnCBD was shifted from 1 to 13.8 μM), mimicking the effect of O1918 (Fig. 1.1).

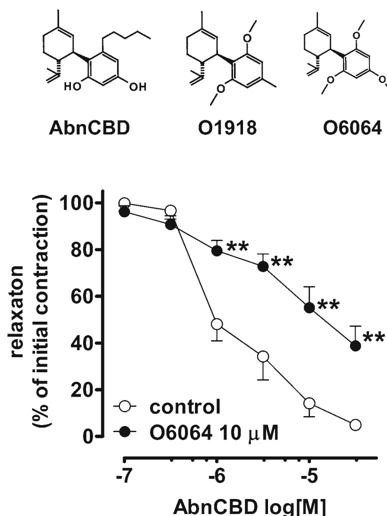

Fig. 1.1 Influence of O-6064 on the vasorelaxant effect of AbnCBD in endothelium-intact rat mesenteric arteries. Third-order segments of mesenteric arteries (200–300 μm in diameter) were isolated from male Sprague–Dawley rats (200–300 g) and mounted in a wire myograph, as described previously (Godlewski et al. 2009a). AbnCBD was added cumulatively to the tissue bath alone (control) or in the presence of O-6064, 10 μM. Values are means ± SEM from six experiments. $^{**}P<0.01$ compared to control

As shown in Table 1.1, signaling mechanisms activated by the endothelial non-cannabinoid receptor have mostly been explored using primary cultures of human umbilical vein endothelial cells (HUVEC). Thus, in these cells, AbnCBD was found to activate the MAPK/ERK pathway and phosphorylate AKT kinase (Mo et al. 2004; Offertáler et al. 2003). The above effects were inhibited by O1918 or by pertussis toxin, resembling the pharmacology of AbnCBD in rat mesenteric artery (Offertáler et al. 2003). Using an electrophysiological approach, Begg et al. (2003) suggested that the endothelial $G_{i/o}$-coupled receptor is positively coupled to guanylyl cyclase (GC) to raise the intracellular cyclic GMP (cGMP) level, which activates protein kinase G (PKG) (for review, see Begg et al. 2005).

1.3.1.2 Neuronal Receptor

A nonclassical cannabinoid receptor has also been postulated to exist in the CNS. The original evidence came from experiments by Di Marzo et al. (2000) who demonstrated that AEA, unlike Δ^9-THC, could elicit analgesia, catalepsy, and locomotor hypomotility in transgenic mice lacking the CB_1 receptor. This observation was further strengthened by showing that AEA and R-(+)-WIN55212-2, but not Δ^9-THC, CP55940, or HU-210 could stimulate [^{35}S]GTPγS binding in whole brain membranes and in cerebellar homogenates prepared from CB_1 receptor-deficient mice (Breivogel et al. 2001; Di Marzo et al. 2000; Monory et al. 2002). Near maximal concentrations of AEA and R-(+)-WIN55212-2 were not fully additive in the [^{35}S]GTPγS binding assay, supporting the hypothesis that these two agents were acting through a common site on the neuron (Breivogel et al. 2001). The characteristics of this nonclassical cannabinoid receptor, sensitive to AEA and R-(+)-WIN55212-2,

differ from that in endothelium and from established cannabinoid receptors in several ways:

- Coupling to G protein: receptor does not couple to adenylyl cyclase in the mouse cerebellum (Monory et al. 2002).
- Distribution pattern: AEA- and R-(+)-WIN55212-2-stimulated [^{35}S]GTPγS binding were found in brain stem, midbrain, and spinal cord, which express low level of CB_1 receptors (Breivogel et al. 2001; Monory et al. 2002).
- Radioligand binding: specific, high affinity binding sites for [^{3}H]R-(+)-WIN55212-2 were detected in plasma membranes obtained from certain brain regions of CB_1 receptor-deficient mice (Breivogel et al. 2001).
- Potency: AEA and R-(+)-WIN55212-2 stimulated the non-cannabinoid receptor in micromolar concentrations, much higher than those effective at CB_1 receptors (Breivogel and Childers 2000).
- Pharmacology: rimonabant does not appear to be a competitive antagonist of the neuronal non-cannabinoid receptor (Breivogel et al. 2001; Monory et al. 2002).

