The Receptors

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

endoCANNABINOIDS

Actions at Non-CB1/CB2 Cannabinoid Receptors

The 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), $\uparrow Ca_i^{2+}$, PLC	AC stimulation, ↑cAMP, K_{ATP} and Ca_v channels	AC inhibition, ↓cAMP,$\uparrow Ca_i^{2+}$	AC stimulation, ↑cAMP, PKA,$\uparrow Ca_i^{2+}$
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).

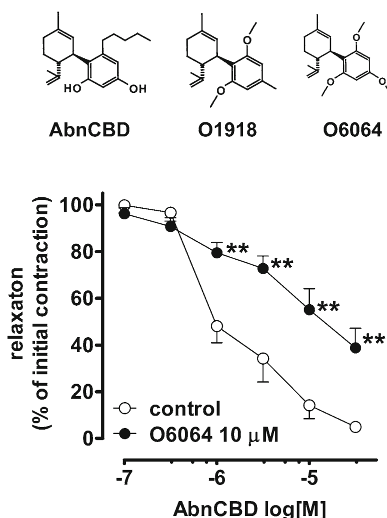

Fig. 1.1 Influence of O-6064 on the vasorelaxant effect of AbnCBD in endothelium-intact rat mesenteric arteries. Third-order segments of mesenteric arteries (200–300 μm in diameter) were isolated from male Sprague–Dawley rats (200–300 g) and mounted in a wire myograph, as described previously (Godlewski 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]GTPγS 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

binding sites. These include opioid κ, μ, and δ receptors (Kathmann et al. 2006), muscarinic acetylcholine M_1 and M_4 receptors (Christopoulos and Wilson 2001; Lanzafame et al. 2004), adenosine A_1 and A_3 receptors (Lane et al. 2009; Savinainen et al. 2003), and α_2- and β-adrenoceptors (Pertwee et al. 2002; Thomas et al. 2004: for review, see Pertwee et al. 2010). Based on this evidence, caution should be exercised whenever using micromolar concentrations of cannabinoid ligands to study drug–receptor interactions. One notable exception was a competitive displacement by rimonabant of a synthetic μ-opioid peptide, [^{3}H]DAMGO, from its receptors (Kathmann et al. 2006). This observation has been verified by others (Cinar and Szucs 2009), suggesting that rimonabant may interact with the μ-opioid receptor in a competitive manner. Additional targets, such as ion channels and transcription factors are covered in Chaps. 8, 9, 10, 11, and 12.

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Chapter 2
Overview of Non-CB_1/CB_2 Cannabinoid Receptors: Chemistry and Modeling

Evangelia Kotsikorou and Patricia Reggio

2.1 Cannabinoid Receptor Ligands

To date, two cannabinoid receptors, CB_1R (Matsuda et al. 1990) and CB_2R (Munro et al. 1993), have been isolated by molecular cloning. The CB_1R and CB_2R cannabinoid receptors are Class A G protein-coupled receptors (GPCRs) that bind constituents of *Cannabis sativa*, such as the classical cannabinoid agonist (−)-*trans*-delta-9-tetrahydrocannabinol [(−)-Δ^9-THC (**1**)]. CB_1R and CB_2R bind four other structural classes of ligands (see Fig. 2.1): nonclassical cannabinoid agonists typified by 2-[5-Hydroxy-2-(3-hydroxy-propyl)-cyclohexyl]-5-(1-methyl-heptyl)-phenol (CP55,940 (**2**)) (Devane et al. 1988; Melvin et al. 1995), endogenous cannabinoids typified by *N*-arachidonoylethanolamine (anandamide, AEA (**3**)) (Devane et al. 1992) and sn-2-arachidonoylglycerol (2-AG, (**4**)) (Mechoulam et al. 1995), aminoalkylindole (AAI) agonists typified by (*R*)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone (WIN55,212-2 (**5**)) (D'Ambra et al. 1992; Ward et al. 1991; Compton et al. 1992), and biarylpyrazole antagonists typified by *N*-(piperidiny-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A (**6**)) (Rinaldi-Carmona et al. 1994) and 1-(2,4-Dichloro-phenyl)-5-(4-iodo-phenyl)-4-methyl-1H-pyrazole-3-carboxylic acid piperidin-1-ylamide (AM251 (**7**)) (Gatley et al. 1996). A large number of physiological processes are controlled by the endogenous cannabinoids (Pertwee 2005). Most of these effects have been attributed to action at either the cannabinoid CB_1R or CB_2R receptors. Yet there are effects that clearly are not CB_1R- or CB_2R-mediated. Some of these cannabinoid effects may in fact not be receptor mediated at all. However, there is mounting evidence

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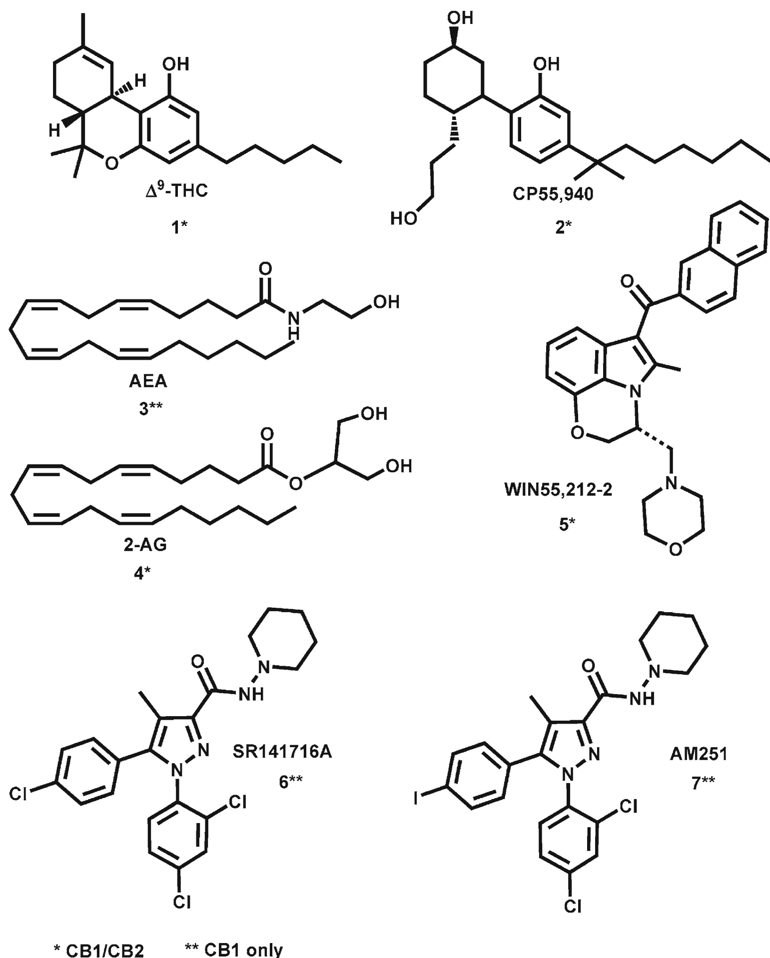

Fig. 2.1 The structures illustrated here are CB_1R/CB_2R ligands

that suggests the involvement of additional GPCRs in cannabinoid effects, as well as the involvement of transient receptor potential (TRP) channels, such as the TRPV1 channel (see also Chap. 8, Di Marzo and De Petrocellis 2010), and peroxisome proliferator-activated receptors (PPARs) (see also Chap. 10, Pertwee et al. 2010). This chapter examines the chemistry of compounds at GPCRs for which a strong link with the other cannabinoid receptors (CB_1R and CB_2R) has been made. A number of orphan GPCRs, including GPR55, GPR18, GPR35, and GPR119, have been proposed as putative cannabinoid receptors (Pertwee et al. 2010). Recent studies have confirmed that GPR18 and GPR55 recognize a range of cannabinoid ligands. Since GPR18 and GPR55 are both Class A GPCRs, we begin by providing some background on the structure and function of GPCRs. We also discuss a

cannabinoid receptor that has been extensively characterized but never cloned, the abnormal cannabidiol (Abn-CBD) receptor. There is emerging data to suggest that this receptor may be, in fact, GPR18 (McHugh et al. 2010).

2.2 GPCR Structure and Function

The number of Class A GPCR/ligand complexes that have been crystallized is growing, but still represents only a handful of receptors. These include rhodopsin (Rho) (Palczewski et al. 2000; Okada et al. 2002; Li et al. 2004), meta-rhodopsin II (Choe et al. 2011), the β_2-adrenergic receptor (β_2-AR) (Cherezov et al. 2007; Rasmussen et al. 2007, 2011; Rosenbaum et al. 2007), the β_1-adrenergic receptor (β_1-AR) (Warne et al. 2008; Moukhametzianov et al. 2011), the adenosine A2A receptor (Jaakola et al. 2008; Lebon et al. 2011), the CXCR4 receptor (Wu et al. 2010), the dopamine D3 (Chien et al. 2010) receptor, the histamine H_1 receptor (Shimamura et al. 2011), and the mu (Manglik et al. 2012) and kappa (Wu et al. 2012) opioid receptors. These crystal structures reveal a common topology that includes: (1) an extracellular N terminus; (2) seven transmembrane alpha helices (TMHs) arranged to form a closed bundle; (3) loops connecting TMHs that extend intra- and extracellularly; and (4) an intracellular C terminus that begins with a short helical segment (Helix 8) oriented parallel to the membrane surface. Ligand binding occurs within the TMH bundle, with additional ligand interactions occurring with extracellular (EC) loop residues in some structures.

Within each Class A GPCR binding pocket, there is thought to be a set of residues that change conformation upon agonist binding. These are called "toggle switch" residues and typically include residue 6.48 of the TMH6 CWXP motif and another residue that interacts with 6.48. The β_2-AR has an aromatic residue at 6.52 (F6.52) that is part of its toggle switch (Shi et al. 2002). The CB_1R and CB_2R receptors have no aromatic residue at 6.52. In CB_1R, the "toggle switch" pair has been shown to be W6.48 and F3.36 (McAllister et al. 2004).

The hallmark of Class A GPCR activation by an agonist is the "tripping" of the toggle switch within the binding pocket that allows TMH6 to flex in the CWXP hinge region and straighten. This straightening breaks the "ionic lock" between R3.50 and E/D6.30 at the intracellular end of the receptor. The result is the formation of an intracellular opening of the receptor, exposing residues that can interact with the C-terminus of the Gα subunit of the G protein (Hamm et al. 1988).

2.3 Abn-CBD Receptor ("Anandamide Receptor")

One effect of cannabinoids that was thought to be mediated by CB_1R initially and later by the CB_2R receptors is vasodilation. However, it became evident that a major player mediating the vasodilation and related effects is a receptor that was identified initially as the endothelial "anandamide receptor," and then as the Abn-CBD

receptor. This receptor has not been isolated and its sequence is not known, however, as discussed later in this chapter, there is increasing evidence that this receptor may be GPR18. In addition to the endothelium, the Abn-CBD receptor has been found to mediate effects in microglia (Walter et al. 2003) and neutrophils (McHugh et al. 2008), the first line of immune defense of the central nervous system and the periphery, respectively.

The therapeutic potential of the endothelial Abn-CBD-sensitive receptor is high. Drugs that act through the Abn-CBD receptor and cause vasorelaxation could reduce hypertension, pulmonary hypertension (Kozlowska et al. 2007, 2008; Su and Vo 2007), and alleviate vascular diseases such as atherosclerosis. Moreover, the Abn-CBD receptor is potentially involved in angiogenesis (Mo et al. 2004), inflammatory diseases of the central nervous system (microglia) (Franklin and Stella 2003; Walter et al. 2003; Kreutz et al. 2009) and the periphery (neutrophils) (McHugh et al. 2008) such as inflammatory diseases of the colon (Schicho et al. 2011). Research implicated the Abn-CBD receptor in intraocular pressure regulation expanding the therapeutic potential of the Abn-CBD receptor to include treatment of eye diseases such as glaucoma and chronic retinitis (Szczesniak et al. 2011).

The Abn-CBD receptor was first identified as the endothelial "anandamide receptor" mediating rat mesenteric vasodilation by Wagner and coworkers because the vasodilation was induced by the endogenous cannabinoid, anandamide (**3**, Fig. 2.1) (Wagner et al. 1999). Vasodilation was also elicited by the metabolically stable anandamide analog icosa-5,8,11,14-tetraenoic acid (2-hydroxy-1-methyl-ethyl)-amide (*R*(+)-methanandamide; **8**, Fig. 2.2), but not by the endocannabinoid 2-AG (**4**, Fig. 2.1), or the potent synthetic CB_1R receptor agonists HU-210 and WIN55212-2 (**5**, Fig. 2.1). The phytocannabinoid, Δ^9-THC (**1**, Fig. 2.1), and arachidonic acid caused vasoconstriction (Wagner et al. 1999). SR141716A (**6**, Fig. 2.1), a CB_1R receptor antagonist, inhibited the mesenteric vasodilation effect, but it reached a plateau despite the increasing concentration of SR141716A suggesting that more than one mechanism is involved in the agonist effect, with only one of them being antagonist-sensitive (Wagner et al. 1999). This was confirmed since SR141716A does not block the AEA-induced vasodilation in endothelium-denuded preparations of the mesenteric arterial bed (Wagner et al. 1999). Finally, the mesenteric vasodilatory effect of the endotoxin, lipopolysaccharide (LPS), and of the calcium ionophore, ionomycin, was reversed by SR141716A. This effect was not present in endothelium-denuded preparations indicating that an endocannabinoid may be released by the endothelium mesenteric arterioles mediating the vasodilatory effect. If AEA is the endocannabinoid released from mesenteric endothelial cells by ionomycin or LPS, its primary site of action is likely the endothelium, which implies its luminal release.

Jarai et al. (1999) showed that AEA-induced mesenteric vasodilation is independent of CB_1R and CB_2R receptors, as AEA-induced vasodilation was present not only in the wild-type mice but also in $CB_1R^{-/-}$ and $CB_1R^{-/-}/CB_2R^{-/-}$ mice. They also showed for the first time that 4-(6-isopropenyl-3-methyl-cyclohex-2-enyl)-5-pentyl-benzene-1,3-diol (Abn-CBD (**9**, Fig. 2.2)), a neurobehaviorally inactive compound, and its synthetic analog 4-(6-isopropenyl-3-methyl-cyclohex-2-enyl)-5-methyl-benzene-1,3-diol (O-1602, **10**) which do not bind to CB_1R and CB_2R receptors

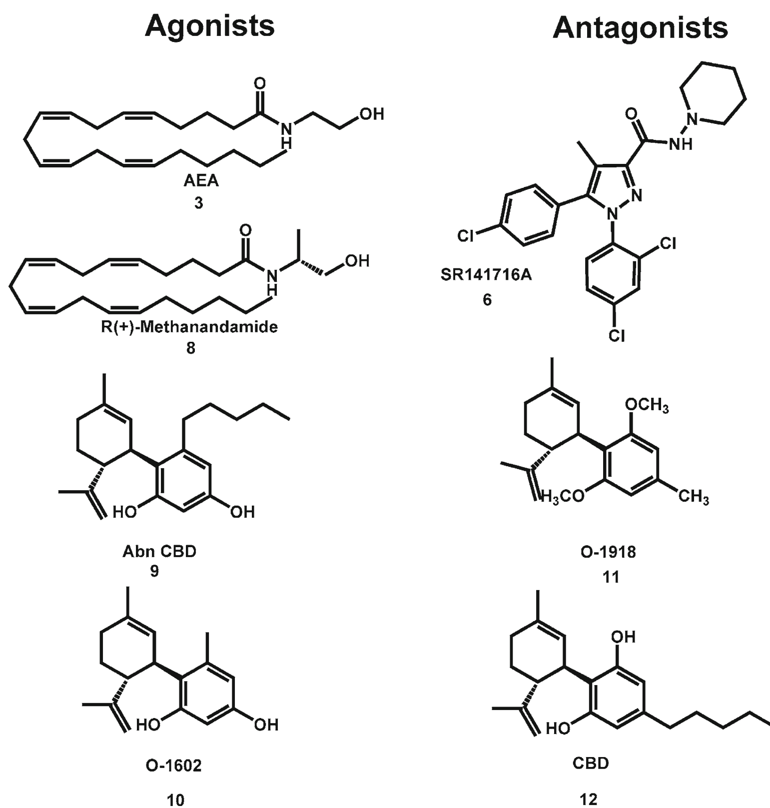

Fig. 2.2 The structures illustrated here have been reported to bind to the Abn-CBD receptor

cause hypotension and mesenteric vasodilation that is inhibited by SR141716A in wild-type mice, as well as in $CB_1R^{-/-}$ and $CB_1R^{-/-}/CB_2R^{-/-}$ mice. 2-(6-Isopropenyl-3-methyl-cyclohex-2-enyl)-5-pentyl-benzene-1,3-diol (Cannabidiol; CBD (**12**)) also inhibited Abn-CBD-induced hypotension, but not AEA- or HU-210-induced hypotension (Jarai et al. 1999). Abn-CBD-induced hypotension was not mediated by nitric oxide (NO) synthase, cyclooxygenase, or TRPV1 receptors, but was abolished by endothelial denudation (Jarai et al. 1999). A combination of K^+ channel toxins inhibited the Abn-CBD-induced vasodilation potentially through blocking the release of an endothelium-derived hyperpolarization factor (EDHF) (Jarai et al. 1999). In this study, the unidentified endothelial anandamide receptor was first described as the “Abn-CBD receptor.”

Bukoski and coworkers showed Ca^{2+} receptor-positive periadventitial nerves in both wild-type and $CB_1R^{-/-}$ mice mesenteric branch arteries that relax when Ca^{2+} is added (Bukoski et al. 2002). They showed that SR141716A (**6**) inhibits (in a concentration-dependent manner) Ca^{2+}-induced relaxation of mesenteric branch

arteries both in wild-type and in CB_1R receptor deficient mice as did the cannabidiol analog 2-(6-Isopropenyl-3-methyl-cyclohex-2-enyl)-1,3-dimethoxy-5-methyl-benzene (O-1918, **11**) (Bukoski et al. 2002). The Ca^{2+}-induced relaxation was not mediated by the CB_2R receptor, gap junctions, large conductance K_{Ca}, or voltage-dependent K^+ channels (Bukoski et al. 2002). These results in combination with the previous findings from the Kunos lab implicating a nonclasssical cannabinoid receptor in the relaxation of isolated rat mesenteric bed activated by AEA (Wagner et al. 1999) and Abn-CBD (Jarai et al. 1999) support the hypothesis that Ca^{2+}-induced relaxation takes place in a hyperpolarization-dependent manner where sensory nerve-dependent release of an endocannabinoid, possibly AEA, causes relaxation via diffusion of AEA to the underlying vascular smooth muscle and activation of the SR141716A-sensitive endothelial "anandamide" receptor (Burstein et al. 2002).

2.4 GPR18

The gene encoding GPR18 was first cloned from mouse in 1996 by Samuelson et al. (1996) and from human and dog in 1997 by Gantz et al. (1997) (see Fig. 2.3). GPR18 is localized on the distal end of mouse Chromosome 14 (Samuelson et al. 1996). In human, GPR18 is localized in Chromosome 13q32, in the same region as

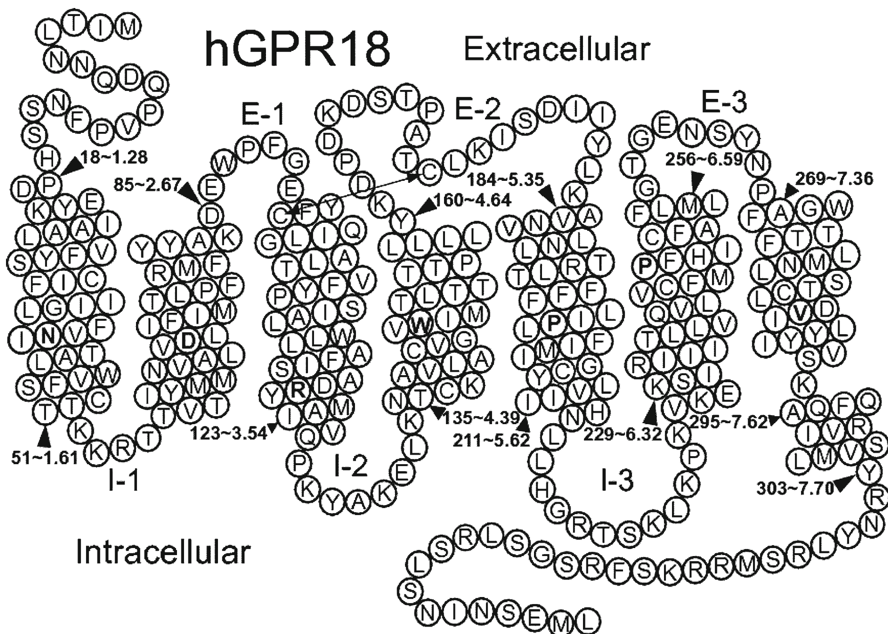

Fig. 2.3 A helix net representation of the GPR18 receptor sequence is illustrated here. The most highly conserved residue in each helix is in *bold*

the GPCR Epstein–Barr virus-induced receptor 2 (EBI2) (Vassilatis et al. 2003; Rosenkilde et al. 2006) and is structurally related to it (Norregaard et al. 2011). Among the published GPCRs, GPR18 shares the greatest nucleotide sequence homology with the rat μ opioid receptor (Genbank L20684) (Samuelson et al. 1996). They have 44.5% nucleotide sequence identity within their coding regions and 34% similarity overall (Gantz et al. 1997).

Northern blot analysis of several human tissues shows that GPR18 is highly expressed in human spleen and testis (Gantz et al. 1997). In addition to the spleen, GPR18 is also expressed in the thymus, peripheral blood leukocytes, and small intestine, all of which play a role in the immune system. This suggests that GPR18 may play an immunomodulatory role (Gantz et al. 1997). Recently, Kohno and coworkers studied the expression of the GPR18 mRNA in lymphoid and non-lymphoid hematopoietic cell lines (Kohno et al. 2006). The hematopoietic cell lines, the HTLV-1-transformed cell lines (HUT 102, MT-2, and MT-4), and lymphoid cell lines (Jurkat, MOLT-4, HUT 78, and Raji) express significant levels of the GPR18 mRNA, while the non-lymphoid hematopoietic cell lines (U937, HL60, and K562) do not express GPR18 mRNA. Analysis of the peripheral lymphocyte subsets ($CD4^+$, $CD4^+CD45RA^+$, $CD4^+CD45RO^+$, $CD8^+$, $CD19^+$, and PHA) showed higher GPR18 mRNA expression than in monocytes (Kohno et al. 2006). These results suggest that GPR18 may be involved in immune system regulation as was suggested by Gantz et al. (1997). In addition to the expression in the testis which has endocrine function, GPR18 is found in the thyroid and the stomach (Gantz et al. 1997). The high expression in testis suggests a possible role in sperm function. Additionally, even though it was not detected in the adult liver, GPR18 is expressed in fetal liver, which is a site of hematopoiesis in utero (Gantz et al. 1997). More recently, GPR18 gene expression was reported by Li and coworkers in primary melanoma cells using in silico analysis (Li et al. 2005), and by Qin and coworkers in melanoma metastases via quantitative PCR (Qin et al. 2011). GPR18 gene expression has also been reported in mouse primary microglia, the mouse microglia cell line BV-2 (McHugh et al. 2010), and in human endometrial cell line HEC-1B via quantitative PCR (McHugh et al. 2011). The pattern of expression makes GPR18 a potential therapeutic target for inflammatory diseases both of the periphery and of the brain. In addition, GPR18 receptor's presence in melanoma cells and endometrial cells makes it a therapeutic target for the aggressive skin cancer melanoma and for endometriosis, a disease that adversely affects millions of women.

Figure 2.3 illustrates a helix net representation of the GPR18 amino acid sequence. GPR18 possesses most of the conserved GPCR Class A patterns in TMHs 1, 2, 4, and 5 (N1.50, D2.50, W4.50, and P5.50). It also has the TMH3 DRY motif. There are some notable GPR18 sequence differences from other Class A GPCRs, including a few notable conserved motif differences: (1) a conservative substitution (CFXP) for the highly conserved TMH6 CWXP motif and (2) a nonconservative substitution (DVILY) for the TMH7 NPXXY motif. The GPR18 extracellular-1 (EC-1) loop is shorter than most (5 amino acids (aa) vs. 6 aa in β_2-AR and Rho). The EC-2 loop in GPR18 is comparable in length (26 aa) to the β_2-AR as is the IC-2 loop (8 aa), but the IC-3 loop in GPR18 (11 aa) is strikingly shortened compared to

the β_2-AR (40 aa). GPR18, like GPR55, has a cysteine at position 3.25 which by analogy with other GPCRs, such as the β_2-AR (Cherezov et al. 2007; Rasmussen et al. 2007, 2011; Rosenbaum et al. 2007), will likely participate in a disulfide bond with the single cysteine found in the EC-2 loop.

TMH6 in most GPCRs has a negatively charged glutamic or aspartic acid in position 6.30. This residue interacts with R3.50 of the conserved DRY motif to keep the intracellular end of the receptor closed. GPR18, however, has a positively charged lysine at 6.30 which cannot form an ionic or a hydrogen bond with R3.50. There is a glutamic acid in position 6.31 that could be involved in the ionic bond with R3.50, thus keeping GPR18's intracellular end closed. GPR18 has an aromatic residue at position 6.52 (H6.52) that presumably can act as a toggle switch for activation along with F6.48. GPR18 has two positively charged residues that face into the binding pocket, R2.60 and R5.42. Although no models of GPR18 have been published, a logical and testable starting place for ligand docking experiments would be these two charged binding pocket residues.

2.5 GPR18 Ligands

In 2006, icosa-5,8,11,14-tetraenoylamino-acetic acid (*N*-arachidonoylglycine; NAGly, **13**, see Fig. 2.4) was identified as the endogenous ligand for GPR18 (Kohno et al. 2006). NAGly is structurally similar to AEA (**3**). The difference between the two ligands is in their head group region where NAGly has a glycine group rather than an ethanolamine. The fact that GPR18 is activated by the atypical cannabinoid Abn-CBD (**9**), and several endo- and synthetic cannabinoids, has led to the proposal that GPR18 should be considered another cannabinoid receptor.