Another type of non-cannabinoid receptor has been proposed to be present in CA1 pyramidal cells of the hippocampus where it is involved in the regulation of glutamatergic neurotransmission (Hájos and Freund 2002; Hájos et al. 2001). This putative receptor, sensitive to R-(+)-WIN55212-2 and CP55940, reduced amplitudes of excitatory postsynaptic potentials (EPSP) in slices obtained from wild-type and CB_1 receptor-deficient CD1 mice (Hájos et al. 2001) and neonatal CB_1 receptor-deficient C57BL/6 mice (Ohno-Shosaku et al. 2002), but not from adult C56BL/6 mice (Hoffman et al. 2005). It was suggested that this receptor may specifically mediate the short-term, rather than long-term, depression of EPSPs by endocannabinoids which were released upon activation of postsynaptic group 1-metabotropic glutamate receptors in the hippocampus (Rouach and Nicoll 2003).

Despite some similarity with the endothelial receptors, the hippocampal non-cannabinoid receptor seems to have a unique pharmacological profile (Table 1.1). Although R-(+)-WIN55212-2 and CP55940 are inactive in endothelium (Járai et al. 1999; Mukhopadhyay et al. 2002), they reduce the EPSP in the hippocampus in a manner sensitive to rimonabant (Breivogel et al. 2001; Hájos and Freund 2002) and pertussis toxin (Misner and Sullivan 1999). Another difference is the sensitivity of both receptors to capsazepine, an antagonist of the TRPV1 receptor. Unlike endothelium-dependent vasodilation, which is unaffected by capsazepine (Ho and Hiley 2003b; Járai et al. 1999; Mukhopadhyay et al. 2002), modulation of glutamate release by R-(+)-WIN55212-2 or CP55940 in the substantia nigra and in the CA1 pyramidal and dentate gyrus granule cells of $CB_1^{-/-}$ animals occurs via a capsazepine-sensitive mechanism (Benninger et al. 2008; Hájos and Freund 2002). It is unlikely, however, that TRPV1 receptor is responsible for this effect for at least two reasons. First, there is evidence that R-(+)-WIN55212-2 does not interact with the cloned TRPV1 receptor (Benninger et al. 2008; Zygmunt et al. 1999), although it may indirectly inhibit the TRPV1 activity at peripheral sites (Jeske et al. 2006) and, second, capsaicin and capsazepine reduce hippocampal glutamatergic neurotransmission similarly in wild-type and TRPV1-deficient mice (Benninger et al. 2008), suggesting an off-target effect.

The true identity of the hippocampal non-cannabinoid receptor still requires elucidation. Early evidence that supported the existence of the receptor in hippocampal neurons was that CB_1 receptor immunoreactivity was not detectable at glutamatergic presynaptic terminals (Hájos et al. 2001). Later, however, Katona et al. (2006) and Kawamura et al. (2006) managed to detect CB_1 receptors in hippocampal glutamatergic neurons using more sensitive approaches. In addition, the selectivity of R-(+)-WIN55212-2 for the non-cannabinoid receptor has been contested. For example, at high concentrations, this compound has been shown to affect the function of ion channels, particularly N-type voltage-gated calcium channels, which are involved in the regulation of presynaptic neurotransmission (Nemeth et al. 2008; Shen and Thayer 1998) (Table 1.1).

1.3.1.3 Glial Receptor

The description of the endothelial nonclassical cannabinoid GPCR (discussed above) coincided with the finding of Sagan et al. (1999), who provided evidence supporting the existence of a nonclassical cannabinoid receptor in glial cells. These authors reported that AEA and R-(+)-WIN55212-2 inhibit the isoproterenol-induced accumulation of cAMP in mouse striatal astrocytes. This response, although similar to responses expected from CB_1 or CB_2 receptor activation in that it was blocked by pertussis toxin, remained insensitive to CB_1 and CB_2 receptor antagonists (Sagan et al. 1999). Subsequent experiments using mouse microglial BV-2 cells showed that the putative receptor shares some common properties with the endothelial non-cannabinoid receptor, such as both are (1) sensitive to activation by AbnCBD and AEA, but not to Δ^9-THC, and (2) susceptible to the blockade by O1918 and CBD (Walter et al. 2003). Moreover, when activated by 2-AG, the chemotactic migration of microglial cells was not inhibited by rimonabant, but was antagonized by nanomolar concentrations of CBD and SR144528, suggesting that the microglial non-cannabinoid receptor interacts with classical CB_2 receptors to trigger its chemotactic response (Walter et al. 2003). This observation was further strengthened by Franklin and Stella (2003) who showed that the CB_1 receptor agonist arachidonylcyclopropylamide increases microglial BV-2 cell migration in a manner sensitive to blockade by pertussis toxin, SR144528, CBD, or O1918, but not by rimonabant. There are also indications that microglial cells may contain additional $G_{i/o}$ protein-coupled receptors for PEA, different from endothelial non-cannabinoid receptors, which potentiate AEA-, but not 2-AG-induced migration in these cells (Franklin et al. 2003), and pertussis toxin-insensitive receptors for R-(+)-WIN55212-2, which inhibit lipopolysaccharide-induced release of proinflammatory cytokines (Facchinetti et al. 2003) (Table 1.1).

1.3.1.4 Additional Atypical Cannabinoid Receptors

Presynaptic nonclassical cannabinoid receptors sensitive to AEA, but distinct from CB_1 receptors, have been hypothesized to be present on nerve terminals in the mouse

vas deferens (Pertwee 1999) and the guinea-pig ileum (Mang et al. 2001), where they inhibit noradrenaline or acetylcholine release, respectively. Other receptors sensitive to AbnCBD and CBD may also be present in the mouse vas deferens, where they attenuate the smooth muscle contraction induced by phenylephrine, norepinephrine, and methoxamine (Pertwee et al. 2002; Thomas et al. 2004).

1.3.2 Orphan Non-cannabinoid GPCRs

1.3.2.1 GRP55

The human orphan GPR55 gene was identified and cloned by Sawzdargo et al. (1999) over a decade ago. The 319 amino acids protein encoded by this gene displays 27–30 % sequence homology with purinergic GPCR subfamily, which comprises purinoreceptor P2Y5 and orphan receptors GPR23 and GPR35 (Fredriksson et al. 2003; Oh et al. 2006; Sawzdargo et al. 1999). High levels of human GPR55 mRNA transcripts have been found in brain regions implicated in the control of memory, learning, and motor functions, such as the dorsal striatum, caudate nucleus, and putamen, and in peripheral tissues, including ileum, testis, spleen, breast, adipose tissue (Brown 2007; Kotsikorou et al. 2011; Sawzdargo et al. 1999), and in some endothelial cell lines (Waldeck-Weiermair et al. 2008). The abundant expression of GPR55 protein has also been documented in large-diameter dorsal root ganglion (DRG) neurons (Lauckner et al. 2008) where it modulates sensory neuronal transmission. Activation of GPR55 by several cannabinoids increased intracellular calcium in HEK293 cells and in isolated DRG neurons (Lauckner et al. 2008), the latter suggesting the involvement of the receptor in pain perception. The same conclusion is supported by the finding that GPR55 receptor-deficient mice lack mechanical hyperalgesia in models of inflammatory and neuropathic pain (Staton et al. 2008). The GPR55 receptor has also been suggested to mediate arthritic joint pain (Schuelert and McDougall 2011), cancer cell proliferation (Hu et al. 2011), and to be a novel pro-angiogenic mediator (Zhang et al. 2010).

Despite the lack of significant alignment of amino acid residues with CB_1 and CB_2 receptors (Sawzdargo et al. 1999), there is a consistent line of evidence in the literature showing that the orphan GPR55 receptor binds certain cannabinoid ligands with high affinity. For example, HU210, a potent synthetic agonist of CB_1 and CB_2 receptors, and JWH015, a selective CB_2 agonist, are both potent agonists at GPR55 (Lauckner et al. 2008; Ryberg et al. 2007), whereas R-(+)-WIN55212-2, a synthetic cannabinoid that is somewhat more potent at CB_2 than CB_1 receptors, is inactive at GPR55 (Johns et al. 2007; Lauckner et al. 2008; Oka et al. 2007; Ryberg et al. 2007). Certain atypical cannabinoids that are not recognized by CB_1 or CB_2 receptors, such as AbnCBD and O1602 (Járai et al. 1999), are potent agonists of GPR55 (Johns et al. 2007; Ryberg et al. 2007; Waldeck-Weiermair et al. 2008), whereas CBD and its analog O1918 act as antagonists. Using the PathHunterTM β-arrestin binding assay, an approach designed to evaluate GPCR-ligand pairing (Yin et al. 2009),