The difference in head groups between NAGly and AEA renders NAGly inactive at the cannabinoid CB_1R and CB_2R receptors (Sheskin et al. 1997). Hence, the antinociceptive and anti-inflammatory effects that NAGly produces in a variety of pain models are likely produced by a different receptor (Huang et al. 2001; Burstein et al. 2002; Succar et al. 2007; Vuong et al. 2008). The biological effects produced by NAGly are consistent with the expression of GPR18 in the peripheral blood leukocytes, in several hematopoietic cell lines (Kohno et al. 2006) and in spleen (Gantz et al. 1997). NAGly was identified as the endogenous ligand of GPR18 by screening 198 lipids of the Bioactive Lipid Library (Kohno et al. 2006) against a stable polyclonal population of GPR18-expressing L929 cells and stably GPR18-transfected K562 and CHO cells with intracellular Ca^{2+} mobilization used as the readout. At a concentration of 10 μM, NAGly induced a significant increase in intracellular Ca^{2+} concentration in GPR18-expressing cells as compared to mock-transfected cells (Kohno et al. 2006). NAGly also inhibited cAMP production in GPR18-transfected CHO cells compared to mock-transfected cells IC_{50} 20 ± 8 nM (Kohno et al. 2006). Pretreatment of the GPR18-transfected CHO cells with pertussis toxin (PTX) abolished the inhibition of forskolin-stimulated cAMP production by NAGly (Kohno et al. 2006). These experiments by Kohno and coworkers indicated that NAGly may

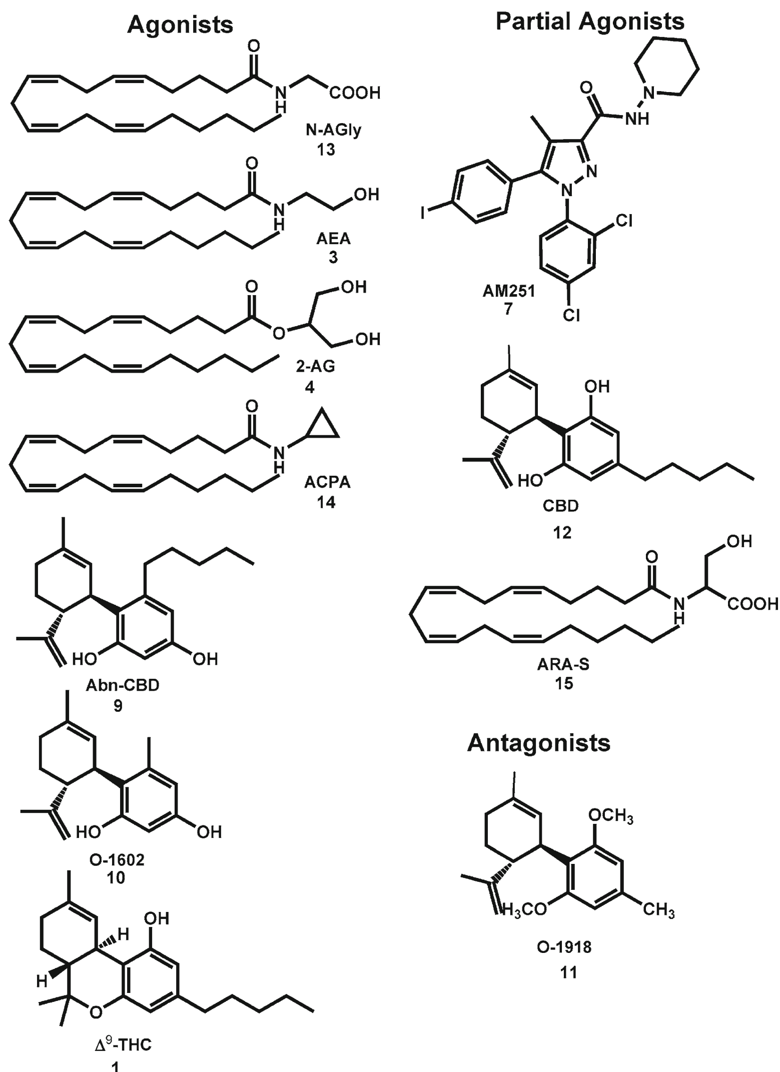

Fig. 2.4 The structures illustrated here have been reported to bind to the GPR18 receptor

act as a natural ligand for GPR18 and that GPR18 couples to $G_{i/o}$ proteins (Kohno et al. 2006). However, recently Yin and coworkers reported that GPR18 showed no activation by NAGly in a β-arrestin Path Finder™ recruitment assay (Yin et al. 2009). However, a negative result in the β-arrestin assay does not definitely prove that GRP18 is not activated by NAGly. Receptor expression or trafficking, ligand solubility or preparation, etc. are some of the reasons why the β-arrestin assay could

fail to identify the true ligand. Yin et al. (2009) reported a 5–30-fold right shift of the agonist titration curves which may have resulted in activation of weak ligands not to be observed.

GPR18 is differentially expressed in melanoma metastatic cells compared to benign nevi (Qin et al. 2011). This is in agreement with an in silico analysis of melanoma cells by Li and coworkers where GPR18 is the only overexpressed GPCR in these cells (Li et al. 2005). Qin and coworkers reported that reducing GPR18 expression using siRNA in human metastatic melanoma cells resulted in enhancement of cell apoptosis (Qin et al. 2011). These results indicate that GPR18 is involved in the signal transduction pathway that mediates inhibition of apoptosis and makes GPR18 a potential anticancer target for treatment of melanoma, the most aggressive human skin cancer (Qin et al. 2011).

Microglia, the first and main form of active immune defense in the central nervous system, respond to chemoattractants produced by damaged cells and move toward them along a concentration gradient of chemoattractants. McHugh and coworkers reported that nM concentrations of NAGly stimulate BV-2 microglia and HEK293-GPR18 transfected cell migration via GPR18 receptor (McHugh et al. 2010). In contrast, NAGly did not produce migration in non-transfected HEK293 or HEK293-GPR55 transfected cells. O-1602 (**10**) and Abn-CBD (**9**) (compounds relevant to the Abn-CBD receptor) also induced BV-2 and HEK293-GPR18 cell migration (McHugh et al. 2010). The NAGly-induced migration of BV-2 and HEK293-GPR18 transfected cells was inhibited or attenuated by the Abn-CBD receptor antagonist O-1918 (**11**) and the low efficacy GPR18 agonists *N*-arachydonoyl-L-serine (ARA-S, **15**) and CBD (**12**). Additionally, NAGly stimulated BV-2 microglia and HEK293-GPR18 cell proliferation via p44/42 MAPK activation (McHugh et al. 2010). The same proliferative response of microglia cells was observed by Carrier and coworkers for 2-AG activation of the CB_2R (Carrier et al. 2004). PTX pretreatment inhibited the NAGly-induced cell migration in both BV-2 and HEK293-GPR18 cells, indicating that NAGly acts via a $G_{i/o}$ coupled GPCR and supporting the theory that NAGly-induced cell migration is mediated by the Abn-CBD receptor (McHugh et al. 2010).

BV-2 cell migration is CB_1R receptor independent (SR141716A had no effect on NAGly-induced microglia migration) but the CB_2R antagonist, SR144528 (Rinaldi-Carmona et al. 1998), which does not bind to GPR18, inhibited NAGly-induced microglia cell migration by approximately 63.5%. This result indicates that SR144528 either produces inverse agonism at constitutively active CB_2R receptors expressed in the BV-2 cells (Rinaldi-Carmona et al. 1998) or blocks the action of ligands activating CB_2R receptors involved in transactivation (cross-modulation of co-expressed receptors such as receptor dimerization and heterologous desensitization). This effect of cross-talk between receptors has been reported before for the CB_2R and Abn-CBD receptors involved in 2-AG-induced BV-2 migration (Walter et al. 2003). Finally, using quantitative PCR, it was demonstrated that BV-2 and primary microglia express large amounts of GPR18 mRNA (McHugh et al. 2010). Immunocytochemical staining showed that GPR18 is expressed in primary microglia, BV-2 microglia, and HEK293-GPR18 transfected cells in actin-rich regions

including the lamellipodia involved in the cell movement (McHugh et al. 2010). The above-mentioned experiments performed by McHugh and coworkers suggest that GPR18 is what the field of cannabinoid research was referring to as "the unidentified Abn-CBD receptor" (McHugh et al. 2010).

Endometriosis is a condition in which uterine cells grow outside the uterus causing chronic pelvic pain, dysmenorrheal, and infertility in women (Bulletti et al. 2010). Fujimoto et al. (1996) and Acconcia et al. (2006) showed that human endometrial cells and human endometrial cell lines (JEC-1A and HEC-1B cells), respectively, migrate toward estrogen and exhibit all the migration-associated cytoskeletal changes. This role of estrogen as chemoattractant for human endometrial cells is likely to participate in endometriosis. McHugh and coworkers demonstrated via PCR experiments that the human endometrial cell line HEC-1B expresses GPR18 receptors. NAGly, AEA, and Δ^9-THC each induced cell migration in HEC-B cells (McHugh et al. 2011). SR141716A had no effect on cell migration, but CBD and the CB_2R antagonist, SR144528, antagonized the effect of AEA. CBD was found to abolish the effect of NAGly (McHugh et al. 2011). These results indicate that CB_2R is involved in endothelial cell migration, but the major player is the GPR18 receptor. To elucidate the pharmacology of the GPR18 receptor, McHugh and coworkers used a MAPK activation assay in HEK293-GPR18 transfected cells (McHugh et al. 2011). NAGly (**13**), O-1602 (**10**), Abn-CBD (**9**), Δ^9-THC (**1**), AEA (**3**), ACPA (**14**), and CBD (**12**) induced p44/42 MAPK phosphorylation (reported in order of potency), but WIN55212-2 (**5**), CP55940 (**2**), R-meth-AEA (**8**), JWH-133, and JWH-015 did not. Finally, PTX and the compounds AM251 (**7**) and CBD (**12**) antagonized the effects of NAGly (**13**) and Δ^9-THC (**1**) on HEC-1B cell migration and MAPK phosphorylation of HEK293-GPR18 cells (McHugh et al. 2011). These experiments demonstrate the role that the GPR18 receptor and cannabinoid compounds play in endometrial cell migration.

The expression pattern of GPR18 suggests it has an immunomodulatory role. Burstein and coworkers published a set of experiments which showed that NAGly has anti-inflammatory activity in vivo in the mouse peritonitis model (Burstein et al. 2011). Administration of NAGly at low doses inhibited the migration of monocytes and neutrophils following injection of pro-inflammatory compounds in the peritoneal cavity. NAGly had the same inhibitory effect on cell culture models including GPR18 transfected HEK293. These results implicate the GPR18 receptor in the anti-inflammatory activity. Treatment of C6 glial cells with NAGly in the presence of BSA (arachidonic acid re-esterification inhibitor) resulted in the release of free arachidonic acid (the precursor of anti-inflammatory eicosanoids such as PGJ (prostaglandin J2) and LXA4 (lipoxin A4)). NAGly-treated HEK293-GPR18 cells produced PGJ and LXA4 and similar results were obtained in four other human cell lines (RAJI, U-937, HL-60, and MOLT-4). In fact, their PGJ levels closely correlated with the amount of GPR18 expression levels quantified using quantitative PCR, suggesting that the effect of NAGly is mediated by the GPR18 receptor. The experiments suggest that NAGly activates GPR18 causing an elevation of free arachidonic acid (released from cellular storage sites) which then gets converted to the anti-inflammatory PGJ by cyclooxygenases. Use of a GPR18 receptor-specific

antibody reduced the NAGly-induced PGJ synthesis (Burstein et al. 2011). Finally, the NAGly treatment of U-937 and HEK293-GPR18 cells showed an increased number of cells stained with Trypan Blue which is a possible indicator of programmed cell death. Addition of PGJ caused a similar response suggesting that PGJ is a mediator in cell death. The GPR18 antibody was able to block that action in the HEK293 cells, further supporting the implication of GPR18 in cell death of inflammatory cells which is important for the resolution of inflammation.

2.6 GPR55

GPR55 is a rhodopsin-like (Class A) GPCR, highly expressed in human striatum (Sawzdargo et al. 1999) (Genbank accession # NM-005683; see helix net representation in Fig. 2.5), that was de-orphanized as a cannabinoid receptor (Brown and Wise 2001; Drmota et al. 2004). GPR55 has been reported to couple to Gα13 (Henstridge et al. 2009; Ryberg et al. 2007), Gα12, or Gαq (Lauckner et al. 2008) proteins. In the human CNS, GPR55 is found predominantly in the caudate, putamen, and striatum (Sawzdargo et al. 1999). GPR55$^{-/-}$ mice have been shown to be protected in models of inflammatory and neuropathic pain, suggesting that GPR55 antagonists may have therapeutic potential as analgesics for both these types of pain (Staton et al. 2008). GPR55 also has physiological relevance in bone resorption,

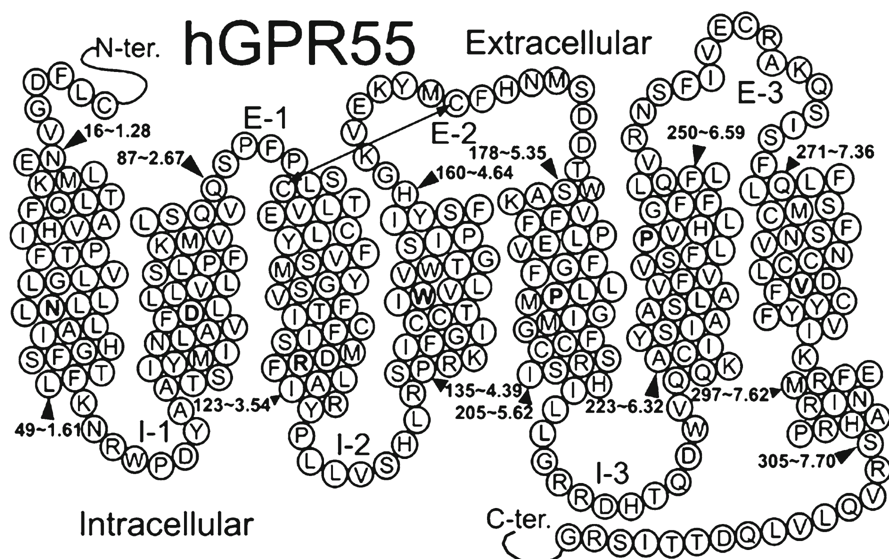

Fig. 2.5 A helix net representation of the GPR55 receptor sequence is illustrated here. The most highly conserved residue in each helix is in *bold*

suggesting a possible therapeutic use for GPR55 antagonists in the treatment of osteoporosis (Whyte et al. 2009). Other studies indicate that GPR55 activation is pro-carcinogenic (Andradas et al. 2010; Ford et al. 2010; Pineiro et al. 2010). The GPCR proteins most homologous to GPR55 are GPR35 (27%), P2Y5 (29%), GPR23 (30%), and CXCR4 (26%) (Sawzdargo et al. 1999). GPR55 exhibits lower amino acid identity to the cannabinoid CB_1R (13.5%) and CB_2R (14.4%) receptors.