GPR55 was confirmed to be activated by endocannabinoids, by the CB_1 antagonists rimonabant and AM251, by lysophosphatidylinositol (LPI) (Yin et al. 2009) and, importantly, by 2-arachidonoyl-sn-glycero-3-phosphoinositol (2-ARA-Gly) (Oka et al. 2009), which is now believed to be the cognate endogenous ligand of GPR55 (Oka et al. 2007, 2009; Okuno and Yokomizo 2011). These agonists, particularly rimonabant, AM251 and LPI, have been reported to increase $[^{35}S]GTP\gamma S$ binding with nanomolar potencies in membrane fractions prepared from HEK293 cells transfected with the human GPR55 gene (Oka et al. 2007; Ryberg et al. 2007). GPR55 was found to couple to $G_{\alpha 13}$ and activate small GTPases (RhoA, cdc42, and rac1) (Ryberg et al. 2007), resulting in oscillatory release of intracellular calcium (Ca_i^{2+}) and downstream activation of transcription factors that regulate gene expression (Henstridge et al. 2009; Ryberg et al. 2007: reviewed by Henstridge et al. 2010). Others have reported ligand-induced interactions of GPR55 with $G_{\alpha 12}$ and $G_{\alpha q}$, resulting in the activation of phospholipase C (PLC) and an increase in intracellular calcium mediated through inositol triphosphate receptor-gated stores (Lauckner et al. 2008), which promotes pain perception (Staton et al. 2008) or endothelium-mediated hyperpolarization (Busse et al. 2002). Waldeck-Weiermair et al. (2008) suggested that preferential activation of CB_1 or GPR55 receptors by AEA in the endothelial cell line EA.hy926 may depend on the activity of integrins, cell surface receptors for adhesion molecules (Table 1.1).

A question arising from these studies is whether GPR55 is identical with the putative endothelial receptor and whether there is evidence beyond pharmacological bioassays. Possible support for this notion comes from studies of ARA-S, an endogenous lipid that causes O1918-sensitive mesenteric vasodilation through endothelium-dependent (Milman et al. 2006) and independent (Godlewski et al. 2009a) mechanisms; the former being sensitive to pertussis toxin. It has been shown that nanomolar concentrations of ARA-S promote angiogenesis and wound healing in human dermal microvascular endothelial cells and that these effects could be partly inhibited by knocking down GPR55 expression with siRNA (Zhang et al. 2010). Additional studies suggest that a putative receptor with the same or similar pharmacology is also involved in regulating microglia migration (Walter et al. 2003) and microglia-mediated neuroprotection (Kreutz et al. 2009), endothelial cell (Mo et al. 2004) and neutrophil migration (McHugh et al. 2008), endothelial cell transformation induced by Kaposi sarcoma-associated herpesvirus infection (Zhang et al. 2007), and decreased cardiac contractility (Ford et al. 2002). Two key differences, however, imply that GPR55 and the endothelial cannabinoid receptor are distinct molecular entities. First, the endothelium-dependent vasodilatory effect of AbnCBD and AEA is pertussis toxin-sensitive (Járai et al. 1999; White and Hiley 1997), suggesting the involvement of $G_{i/o}$ proteins, whereas GPR55 receptor signals through $G_{\alpha 12}$, $G_{\alpha 13}$, or $G_{\alpha q}$ in a cell-specific manner. Second, the hypotensive/vasodilatory actions of AbnCBD persist in GPR55 receptor-deficient mice (Johns et al. 2007) (Table 1.1). However, one may not exclude the possibility that GPR55 mediates localized vasodilation rather than systemic hypotension or that it could be more than just one receptor, e.g., GPR55 and GPR18 that are involved in the net tissue response to cannabinoid ligands. These questions need to be addressed in future experiments.

1.3.2.2 GRP119

The orphan GPR119 receptor was found through a bioinformatic search of the human genome database and assigned to the receptor cluster encompassing the cannabinoid receptors (Fredriksson et al. 2003; Oh et al. 2006). The GPR119 gene encodes a 335 amino-acid protein (Fredriksson et al. 2003; Takeda et al. 2002) that is primarily expressed in pancreatic and intestinal tissues (Chu et al. 2007; Lauffer et al. 2009; Soga et al. 2005). GPR119 receptor immunoreactivity was detected in β-cells of the pancreatic islets of Langerhans (Chu et al. 2007; Reimann et al. 2008) and in proglucagon positive cells of the small intestine (Lauffer et al. 2009; Semple et al. 2008). Consistent with these reports, the GPR119 receptor was found to be involved in the secretion of glucagon-like peptide 1 (GLP-1) from intestinal enteroendocrine cells (Chu et al. 2007, 2008; Lan et al. 2009; Lauffer et al. 2009; Semple et al. 2008) and in the regulation of incretin-dependent insulin release (Chu et al. 2007, 2008; Flock et al. 2011) and, therefore, in the control of energy balance and metabolic homeostasis (Chu et al. 2007, 2008; Hughes 2009; Lauffer et al. 2009; Oh da and Lagakos 2011; Shah 2009; Soga et al. 2005).