2.7 Cannabinoid Ligands Recognized at GPR55

The de-orphanization of GPR55 as a cannabinoid receptor and the potential utility of cannabinoids in therapy prompted a wider search for GPR55 ligands among known cannabinoid receptor compounds (Henstridge et al. 2009). Initial studies conducted in several labs (Johns et al. 2007; Lauckner et al. 2008; Ryberg et al. 2007) confirmed that GPR55 is activated by various cannabinoid and atypical cannabinoid compounds. However, in some cases GPR55 ligand results from lab to lab were inconsistent with each other. Observations using a GTPγS functional assay indicated that GPR55 is activated by nanomolar concentrations of the endocannabinoids 2-AG (**4**), virodhamine, noladin ether, oleoylethanolamide and palmitoylethanolamide, and the atypical cannabinoids CBD (**12**) and Abn-CBD (**9**) (Ryberg et al. 2007). Using a GTPγS functional assay in a separate study, Abn-CBD (**9**) and O-1602 (**10**) were found to act as agonists at GPR55, while the cannabinoid aminoalkylindole, WIN55,212-2 (**5**), produced no effect (Johns et al. 2007). Δ^9-THC (**1**), the aminoalkylindole, JWH-015, anandamide (AEA; **3**), and R-methanandamide (**8**) acted as agonists, producing an increase in intracellular Ca^{2+}, while the CB_1R antagonist, SR141716A (**6**), acted as an antagonist at GPR55 (Lauckner et al. 2008). The CB_1R antagonist, AM251 (**7**), was reported to act as an agonist at GPR55, evoking GPR55-mediated Ca^{2+} signaling in one study (Henstridge et al. 2009) and an increase in GTPγS binding in another study (Ryberg et al. 2007). Subsequent studies of GPR55 pharmacology suggested that lysophosphatidylinositol (LPI, **16**; see Fig. 2.6) compounds are endogenous GPR55 agonists (Oka et al. 2007), with 2-arachidonoyl-sn-glycero-3-phosphoinositol (2-AGPI; **17**) possessing the best activity observed to date (Oka et al. 2009). Neither LPI nor 2-AGPI binds to CB_1R or CB_2R receptors. Using a β-arrestin green fluorescent protein biosensor to assess a cohort of CB_1R/CB_2R ligands for GPR55 activity, Kapur et al. (2009) confirmed LPI (**16**) as a GPR55 agonist, while observing that the cannabinoid antagonists AM251 (**7**) and SR141716A (**6**) were also GPR55 agonists. These GPR55 ligands exhibited comparable efficacy in inducing β-arrestin trafficking, and moreover, activated the G protein-dependent signaling of PKCβII. Conversely, the potent synthetic cannabinoid agonist CP55940 (**2**) acted as a GPR55 antagonist/partial agonist, inhibiting GPR55 internalization, the formation of β-arrestin GPR55 complexes, and the phosphorylation of ERK1/2 (Kapur et al. 2009).

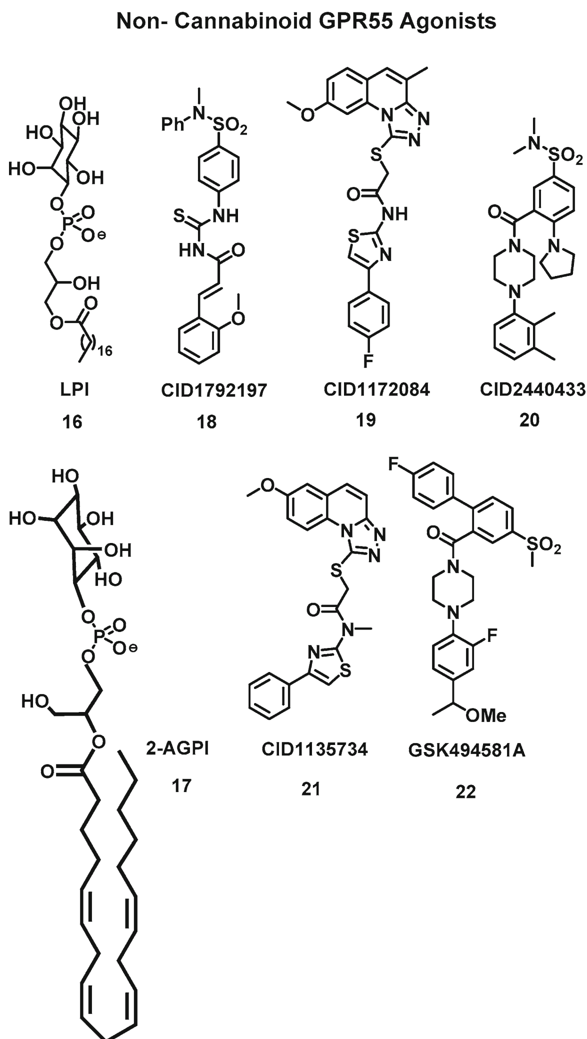

Fig. 2.6 The structures illustrated here are non-cannabinoid compounds that have been reported to bind to the GPR55 receptor

2.8 Other GPR55 Ligands

Despite the identification of numerous cannabinoids acting at GPR55 (see above), it is important to note that no cannabinoid ligand has been confirmed to have low nanomolar potency at GPR55. During a collaborative project between the Abood laboratory and the Sanford-Burnham screening center of the Molecular Libraries Probe Production Centers Network (MLPCN), a series of GPR55 agonists

that belong to novel, unreported GPR55 agonist chemotypes were identified (Heynen-Genel et al. 2010). These were discovered by a high content, high throughput β-arrestin screen (see http://mli.nih.gov/mli/mlp-probes/). Three of the novel agonists **18** (EC_{50} = 0.11 μM), **19** (EC_{50} = 0.16 μM), and **20** (EC_{50} = 0.26 μM) obtained from the screen are illustrated in Fig. 2.6. These compounds constitute excellent templates to design nanomolar efficacy ligands.

2.9 GPR55 Model

Figure 2.5 illustrates a helix net representation of the GPR55 amino acid sequence. GPR55 possesses most of the conserved Class A patterns in TMHs 1, 2, 4, and 5 (N1.50, D2.50, W4.50 and P5.50), with a few notable conserved motif differences: (1) a conservative substitution (DRF) for the TMH3 DRY motif, (2) a conservative substitution (SFLP) for the TMH6 CWXP motif, and (3) a nonconservative substitution (DVFCY) for the TMH7 NPXXY motif. Finally, the GPR55 extracellular-1 (EC-1) loop is shorter than most (3 amino acids (aa) vs. 6 aa in $β_2$-AR and Rho) and the GPR55 EC-3 loop is noticeably longer than most (14 aa long vs. 5 aa in $β_2$-AR, 6 aa in rhodopsin and CB_1/CB_2). Like most Class A GPCRs, GPR55 also has a cysteine in the EC2 loop (C168) that can form a disulfide bridge with C3.25 (94) (CB_1R and CB_2R are exceptions). TMH6 in most GPCRs has a negatively charged glutamate or aspartate in position 6.30. This residue interacts with R3.50 of the conserved DRY motif to keep the intracellular end of the receptor closed. GPR55 does not have a negatively charged residue at 6.30, but has a glutamine that can still form a hydrogen bond with R3.50 to keep the receptor closed. Like GPR18, GPR55 has an aromatic residue at position 6.52 (H6.52) that presumably can act as a toggle switch for activation with F6.48. Conformational Memories calculations have indicated that residues F6.48 and H6.52 do have correlated conformational states (Kotsikorou et al. 2011). The CB_1R and CB_2R receptors have a single positively charged residue within the transmembrane helix domain, K3.28. K3.28 in CB_1R has been shown to be the primary ligand interaction site for classical, nonclassical, and endo-cannabinoids, as well as for the biarylpyrazole inverse agonists (Song and Bonner 1996; Hurst et al. 2002). GPR55 has one positively charged residue, K2.60, which mutation studies indicate to be important for ligand binding (Kotsikorou et al. 2011). GPR18 has an arginine at this same position (Gantz et al. 1997).

Recently, the GPR55 agonist binding site was explored using models of the GPR55 inactive (R) and activated (R*) states and a novel set of high potency GPR55 selective ligands (Kotsikorou et al. 2011). These models were initially based on the 2.4 Å crystal structure of the $β_2$-AR (Cherezov et al. 2007) and then modified to reflect sequence dictated conformational differences in TMHs 1, 2, 5, 6, and 7 (for a complete discussion, see Kotsikorou et al. 2011). Because GPR55 has considerable sequence similarity with the CXCR4 receptor (26%) (Sawzdargo et al. 1999) and their sequences share key cysteines in the N terminus (C(10) in GPR55; C(28)

in CXCR4) and at the TMH7 extracellular (EC) end (C7.25 in both GPR55 and CXCR4), the current GPR55 model generated by the Reggio lab has been updated to include an N-terminus/TMH7 disulfide bridge that helps to open up the extracellular region of the receptor (Kotsikorou et al. 2011).

GPR55 model activated form (*R**). The hallmark of GPCR activation is the breaking of the ionic lock between R3.50 and E/D6.30 which allows TMH6 to flex in the CWXP hinge region and straighten. The result is an intracellular opening of the receptor, exposing residues that can interact with the C-terminus of Gα (Hamm et al. 1988). To create a GPR55 R* (activated state) model, a straighter TMH6 helix was chosen from the output of the GPR55 TMH6 Conformational Memories (Guarnieri and Weinstein 1996; Guarnieri and Wilson 1995; Whitnell et al. 2008) study and substituted for TMH6 in the R state model. This R* model was equilibrated via a 70 ns NAMD2 molecular dynamics (MD) simulation in a fully hydrated POPC bilayer environment (Kotsikorou et al. 2011). The major change seen in this simulation was that D7.49 (normally N7.49 in Class A), along with D2.50, recruits water and this water perturbs the backbone structure on TMH7 by hydrogen bonding to backbone carbonyls in the S7.42 to D7.49 region of TMH7. The net result is an increase in flexibility allowing TMH7 to bend at S7.42 and compensate for the lack of a proline at position 7.50.

2.10 Ligand Docking Studies

The GPR55 R* model described above was used to evaluate the binding of LPI (**16**) and three novel agonists obtained from the Sanford-Burnham screen, **18** (EC_{50} = 0.11 μM), **19** (EC_{50} = 0.16 μM), and **20** (EC_{50} = 0.26 μM) (Kotsikorou et al. 2011). These structures are shown with PubChem Compound IDs in Fig. 2.6 (Kotsikorou et al. 2011). The closely related compound **21** (EC_{50} > 32 μM), that does not bind to GPR55, served as negative control. Complete conformational analyses of all ligands were performed using ab initio Hartree–Fock calculations at the 6–31 G* level as encoded in Jaguar (version 9.0, Schrodinger, LLC, New York, NY). The automatic docking program, Glide (Schrodinger, LLC, New York, NY), was used in docking experiments and the dock with the best docking score was used. Jaguar results were also used to determine energy penalties paid by ligands in the final docked complex.

Modeling data indicated that the similarity between **18**, **19**, **20** and LPI (**16**) enables all of these compounds to be recognized by a single GPR55 binding pocket. The shape of the GPR55 R* binding site accommodates ligands that are inverted L shapes or T shapes with long, thin profiles that can fit vertically deep in the receptor binding pocket, while their broad head regions occupy the horizontal binding pocket opening near the EC loops. The vertical pore is narrow enough that it cannot accommodate the *N*-methyl group of **21**. For GPR55 agonist ligands (**18**–**20**), the highest

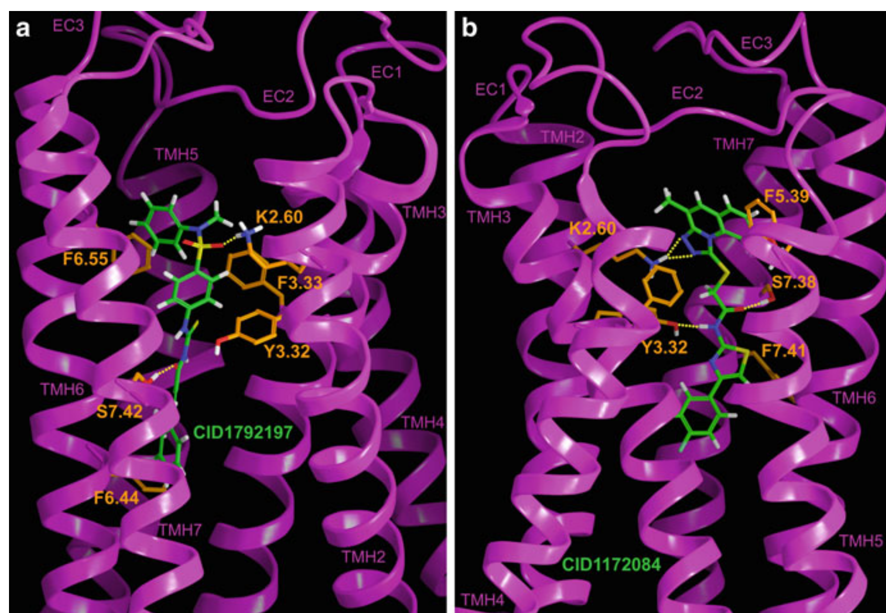

Fig. 2.7 The complexes formed between GPR55 R* and (**a**), the L-shaped CID1792197 (**18**) and (**b**), the T shaped CID1172084 (**19**) are illustrated here. Labeled amino acids in this figure are those with which each ligand has significant interactions

negative electrostatic potential region is exposed either at the "elbow" of the L or at one end of the T cross bar (see red regions in Fig. 2.5 in the paper (Kotsikorou et al. 2011)). It is this region that interacts with K2.60 in each of the docks. Figure 2.7 illustrates the complexes formed between GPR55 R* and (a), the L-shaped CID1792197 (**18**) and (b), the T shaped CID1172084 (**19**) Labeled amino acids in this figure are those with which each ligand has significant interactions. For the sake of brevity here, the reader is referred to our published paper which has an in depth discussion of each binding site (Kotsikorou et al. 2011).

Tests of GPR55 model using other reported GPR55 agonists. Other GPR55 agonists have been docked in this GPR55 R* model to test the model. The CB_1R antagonist/GPR55 agonist, AM251 (**7**) (Kapur et al. 2009), was found to adopt a T-shape in the GPR55 R* binding pocket, with the 2,4-dichlorophenyl and pyrazole rings forming the cross bar of this T-shape and the 4-iodophenyl ring penetrating into the vertical section of the binding pocket. The structure of benzoylpiperazine GPR55 agonist (GSK494581A; **22**) (Brown et al. 2011) is similar to **20**. The GPR55 R* docking study shows that **22** adopts a T-shape at GPR55 R*, binding in the same receptor region and in a similar orientation as **20** with comparable energy of interaction (see Supplementary Information in the recent paper) (Kotsikorou et al. 2011).

2.11 Conclusions

A large number of physiological processes are controlled by the endogenous cannabinoids (Pertwee 2005). Most of these effects have been attributed to action at either the cannabinoid CB_1R or CB_2R receptors. Yet there are effects that clearly are not CB_1R- or CB_2R-mediated. Some of these cannabinoid effects may in fact not be receptor mediated at all. However, there is mounting evidence that suggests the involvement of additional GPCRs in cannabinoid effects, as well as the involvement of TRP channels, such as the TRPV1 channel (see also Chap. 8 and Di Marzo and De Petrocellis 2010), and PPARs (see also Chap. 10 and Pertwee et al. 2010). The present chapter explored additional GPCRs, GPR18 and GPR55, that recognize a subset of cannabinoid ligands. Action at these receptors may contribute to the collective pharmacology attributed to cannabinoids.

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Part II
G Protein-Coupled Receptors

Chapter 3
GPR55 in the CNS

Hui-Chen Lu, Jane E. Lauckner, John W. Huffman, and Ken Mackie

3.1 Introduction

The observation that cannabinoids produced effects independent of CB_1 and CB_2 cannabinoid receptors has promoted the search for additional cannabinoid receptors (Pertwee et al. 2010). The first metabotropic receptor put forward as being an additional cannabinoid receptor was the orphan G protein-coupled receptor (GPCR), GPR55. GPR55 was hypothesized to be a cannabinoid receptor based on the observation that a subset of cannabinoid ligands bound to and activated this receptor (Brown and Wise 2003; Drmota et al. 2004). Following these initial observations in the patent literature, several studies have been undertaken to better understand this receptor's function, pharmacology, and distribution, as summarized in recent reviews (Pertwee et al. 2010; Sharir and Abood 2010). In this chapter we briefly review the distribution of GPR55 in the nervous system, its complicated pharmacology and intracellular signaling, GPR55's potential role in mediating chronic neuropathic and inflammatory pain, and speculate on possible additional functional roles.

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3.2 Distribution

The report describing the initial cloning of GPR55 determined its distribution in the human brain by Northern analysis and in rat brain by in situ hybridization (ISH) (Sawzdargo et al. 1999). In human brain, GPR55 mRNA was found in caudate and putamen, but not in frontal cortex, hippocampus, thalamus, pons, or cerebellum. Thus, in human brain GPR55 appears to be enriched in the basal ganglia, suggesting a possible role in the control of movement. Sawzdargo et al. stated that their preliminary ISH studies in rat brain found that GPR55 was expressed in hippocampus, some thalamic nuclei, and some midbrain regions, although no data were shown (Sawzdargo et al. 1999). The ISH studies available on the Allen Brain Atlas (http://mouse.brain-map.org/) show uniform, low-level GPR55 expression in mouse (developing or adult) brain and spinal cord. In addition, the limited human brain transcriptome data for GPR55 does not reveal a consistent pattern of expression between the two probes used (http://human.brain-map.org/). These results suggest that wherever GPR55 is expressed in the CNS, it is expressed at moderately low levels. GPR55 expression in mouse tissues has been surveyed by quantitative PCR (Ryberg et al. 2007). This study found the following order of expression: Frontal cortex > striatum > hypothalamus > brain stem > cerebellum = hippocampus > spinal cord. There was approximately a tenfold difference between expression levels of GPR55 in frontal cortex compared to spinal cord. Interestingly, while levels of *CB1* mRNA were about 100 fold higher than levels of GPR55 in cerebellum, expression levels measured in this study of the two genes were similar in hypothalamus and brain stem. The only published immunocytochemical study of GPR55 expression in the nervous system was restricted to examining mouse dorsal root ganglion (DRG) neurons. In this study GPR55 immunoreactivity was primarily present in medium diameter neurons (Lauckner et al. 2008), which normally are involved in proprioception. However, these neurons apparently can be recruited to serve a nocioceptive function in some chronic pain states (Neumann et al. 1996; Ruscheweyh et al. 2007). Given the observation that GPR55 appears to play a mandatory role in the establishment of certain chronic pain states (see below), it is interesting to speculate that GPR55 is involved in the recruitment of medium diameter DRG neurons to a nocioceptive function that occurs in some preclinical models of chronic pain.

Taking the nervous system expression data as a whole, there are fundamental inconsistencies in the mapping of GPR55 expression by molecular techniques. These may be due to species differences and to differences in methodologies and their sensitivities (e.g., northern blotting vs. ISH vs. rt-PCR). A thorough understanding of CNS GPR55 expression will be critical for focusing, generating, and testing hypotheses on the role of GPR55 in the nervous system.

3.3 GPR55 Ligands and Their Signaling

3.3.1 Introduction and General Considerations

GPR55 signaling is complicated and varied. Like most lipid receptors, it binds to and is activated by an impressive array of structurally diverse ligands. Conversely, all well-characterized and frequently used GPR55 ligands interact with other receptors, channels, and/or signaling molecules at concentrations similar to those that activate or antagonize GPR55. Thus, at the present time, referring to *any* GPR55 ligand as specific or selective is inaccurate, misleading, and only serves to confuse the literature. Conversely, the identification of potent and selective GPR55 ligands will be important for determining the physiological role(s) of this receptor. There has been some recent progress in this direction (Kotsikorou et al. 2011a), although establishing that a ligand is specific requires a substantial investment of time and resources. Furthermore, with the continued identification of drug targets, "specificity" is a constantly moving target, and a "specific drug" is only specific until another target is identified.

Why has the identification of GPR55 ligands been so difficult? In part, the reasons are historical. The first published pharmacological studies of GPR55 took the approach of identifying GPCRs that were activated by cannabinoid ligands. Thus, there was a built in bias towards examining ligands related to cannabinoids as these compounds either interacted with cannabinoid receptors or were closely related to ligands that did. Thus, GPR55-interacting compounds identified in this fashion are likely to interact with GPCRs other than GPR55. This contrasts to the approach of identifying a receptor and then performing unbiased high-throughput screens to identify potent ligands. This unbiased approach has recently been taken for GPR55, and three medium affinity GPR55 ligands were identified (CID1792197, CID1172084, and CID2440433) (Brown et al. 2011; Kotsikorou et al. 2011b).

Another issue that has confused the identification and characterization of GPR55 agonists is the measurement of GPR55 activity. As discussed below, GPR55 most readily signals through G proteins belonging to the Gα12/13 family (Ryberg et al. 2007; Lauckner et al. 2008). These G proteins are notoriously promiscuous and diffusely couple to many signaling pathways, making them particularly prone to agonist selective signaling (that is, functional selectivity (Urban et al. 2007) where specific ligands will activate the receptor to signal via different effector molecules) (Kelly et al. 2007; Suzuki et al. 2009). Thus, choice of assay is immensely important when screening potential GPR55 ligands. Consequently, when discussing whether a compound is or is not a ligand (both agonists and antagonists, especially inverse agonists, can show functional selectivity) for GPR55, it is important to specify the signaling pathway being studied and to appreciate that certain assays

that are amenable to high-throughput screening (e.g., increases in intracellular calcium) may overlook GPR55 agonists that only activate a limited repertoire of signaling pathways. As an example, several agonists that activate GPR55 to increase intracellular calcium do not activate GPR55 appropriately to stimulate ERK1/2 (extracellular signal-regulated kinase types 1/2) phosphorylation (Oka et al. 2007; Lauckner et al. 2008).

GPR55 appears to primarily signal through the G proteins, Gq, Gα12, and Gα13 (Ryberg et al. 2007; Lauckner et al. 2008). More downstream signaling pathways of GPR55 include: increases in intracellular calcium, stimulation of ERK1/2 phosphorylation, activation of Rho kinase, the small G proteins: RhoA, cdc42, and rac1, and stimulation of several transcriptional networks, including those mediated by NFAT, NFκB, and CREB (Ryberg et al. 2007; Lauckner et al. 2008; Henstridge et al. 2010). Note that the increase in intracellular calcium may be gradual and low in magnitude (100–200 nM), or it can be abrupt and of considerable amplitude (>1,000 nM) (Oka et al. 2007; Lauckner et al. 2008; Henstridge et al. 2009). This may be due to different levels of receptor expression, different expression systems (e.g., transient vs. stable), different interacting proteins, or trafficking to different cellular compartments. GPR55 activation can also lead to beta arrestin recruitment and the receptor's internalization (Kapur et al. 2009; Henstridge et al. 2010). Little is known about the consequences of chronic GPR55 stimulation. In particular, the mechanisms supporting GPR55 internalization, desensitization, trafficking, and recycling are all unknown.

Finally, there is much interest in identifying and understanding the role(s) of the endogenous ligands for GPR55. At best, the two major endogenous cannabinoids (endocannabinoids), anandamide and 2-arachidonoyl glycerol (2-AG), have modest and minimal activity at GPR55, respectively (Ryberg et al. 2007; Lauckner et al. 2008). Lysophosphatidyl inositol (LPI) has been identified as an endogenous compound that potently (mid-nanomolar) activates GPR55 to stimulate a wide variety of signaling pathways (Oka et al. 2007). Thus, LPI is an endogenously occurring compound that *can* activate GPR55. However, to demonstrate that it is a physiologically relevant endogenous activator, two additional criteria need to be met. The first is the demonstration that endogenously produced LPI activates GPR55 in situ. Experiments supporting the involvement of endogenous LPI in a response should use some combination of inhibiting LPI production or degradation, while also demonstrating that LPI levels are appropriately changing. The canonical pathway for LPI production is via phospholipase A_1 (PLA_1—to synthesize the corresponding *sn-2* LPI) or phospholipase A_2 (PLA_2—to produce the corresponding *sn-1* LPI). The biology of the PLAs is rich and varied, with multiple cytosolic, membrane-bound, and secreted forms reported. There are also a number of inhibitors of these enzymes of varying specificity. Together, the richness of the biology and the complexity of the pharmacology mandate careful experimentation and the thorough use of appropriate controls. The second criterion is that responses seen following LPI production (or administration) are actually mediated via GPR55 receptors and not other LPI targets, for example two-pore or calcium-activated potassium channels (Danthi et al. 2003; Bondarenko et al. 2011). This second criterion can be met by careful

experimental design using the appropriate antagonists (when they become available) or GPR55 knockout mice (acknowledging that compensatory changes may be occurring). In vivo experiments lacking these key controls that claim GPR55 as the target of LPI should be viewed skeptically.

3.3.2 A Brief Review of GPR55 Ligands

An extensive review of the pharmacology of GPR55 ligands has recently been published (Sharir and Abood 2010). In the section below we briefly consider some highlights of GPR55 pharmacology with an emphasis on caveats that should be employed when using these ligands. Those wanting a more in-depth treatment of the subject should consult with the excellent review cited above.