Because of the close phylogenetic proximity of GPR119 and the cannabinoid receptors, substances related to endocannabinoids were among the first to be considered as potential GPR119 ligands. Using a reporter-based assay, Overton et al. (2006) reported that endogenous acylethanolamides, structural analogs of AEA, could induce a fluorescent signal in yeast cells transfected with human or mouse GPR119. *N*-oleoyl dopamine was found to be most potent, followed by OEA and PEA, whereas AEA itself displayed only residual activity (Chu et al. 2010; Overton et al. 2006). This observation was further strengthened by Lauffer et al. (2009) who found that OEA could stimulate cAMP production in cells expressing native or recombinant GPR119 receptors, while cells lacking GPR119 receptors failed to respond to OEA. The GPR119 receptor is not the only target activated by OEA. The compound has also been shown to exhibit high affinity for the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα; see Chap. 10 for details), which controls feeding and body weight (Fu et al. 2003) and, therefore, OEA serves as fat-induced satiety factor (Gaetani et al. 2010). Nanomolar and low micromolar concentrations of lysophospholipids, e.g., palmitoyl-, oleoyl-, stearoyl-lysophosphatidylcholine (palmitoyl-LPI, oleoyl-LPI, stearoyl, respectively) have also been reported to activate the GPR119 receptor (Soga et al. 2005). Recently, 2-oleoyl glycerol has been postulated to be a GPR119 agonist that signals through GLP-1 secretion from human intestine (Hansen et al. 2011). These diverse responses place into question which compound is the true endogenous ligand of GPR119 receptors. Regardless, these ligands have been shown to stimulate AC, increase cAMP, and enhance protein kinase A (PKA) activity, which implies coupling of GPR119 receptor to the protein G_s (Chu et al. 2007; Lauffer et al. 2009; Overton et al. 2006, 2008; Reimann et al. 2008; Semple et al. 2008; Soga et al. 2005). There is also evidence for the involvement of ATP-sensitive potassium (K_{ATP}) channels and voltage-dependent Ca^{2+} (Ca_v) channels in GPR119-mediated responses (Ning et al. 2008), and for the presence of high constitutive activity of GPR119 receptors regardless of activation by

ligands (Chu et al. 2007) (Table 1.1). Cox et al. (2010) have also shown that peptide YY is a critical factor in the gastrointestinal mucosal responses mediated by GPR119 receptor.

1.3.2.3 GRP18

The human GPR18 receptor gene was originally cloned in 1997 and described as an orphan GPCR encoded by a gene on chromosome 13. This 331 amino acid protein is expressed at high level by testis, thymus, spleen, peripheral blood leukocytes, and hematopoietic cells (Gantz et al. 1997). Subsequent studies suggested *N*-arachidonoyl glycine (ARA-Gly) to be the candidate endogenous ligand for this receptor (Kohno et al. 2006). These authors showed that in GPR18-transfected cells, ARA-Gly mobilizes intracellular Ca^{2+} (Ca_i^{2+}) and concentration dependently inhibits the forskolin-stimulated accumulation of cAMP with nanomolar EC_{50} values. The effects were sensitive to pertussis toxin, supporting the hypothesis that GPR18 coupled to $G_{i/o}$ protein (Kohno et al. 2006) (Table 1.1). GPR18 has also been shown to cluster on chromosome 13 with orphan receptor GPR17 (Rosenkilde et al. 2011) and with the recently deorphanized, structurally related Epstein–Barr virus active receptor 2 (EBI2 or GPR183) (Norregaard et al. 2011; Rosenkilde et al. 2006), key players in immune responses. This may suggest that they function as heterodimers and/or have similar endogenous ligands.