3.3.3 Lysophosphatidyl Inositol

LPI was identified as an endogenous GPR55 agonist by Takayuki Sugiura and his colleagues based on its ability to promote ERK1/2 phosphorylation in GPR55-expressing HEK293 cells (Oka et al. 2007). They also found that it increased intracellular calcium in HEK293 cells expressing GPR55, but not in non-transfected cells. It is important to note that "LPI" is not a single compound, but is a family of glycerol lipids, containing a single acyl chain esterified to either the 1 or 2 position of glycerol, with inositol phosphate in the 3 position. Given the variety of possible acyl chains that comprise the family of LPIs, it can be expected that different LPIs will vary in their ability to activate GPR55. In examining the effect of the fatty acid moiety on LPI activity at GPR55, the Sugiura group has reported that 2-arachidonoyl LPI was more potent than LPI containing shorter or less saturated fatty acids (Oka et al. 2009). It should be noted that often commercially available LPI is from soybean, which does not contain arachidonate as the acyl group. In brain the most abundant forms of LPI have either stearic or palmitic acid as the acyl chain (Oka et al. 2009). There may be considerable regulation of LPI activity by enzymatic control of its synthesis via PLA_1 vs. PLA_2. For example, since arachidonic acid is esterified primarily to the second position in glycerol, then activation of PLA_1 will produce *sn-2* arachidonoyl LPI. There is a need for a careful survey of the forms of LPI present in the mammalian brain and the regulation of their synthesis. This will be all the more important if LPI activates GPR55 to modulate synaptic transmission (see below). In summary, at the present time LPI is the most widely accepted GPR55 agonist, both because of its ability to activate what is currently understood to be the complete range of signaling pathways utilized by GPR55 and also because of its relatively high potency of endogenously occurring agonists. However, considerable work remains to be done to understand the forms of LPI most relevant in the brain for GPR55 activation and how their synthesis and metabolism are controlled.

3.3.4 Aryl Pyrazoles

Diarylpyrazoles, such as rimonabant, are well known in the cannabinoid field as having been the basis for the development of widely used CB_1 and CB_2 receptor antagonists (e.g., SR141716 and SR144258, respectively), and their brief clinical use as an antiobesity drug. However, as initially shown by Andy Irving and his colleagues, several of these compounds efficaciously activate GPR55 signaling to stimulate calcium transients in HEK293 cells stably expressing GPR55 (Henstridge et al. 2009, 2010). A cursory inspection of the structure–activity relationship (SAR) of these compounds suggests that substitutions on the 3-carboxamide play an important role in determining GPR55 affinity. With a piperidine group present (e.g., SR141716A and AM251) the diarylpyrazoles are agonists with medium affinity. However, potency is strongly diminished when a morpholino group is present (e.g., AM281). In contrast, modification of the substituted phenyl (e.g., T1118) is well tolerated (Daly et al. 2010). This crude SAR predicts that SR144258 will be a particularly poor GPR55 agonist, but this remains to be tested. It is likely that (given the extensive synthetic chemistry of the diarylpyrazoles pursued during the development of these compounds as antiobesity agents) this chemotype could serve as a route to the synthesis of specific GPR55 agonists. As a side note, the plasma concentrations achieved during the therapeutic use of rimonabant are of sufficient magnitude to activate GPR55, suggesting the intriguing notion that some of the effects, or side effects, of rimonabant might be mediated by GPR55. The effects of structurally dissimilar CB1 antagonists, such as taranabant (Addy et al. 2008) or LY320135 (Felder et al. 1998), on GPR55 activity have not been studied, but would be interesting to investigate.

3.3.5 O-1602

O-1602 (see Chap. 2) is an abnormal cannabidiol analog that has been shown to be a GPR55 agonist in a number of studies (Johns et al. 2007; Schuelert and McDougall 2011). However, this compound has clear biological activity in GPR55 knockout mice (Schicho et al. 2011), and potently activates GPR18 (McHugh et al. 2010), so its usefulness in complex biological systems to determine GPR55 involvement is limited. Similarly, the structurally related compound, O-1918, has been claimed to be a selective GPR55 antagonist (Schuelert and McDougall 2011); however, later studies showed that it also potently antagonizes GPR18 (McHugh et al. 2010). These results suggest that abnormal cannabidiol analogs should be used sparingly in studies examining GPR55 signaling in complex systems unless actions at non-GPR55 targets can be specifically excluded.

3.3.6 PEA, Virodhamine, 2-AG, and CP55,940

The endogenous ligands 2-AG, palmitoylethanolamine (PEA), and virodhamine (the ester of arachidonic acid and ethanolamine), and the cannabinoid receptor agonist CP55,940 (see Chap. 2), were all reported to increase GTPγS binding in HEK293 cells expressing GPR55 with quite high potency, and in the case of virodhamine, efficacy (Ryberg et al. 2007). However, subsequent studies have found that these compounds generally do not activate GPR55 to increase intracellular calcium, ERK1/2 phosphorylation, or β-arrestin$_2$ recruitment (Oka et al. 2007; Lauckner et al. 2008; Henstridge et al. 2009; Kapur et al. 2009), suggesting they activate only a few GPR55 signaling pathways and/or they exhibit low efficacy in the expression systems examined in these studies. Determining whether these compounds are physiologically relevant GPR55 agonists will be important as some of them, for example PEA (as an anti-inflammatory agent) have marked biological activities via un- or partially characterized signaling pathways (Calignano et al. 1998).

3.3.7 Cannabidiol

Cannabidiol was identified as a GPR55 antagonist in the receptor's initial characterization (Ryberg et al. 2007). However, the lack of a practical receptor binding assay for GPR55 precludes determination of the affinity of cannabidiol (or any other ligand for that matter) for GPR55. Nonetheless in a functional assay, mid- to high-nanomolar concentrations of cannabidiol were reported to antagonize low nanomolar concentration-CP55,940-stimulated GTPγS binding (Ryberg et al. 2007) and many in vitro studies (e.g., see Whyte et al. 2009) have used cannabidiol as a low affinity GPR55 antagonist. However, cannabidiol clearly interacts with a number of non-GPR55 targets. Thus, like the abnormal cannabidiol analogs, the use of cannabidiol as a GPR55 antagonist in complex biological systems should be viewed skeptically in the absence of comprehensive controls.

3.3.8 Anandamide

Anandamide was initially proposed to be a GPR55 agonist based on GTPγS binding (Ryberg et al. 2007) and calcium release studies (Lauckner et al. 2008). Subsequent examination of this compound in different expression systems failed to confirm these initial results, at least for increases in intracellular calcium (Oka et al. 2007; Henstridge et al. 2009). However, a possible explanation for this discrepancy comes from a study by Markus Waldeck-Weiemair and colleagues who found that if integrins are clustered, CB_1 inhibits GPR55 activation by anandamide (Waldeck-Weiermair et al. 2008). It is possible that this is a general property of Gi-linked receptors in addition to the CB_1 receptors that were the focus of the Waldeck-Weiermair study. Thus, inhibition of GPR55 signaling mediated by Gi-activation of

spleen tyrosine kinase (Syk) culminating in the inhibition of PI3 kinase may be quite wide spread. For example, constitutively activated Gi/o-linked receptors might tonically inhibit GPR55 signaling. Indirect support for this hypothesis comes from the observation that treating GPR55 expressing cells with pertussis toxin to inactivate Gi/o signaling increases the magnitude of GPR55-stimulated calcium transients (J.L., B.H., and K.M., unpublished observations). If this Gi-mediated tonic inhibition (or inhibition via incidentally activated GPCRs in an assay system) is commonplace, then it will greatly increase the complexity of studying GPR55 signaling. However, it will also greatly enrich the flexibility of GPR55 signaling by serving to integrate Gi-coupled GPCR and GPR55 signaling, and thus will be quite interesting to study further.

3.3.9 Tetrahydrocannabinol (THC)

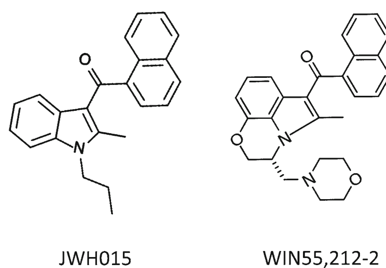
As the prototypical cannabinoid, THC's ability to stimulate GPR55 activation has been widely studied. In the initial characterization of GPR55, THC was found to potently stimulate GTPγS binding (Ryberg et al. 2007). Extension of these results to downstream signaling by GPR55 has yielded conflicting results, with some studies showing activation of various signaling pathways and others showing no activation (Oka et al. 2007; Lauckner et al. 2008; Henstridge et al. 2010; Anavi-Goffer et al. 2012). Whether this is due to differences in experimental systems or other factors remains to be determined.

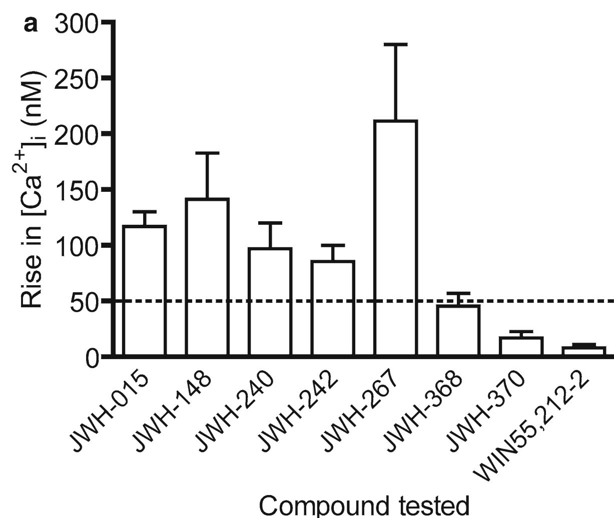
3.3.10 Aminoalkylindoles

The aminoalkylindoles (the CB_1, CB_2 agonist WIN55,212-2 is the best known of this class of compound) offer interesting insights into GPR55 ligand specificity. In the initial characterization of GPR55 as a putative novel cannabinoid receptor, WIN55,212-2 failed to stimulate GTPγS binding (Ryberg et al. 2007). In contrast, the structurally related aminoalkylindole, JWH015, potentially and efficaciously stimulated GTPγS binding (Ryberg et al. 2007). Additional studies have uniformly found that WIN55,212-2 fails to activate GPR55, while other studies support the notion that JWH015 can be a GPR55 agonist, at least for stimulating calcium release (Lauckner et al. 2008). Structurally, the two compounds are identical, except for the modification of the indole ring. JWH015 contains a simple propyl side chain on the nitrogen, while WIN55,212-2 contains a morpholinoethyl group rather than a simple alkyl substituent. A six-membered ring containing an oxygen atom is fused between C7 of the indole and the indole nitrogen (Fig. 3.1).

We took advantage of the distinct differences between these two compounds to develop a SAR among different aminoalkylindoles and their ability to stimulate calcium release. The results were quite striking as shown in Fig. 3.2. Substitutions on the napthoyl moiety were relatively well tolerated (JWH148, JWH240, JWH242,

Fig. 3.1 Comparison of the structures of the aminoalkylindoles JWH015 (*left*) and WIN55,212-2 (*right*)





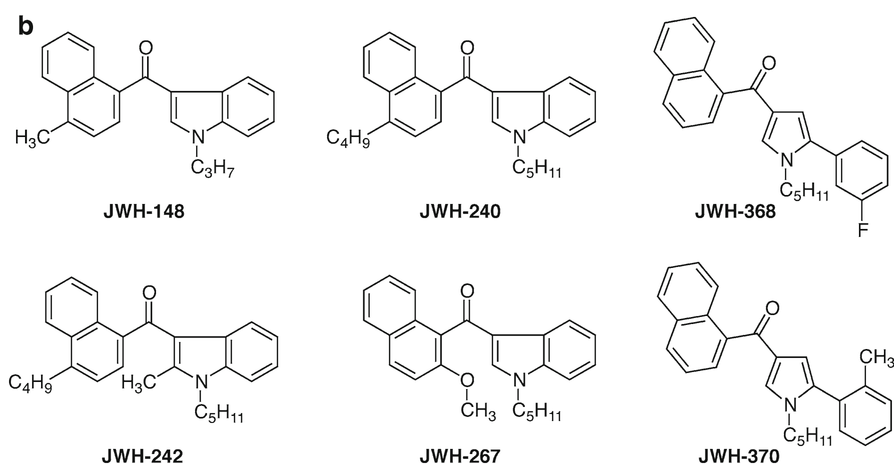


Fig. 3.2 Increase in intracellular calcium by various aminoalkylindoles. (**a**) Modification of the naphthoyl group, varied substitutions on the indole nitrogen, and methylation of C2 on the indole group had minor effects of GPR55 agonism. However, manipulation of the six-carbon aromatic ring of the indole caused a significant decrease in GPR55-mediated calcium signaling. Dotted line corresponds to a 50 nM increase in intracellular calcium. Data are displayed as mean increase in intracellular calcium ± SEM, $N = 3$–8 for each condition. (**b**) Chemical structures of the aminoalkylindoles tested

and JWH267), as was a butyl or propyl alkyl chain on the indole nitrogen (JWH015 and JWH148 vs. JWH240, JWH242, and JWH267). The presence or absence of a methyl group on C2 of the indole also did not produce a major effect on activity (JWH015, JWH242, JWH148 compared to JWH240 and JWH267). In contrast, transforming the indole to a pyrrole with substituted phenyl groups (JWH368 and JWH370) led to an almost complete loss of activity, very similar to WIN55,212-2. Since modifications of the naphthoyl group can strongly diminish binding of aminoalkylindoles to CB_1 or CB_2 receptors (Huffman et al. 2005), the aminoalkylindoles may offer a route for the development of GPR55 agonists specifically lacking activity at CB_1 or CB_2 receptors.

3.3.11 Summary of GPR55 Ligands

From the above discussion, it is clear that GPR55 pharmacology is complicated and conflicting. LPI is clearly the most widely accepted ligand; however as discussed above, even this compound must be used with care. Until more specific ligands are identified, careful experimentation is necessary to draw valid results when using any of these compounds to activate or antagonize GPR55 receptors.

3.4 CNS Effects of GPR55 Activation

Very little has been reported on the effects of GPR55 signaling on neuronal physiology or behavior. A major reason for this is the lack of selective pharmacological tools. However, there is reason to expect this is changing and it is likely that in the near future a number of studies will come out that will help us understand how and where GPR55 participates in CNS physiology and function, as well as its potential role(s) in pathological states. In the sections below we examine the role of GPR55 on neuronal and synaptic function as well as in the development of chronic pain states.

3.4.1 Effects of GPR55 on Neuronal Function

The ability of GPR55 to stimulate Rho-mediated pathways and increase intracellular calcium suggests that it may play a role in cell migration and neuronal excitability. While not much is known about the role of GPR55 in neuronal migration, LPI can stimulate migration in neutrophils and breast cancer cell lines (Ford et al. 2010; Balenga et al. 2011). Emerging evidence supports a role for GPR55 in enhancing neuronal excitability (Lauckner et al. 2008; Jensen et al. 2011; Sylantyev et al. 2011). A variety of agonists, including LPI stimulate GPR55 to increase intracellular calcium in DRG neurons expressing the receptor (Lauckner et al. 2008).

GPR55 activation also inhibits the potassium M-current (a depolarizing effect) in HEK293 cells expressing GPR55 (Lauckner et al. 2008).

A recent report examined the role of GPR55 in neurotransmission in the Schaeffer collateral synapse in acutely prepared brain slices (Jensen et al. 2011; Sylantyev et al. 2011). These investigators found that GPR55 agonists caused a slow release of calcium from intracellular stores, an effect absent in GPR55 KO mice. The increased calcium facilitated synaptic transmission (via an increased in the probability of release) in a completely presynaptic fashion. Interestingly, cannabidiol antagonized the slow increase in terminal calcium accompanying posttetanic potentiation (PTP), consistent with the idea that PTP stimulation causes the synthesis of LPI, which then acts to release calcium from intracellular stores thereby facilitating subsequent neurotransmitter release. It will be interesting to ascertain whether this is a widespread phenomenon, the route(s) of LPI synthesis, and if antagonizing GPR55 underlies some of the behavioral effects of cannabidiol.

3.4.2 Behavioral Effects Mediated by GPR55

As discussed above there is a lack of selective GPR55 agonists and antagonists, and thus it has not been possible to reliably determine their effects on animal behavior. Therefore it has been necessary to infer the functional role of GPR55 from studies examining GPR55 knockout mice. So far, only one paper has been published examining the role of GPR55 in behavior (Staton et al. 2008). This paper reported the initial characterization of a GPR55 null mouse made by deleting amino acids between residues 38 and 282 (corresponding to the first through sixth transmembrane domains). These mice are fertile and do not exhibit gross deficits in the following behaviors—cage behavior, righting reflex, locomotor activity (including rotarod), corneal and pinna reflexes, fear and irritability, tremor, or limb grasping (Staton et al. 2008). In the hotplate test there were no differences at any temperature between wildtype and knockout males, but GPR55 knockout females were more sensitive to less noxious heat (50°C). Interestingly, there was no difference in withdrawal latency between the female genotypes at higher temperatures (52.5 and 55°C). These results suggest that GPR55 may regulate thermal sensitivity thresholds in a sex-dependent fashion.

Considerably greater differences between wildtype and GPR55 KO mice were found in preclinical models of chronic pain (Staton et al. 2008). In this study, two different models were assessed. The first was the Freund's adjuvant test investigating chronic inflammatory pain whereas the second was partial ligation of the sciatic nerve as a neuropathic pain model. In both of these studies elimination of GPR55 function greatly attenuated the development of chronic pain behaviors. If confirmed, these results provide strong suggestive evidence that GPR55 antagonists may have clinical usefulness in preventing or treating chronic pain.

In the inflammatory model, Complete Freund's Adjuvant (CFA) was injected in one hindpaw and mechanical thresholds for paw withdrawal were measured regularly for the next 2 weeks. There were no differences in baseline mechanical thresholds between the two genotypes. Prominent mechanical hyperalgesia developed in the injected paw in the wildtype mice (as expected), but no significant hyperalgesia developed in the GPR55 knockout mice (Staton et al. 2008). Of note, in this experiment female mice were sacrificed 1 day after CFA injection, and only males tested, so it is not possible to draw any conclusions on the role of GPR55 in the development of mechanical hyperalgesia in female mice from this experiment. Ample evidence shows that different nociceptive modalities are subserved by different molecular mechanisms (Caterina et al. 2000; Story and Gereau 2006; Rahn and Hohmann 2009), yet it will be important to carefully evaluate heat and cold hyperalgesia in the GPR55 mice. Paw cytokine levels were measured to determine if the differences in mechanical hyperalgesia might be explained by differences in cytokine expression following CFA administration. One day after injury (female mice) there were no significant differences in cytokine elevations between the two genotypes. However, 2 weeks after injury (male mice), IL-4, IL-10, IFNγ, and GM-CSF all were elevated to a greater extent in the GPR55 knockout mice compared to the wildtype mice. The persistent elevation of IL-4 and IL-10 is intriguing as these cytokines are generally considered anti-inflammatory (Opal and DePalo 2000) and produce analgesia in various preclinical pain models (Verri et al. 2006).

The Seltzer model (Seltzer et al. 1990) of partial sciatic nerve ligation was used to evaluate the development of neuropathic pain in the GPR55 KO mice. Prominent hyperalgesia developed in the paw ipsilateral to the nerve injury in the wildtype mice within 3 days of the surgery and was sustained for at least 4 weeks. However, hyperalgesia failed to develop in the ipsilateral paw at any time during the 4 weeks of observation. In the neuropathic experiment, GPR55 knockouts of both sexes showed identical resistance to the development of neuropathic pain.

The striking disruption by GPR55 deletion of the development of mechanical hyperalgesia in both the CFA and partial nerve ligation models suggests that GPR55 antagonists might be clinically useful in the treatment of chronic pain. However, whether GPR55 facilitates the development of mechanical hyperalgesia via its expression on neuronal, glial, immune cells, or some combination of these, is unknown. The detection of GPR55 on large diameter DRG neurons is interesting as these cells are involved in mediating pathological excitation of spinal nociceptive pathways in various preclinical chronic pain models (Neumann et al. 1996; Ruscheweyh et al. 2007). An intriguing possibility is that GPR55 activation on large diameter DRG neurons is necessary for the development of mechanical hyperalgesia. However, an alternative (though not mutually exclusive) possibility is that GPR55 activation on immune cells (peripheral or microglia) is necessary for the developmental of mechanical hyperalgesia following CFA or nerve ligation. Evidence in support of this notion is the expression of GPR55 in microglia (Pietr et al. 2009) and a variety of peripheral blood cells (Oka et al. 2010; Balenga et al. 2011). No matter its mechanism of action, further study into the role of GPR55 in chronic pain states is certainly warranted.

3.5 Current Status and Future Directions

Our understanding of the function of GPR55 in the CNS at present is quite rudimentary. The study of Staton et al. (2008) strongly suggests that GPR55 plays a role in the establishment of mechanical hyperalgesia following inflammatory or neuropathic insults. It will be important to confirm and extend this report to determine the molecular mechanism(s) involved. The widespread, albeit modest, level of GPR55 expression in the nervous system suggests that it is likely involved in other, as yet unknown behaviors and CNS functions. In the absence of suitable agonists and antagonists, studies investigating CNS activity of GPR55 will have to rely primarily on GPR55 knockout mice. The initial characterization of a GPR55 knockout (Staton et al. 2008) failed to reveal a notable behavioral phenotype; however, it is quite likely that behavioral or neurological phenotypes will emerge with more precise testing. Hopefully, potent and selective agonists and antagonists will soon be developed to complement these knockout studies.

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Chapter 4
The Role of GPR55 in Bone Biology

Lauren S. Whyte and Ruth A. Ross

4.1 Introduction

Cannabinoid receptor pharmacology has been extensively studied over the past 30 years. Many actions of endocannabinoids (2-Arachidonylglycerol (2-AG) and anandamide (AEA)), phytocannabinoids (Δ^{9-}THC) and synthetic cannabinoids (CP 55,940) are mediated by the cannabinoid receptors, CB_1 and/or CB_2. In particular, a clear role for both CB_1 and CB_2 receptors has been demonstrated in the regulation of mouse osteoclast formation and function from the observation that cannabinoid receptor knockout mice display significant bone phenotypes. However, there is considerable evidence to suggest that the effects of certain cannabinoids are mediated through non-CB_1, non-CB_2 receptors (McHugh et al. 2008; Jarai et al. 1999). The following pharmacological evidence substantiates the proposal for such novel cannabinoid receptors.

Several studies have demonstrated non-CB_1/non-CB_2 effects for a range of cannabinoid ligands at a number of sites, including vasculature, central nervous system (CNS) and immune cells (for review, see Begg et al. 2005). The suggestion of a non-CB_1, non-CB_2 cannabinoid receptor is primarily based on the unexplained AEA in cannabinoid receptor knockout mice in which it was shown that anandamide induces vasodilation that was inhibited by the CB_1 antagonist SR141716A at higher

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concentrations than needed to inhibit CB_1 and that persisted in CB_1 and $CB_{1/2}$ double knockout mice (Jarai et al. 1999). It was initially thought that the non-CB_1/non-CB_2 vascular receptor may be the G-protein-coupled receptor, GPR55, as the GPR55 ligand O-1602 induced vasodilatory responses. However, these responses were also seen in $GPR55^{-/-}$ mice showing that the effects of O-1602 in the vasculature are not mediated by GPR55 (Johns et al. 2007).

For many years the suggestion of an elusive third cannabinoid receptor has been rife. Much attention is currently focused on the search for additional cannabinoid receptor members, of which it has been proposed that there are at least three yet to be characterised (Mackie and Stella 2006). GPR55 has recently been identified as one of these additional putative cannabinoid receptors. As CB_1 and CB_2 receptors are both G-protein coupled receptors (GPCR) known to have an effect on bone turnover, the possibility that non-CB_1/non-CB_2 cannabinoid receptors, such as GPR55, are also present in bone could significantly aid in deciphering the true effects of cannabinoid ligands in bone.

4.2 Bone

4.2.1 Function of Bone

Bone is a highly specialised tissue that serves three main functions: mechanical, chemical and haematological. Bone provides structural support, acting as a site of attachment for muscles and protecting vital organs. In its chemical function, bone is a store for calcium and phosphate, helping to maintain ion homeostasis. Bone also aids in the production of blood cells through the marrow contained within the medullary cavity of long bones.

4.2.2 Types of Bone

The skeleton contains five different types of bone, all serving different functions that relate to their characteristic structure. The long bones, such as the tibia and femur, are associated with large movement. These bones have a long cylindrical midshaft called the diaphysis and bony protrusions at either end called the epiphysis. In comparison to long bones, short bones, such as the carpals in the hand, are associated with small movements. Flat bones such as the skull, ribs and pelvis all protect vital organs, and similarly, sesamoid bones such as the patella are small rounded bones that develop in tendons and help to protect them from wear and tear. Bones that do not fit into any of these categories are termed irregular bones and are generally found in sites requiring extra strength such as the vertebrae.

Within these different types of bones are two different types of bone tissue, cortical (compact) and trabecular (cancellous). Long bones are composed of an outer shell of cortical bone, giving bones their hard outer surface, and an inner cavity containing a second type of bone called trabecular bone filled with bone marrow. Trabecular bone, found mainly at the interior ends of long bones, is more porous and less dense than cortical bone giving trabecular bone its characteristic honeycomb appearance. Individual trabeculae can readily adapt and compensate for changes in the amount or distribution of intermittent load deformities by orientating and connecting in a manner that provides maximal structural support. The proportion of each type of bone tissue varies in different parts of the body depending on its specific role. Cortical bone is denser, harder and stiffer than trabecular bone and contributes to around 80% of the weight of the human skeleton, fulfilling a mechanical and protective function. Despite only making up the remaining 20%, trabecular bone is also important for bone strength but mainly serves a metabolic function.

4.2.3 Organisation

Cortical bone is arranged in Haversian systems (osteons) composed of concentric rings of collagen fibres (lamellae) which enclose a central canal, called the Haversian canal, which contains blood vessels and nerves. Trabecular bone is spongy in appearance with lamellae that run parallel rather than concentrically to the bone surface. Trabecular bone has a larger surface area than cortical bone and is more prone to changes in remodelling induced by ageing, disease or therapeutic agents (Fig. 4.1).

4.2.4 Composition

Bone is composed of around 65% mineral and 35% organic matrix making it light weight yet strong. The organic matrix contains type I collagen, giving bone its strength, and lesser amounts of non-collagenous extracellular matrix proteins such as osteocalcin, osteopontin and fibronectin (Young 2003). These extracellular matrix proteins guide specific interactions between bone cells, and together with the collagen fibrils, produce a framework upon which bone mineral is deposited. Bone mineral contains calcium and phosphate chemically arranged as hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$], providing bone with its rigidity (Blair 1998). Bone also contains specialised cells unique to bone that regulate bone turnover. Two of the major cellular elements of bone are the osteoclasts and the osteoblasts. Osteoclasts destroy bone in a process termed resorption, and they work in a highly controlled manner with osteoblasts, the cells responsible for laying down new bone, to regulate bone growth in early development and to maintain bone throughout life.