Recently, GPR18 has emerged as a possible candidate for the glial non-cannabinoid receptor. This evidence came from McHugh et al. (2010) who found that the putative endogenous GPR18 ligand, ARA-Gly strongly stimulates the migration of GPR18 transfected HEK293 cells and mimics the effects of AbnCBD and O1602 in BV-2 microglial cells. Furthermore, the pro-migratory effect of the above compounds was sensitive to blockade by pertussis toxin and to inhibition by O1918 and CBD (McHugh et al. 2008, 2010; see also Table 1.1).

ARA-Gly differs from AEA in the oxidative state of the carbon beta (carboxyl- vs. hydroxyl-group, respectively) of the moiety linked with arachidonic acid through an amide bond. Burstein's group (Burstein et al. 2000, 2002) suggested that AEA may be oxidatively metabolized in tissues to form ARA-Gly, a biologically active endogenous ligand whose effects are not mediated through cannabinoid receptors. This implied that ARA-Gly, and perhaps its analogs, may be part of a broader endocannabinoid family. In support for a non-cannabinoid receptor action, ARA-Gly was shown to produce antinociceptive and anti-inflammatory effects in a variety of pain models (Burstein et al. 2000, 2002; Huang et al. 2001; Succar et al. 2007; Vuong et al. 2008) and in the mouse peritonitis model, where it reduced the migration of activated leukocytes (Burstein et al. 2011). It was also reported to cause migration in the human endometrial cell line (McHugh et al. 2011) and promote insulin release in pancreatic beta cells (Ikeda et al. 2005). The hypothesis that GPR18 is a true alternative receptor for ARA-Gly still requires verification, however. In a study which utilized the β-arrestin PathHunter assay to verify the pairing of ligands with deorphanized receptors, ARA-Gly failed to activate GPR18 receptors, but was found to be a weak agonist of GPR92 receptors (Oh et al. 2008).

ARA-Gly is just one example of the growing family of endogenous *N*-acyl-amides, which is also represented by its analog, *N*-palmitoyl glycine (PAL-Gly) (Rimmerman et al. 2008). PAL-Gly was shown to play a role in sensory neurotransmission and its level was found to be regulated by FAAH (Rimmerman et al. 2008: for review of ligands see Bradshaw et al. 2009). It has been suggested that the anti-nociceptive signaling pathway activated by PAL-Gly may resemble those activated by GPR37, for which neuropeptide "head activator" serves as a high affinity endogenous ligand (Rezgaoui et al. 2006). This pertussis toxin-sensitive cascade also involves generation of nitric oxide and activation/translocation of the growth factor-regulated calcium channel (Boels et al. 2001), suggesting that structurally similar ligands may function through entirely separate targets to regulate physiological processes.

1.3.2.4 GPR92

GPR92, a relative of GPR23 by amino acid homology, was originally identified as a lysophosphatidic acid (LPA) receptor expressed in brain, spleen, gastrointestinal tract, platelets, lung, and liver (Amisten et al. 2008; Kotarsky et al. 2006; Lee et al. 2006). Particularly high levels of GPR92 mRNA were detected in the DRG, suggesting that this receptor may play a role in sensory neurotransmission (Lee et al. 2006). GPR92 has also been implicated in platelet activation (Williams et al. 2009), formation of atherosclerotic plaque (Khandoga et al. 2011), and nutrient sensing (Wellendorph et al. 2010). Farnesyl pyrophosphate (FPP) and ARA-Gly are more potent endogenous ligands of GPR92 than LPA (Oh et al. 2008; Williams et al. 2009), yet these ligands vary with respect to their downstream signaling actions. LPA and FPP induce both $G_{q/11}$ and G_s-mediated pathways, whereas ARA-Gly activates only the latter (Lee et al. 2006; Oh et al. 2008) (Table 1.1). The reason for such ligand-specific pathway selectivity is not yet clear (for review, see Bradshaw et al. 2009).

1.3.2.5 Other Orphan GPCRs

Several other orphan GPCRs have been considered as possible non-cannabinoid receptor candidates due to their close phylogenetic proximity with existing cannabinoid receptors or from deorphanization results that show fatty acids and their derivatives as matching ligands. However, conclusive evidence has not been provided. These receptors include GPR3, GPR6, GPR12, GPR23, GPR40, GPR41, GPR43, GPR84, and GPR120 (for review, see Pertwee et al. 2010).

1.3.3 Established GPCRs as Targets for Cannabinoid Ligands

Low micromolar concentrations of cannabinoids have been shown to interact with a number of established GPCRs, in most cases by targeting allosteric sites on these receptors and non-competitively modifying the access of other ligands to their