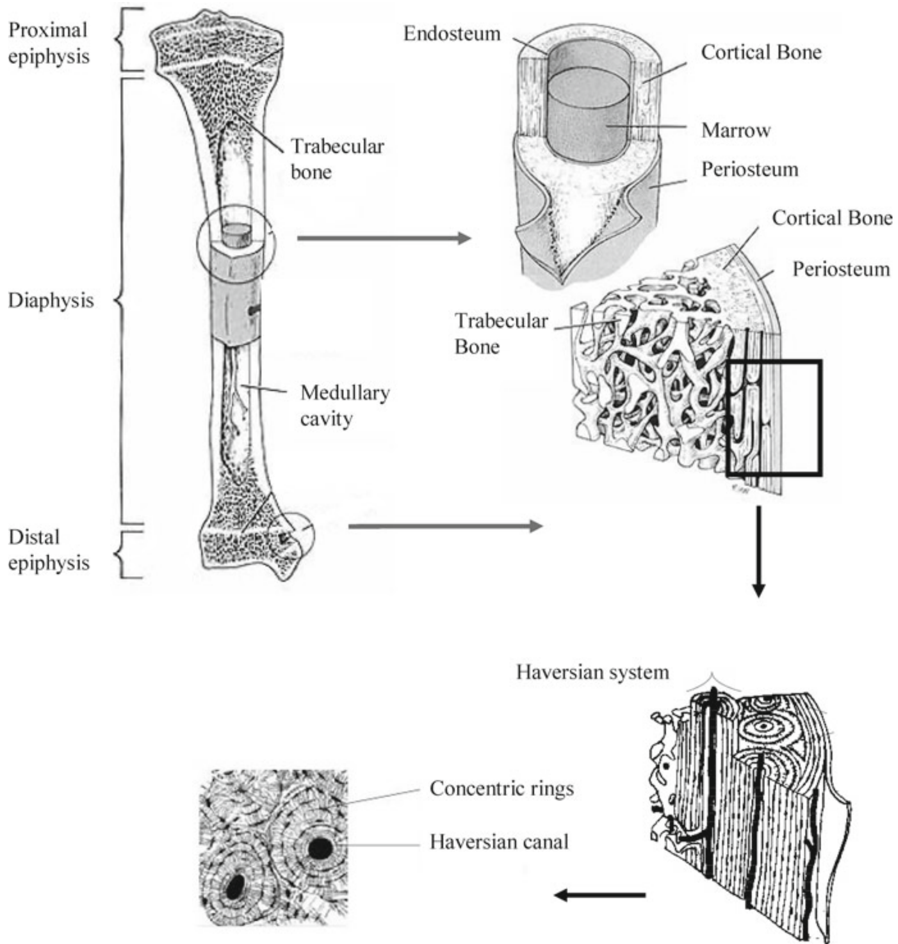

Fig. 4.1 Longitudinal section of long bone showing cortical and trabecular bone with Haversian systems. This image was taken and adapted from Basic Medical Anatomy, by Alexander Spence (1990) and Anatomy and Physiology of Animals (wikibooks—http://en.wikibooks.org/wiki/Anatomy_and_Physiology_of_Animals/The_Skeleton)

4.3 Remodelling

Bone is a highly dynamic tissue that once formed undergoes continuous remodelling through a process that entails the resorption of old bone by the osteoclasts and the formation of new bone by the osteoblasts. It is estimated that at any one time approximately 10% of the skeleton is undergoing active remodelling (Alliston and Derynck 2002). Under normal circumstances, remodelling is a tightly coupled process whereby osteoblasts lay down bone until all of the resorbed bone has been completely replaced in what is described as the basic multicellular unit (BMU). Bone remodelling (Fig. 4.2) is vitally important as it helps to maintain normal bone

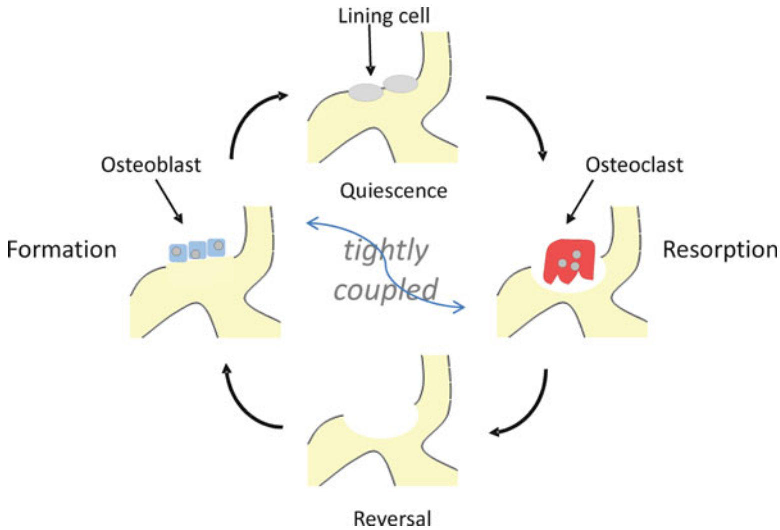

Fig. 4.2 The bone remodelling cycle. Osteoclasts and osteoblasts function in a tightly controlled coupled manner in order to preserve skeletal mass through the process of the bone remodelling cycle. Lining cells cover inactive bone surfaces and bone remodelling starts upon the recruitment of bone marrow macrophages (BMMs) that fuse and differentiate into osteoclasts through direct contact with osteoblasts or their stromal precursor cells that express RANKL. Osteoclasts then adhere to the bone surface and begin the process of resorption—the exact cue(s) to initiate this process, or the existence of such a cue is still the subject of much debate; however, microfractures that accumulate in bone are proposed to initiate bone remodelling in an attempt to preserve healthy bone architecture. Once recruited, osteoclasts then excavate a resorption pit through the secretion of enzymes and acid into a tightly sealed resorption lacuna. In healthy individuals, once the osteoclasts have resorbed an area, mesenchymal cells within the marrow differentiate into osteoblasts and completely replace the bone resorbed by osteoclasts. In conditions such as osteoporosis, osteoclast activity is increased and osteoblast activity cannot compensate resulting in a net negative balance and bone loss

structure by repairing microfractures that accumulate in bone over time. Remodelling also aids in the maintenance of calcium homeostasis (Parfitt 2002). Thus, the concerted actions of the osteoclasts and the osteoblasts are key players in the bone remodelling cycle.

Remodelling is a lengthy process that begins with exposure of the mineralised bone surface by the bone lining cells. This is followed by the recruitment of mononuclear precursors to the resorption site. The mononuclear cells fuse to form osteoclasts that then attach to the bone surface and become activated to resorb. Osteoclasts typically excavate a resorption pit over a period of 7–10 days and, subsequently, undergo apoptosis making way for a team of osteoblasts to then move in and secrete unmineralised bone matrix (collagen) to refill the lacuna over a period of 2–3 months. The mineralisation process is completed by the deposition of calcium and phosphate (hydroxyapatite crystals) between the collagen fibrils to form mature bone (Grey 2007). Imbalances within the bone remodelling cycle can lead to a variety of disease states including Paget's disease, osteoporosis and osteopetrosis (discussed later and summarised in Table 4.1).

Table 4.1 Osteoclast diseases

Disease	Characteristics	Cause	Outcome
Paget's[a]	Increased bone turnover due to hyperactive osteoclasts	Mutations in SQSTM1, RANK, VCP or decreased OPG	Poor quality bones more prone to fracture
Inflammatory bone loss[b]	Uncoupling of bone formation from bone resorption	Autoimmune—increased TNFα release from T cells that promotes RANKL secretion	Localised periarticular bone erosions Systemic bone loss and severe joint damage
Multiple myeloma[c]	Increased osteoclast activity	Upregulation of RANKL and proresorptive cytokines Downregulation of OPG	Lytic lesions and generalised bone loss associated with pathologic fractures
Metastasis, e.g. breast to bone[d]	Increased osteoclast activity	Breast cancer cells secrete factors that increase RANKL Hypocalcaemia promotes tumour growth—vicious cycle	Decreased BMD and increased fracture risk
Osteoporosis (postmenopausal)[e]	Severe bone loss due to increased osteoclast activity	Drop in estrogen results in increased IL1, IL6 and TNFα, which increases RANKL	Decreased BMD results in increased fracture risk
Osteopetrosis[f]	Increased bone density due to lack of osteoclasts or osteoclast dysfunction	Mutations in V-ATPase, CLCN7, CAII, RANK, RANKL and PLEKHM1	Brittle bones that fracture more readily

[a]Helfrich and Hocking (2008)
[b]Cooper (2009), Gallagher (2008), and Kong et al. (1999)
[c]De Leenheer et al. (2004)
[d]Clines and Guise (2008) and Van Poznak and Nadal (2006)
[e]Riggs (2000) and Compston (2009)
[f]Stark and Savarirayan (2009), Ochotny et al. (2006), Besbas et al. (2009), Waguespack et al. (2003), Guerrini et al. (2008), Sobacchi et al. (2007), Del Fattore et al. (2008a), and Borthwick et al. (2003)

4.3.1 Osteoblasts

Osteoblasts are plump, cuboidal cells responsible for the production of bone. Osteoblasts are derived from pluripotent mesenchymal stem cells present in the bone marrow. Osteoblasts form under the influence of growth factors (FGFs)

(Ornitz 2005), bone morphogenic proteins (BMPs) (Yamaguchi et al. 2000) and Wnt proteins (Glass et al. 2005) that induce transcription factors necessary for commitment toward the osteoblast lineage. Runx2 and osterix are transcription factors that control the expression of characteristic osteoblast genes such as osteocalcin, type I collagen and alkaline phosphatase (ALP) (Shui et al. 2003; Nakashima et al. 2002), the latter being one of the earliest markers of the osteoblast phenotype (Lian et al. 2003).

Osteoblasts can differentiate into lining cells, which are flat cells that line inactive bone surfaces where they act as a barrier between the unmineralised osteoid and the extracellular environment. During bone deposition, osteoblasts become trapped in the bone and become osteocytes. Once located within the bone matrix, osteocytes are in a highly favourable anatomical position to detect strain and are thought to act as sensors of mechanical strains created from bending and compressive forces (Cowin et al. 1991; Klein-Nulend et al. 1995).

4.3.2 Osteoclasts

Osteoclasts are fully differentiated, multinucleated cells unique to bone. They are derived from the fusion of mononuclear cells of the haematopoietic lineage. The primary role of osteoclasts is to resorb bone during both normal and pathological bone turnover (Blair et al. 1986). Bone resorption requires homing of osteoclast precursors to the bone surface followed by their differentiation, fusion and migration to resorption sites.

The capacity to form multinucleated osteoclasts capable of resorbing requires the physical contact of osteoclast precursors with the bone matrix as well as contact with osteoblasts/stromal cells. Osteoclasts attach to the extracellular matrix of the bone surface via cell surface $\alpha_v\beta_3$ integrins, also known as the vitronectin receptor (Duong et al. 2000). Vitronectin, osteopontin and bone sialoprotein are extracellular proteins present in the bone matrix that contain RGD (Asp-Gly-Asp) sequences that are recognised by $\alpha_v\beta_3$ integrins (Helfrich et al. 1992; Fisher et al. 1993). Upon exposure to RANKL, $\alpha_v\beta_3$ integrin expression increases. As such, the expression of the vitronectin receptor is a marker of in vitro osteoclast differentiation from bone marrow mononuclear precursor cells. Upon attachment via integrins, osteoclast binding induces intracellular signals that converge on the organisation of the actin cytoskeleton. Upon activation, osteoclasts polarise whereby podosomes fuse to form an actin ring, the characteristic marker of an actively resorbing osteoclast (Ory et al. 2008). The ability of an osteoclast to successfully resorb bone also depends on its ability to synthesise and secrete electrolytes and degradative enzymes (Fig. 4.3). As discussed in the next section, the main communication pathway that acts to control osteoclast formation and function is the RANK/RANKL signalling system.

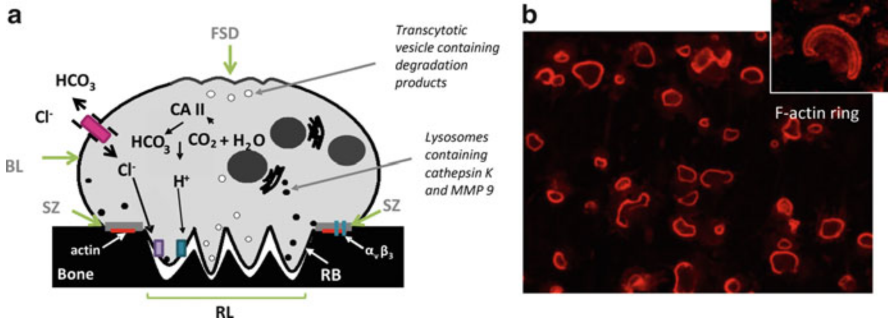

Fig. 4.3 Resorbing osteoclast. (**a**) Actin and the $\alpha_V\beta_3$ integrin facilitate the attachment to bone, forming the sealing zone (SZ). Carbonic anhydrase II (CAII) generates protons and bicarbonate from carbon dioxide and water. Excess bicarbonate is removed through the basolateral membrane (BL) by passive exchange with chloride (*pink box*). Protons are transported through a proton pump (H^+ATPase transporter—*green box*) present in the ruffled border (RB). Chloride channels (ClC-7 -*purple box*) balance the charge of ions across the membrane through coupling with the proton pump. HCl^- is subsequently produced and begins the process of bone demineralisation by acidification of the resorption lacunae (RL). Lysosomes (*black circles*) are inserted into the RB membrane and secrete cathepsin K and MMP9 to degrade the bone matrix. The highly convoluted nature of the membrane serves to increase the surface area through which resorption can occur. Waste products (*white circles*) are transcytosed through the osteoclast and released through the functional secretory domain (FSD). (**b**) Human osteoclasts cultured on dentine and stained for F-actin show the presence of F-actin rings—characteristic cytoskeletal feature of polarised, functionally-active osteoclasts

4.4 Regulation of Osteoclasts

The RANK/RANKL/OPG signalling system, discovered through three seminal papers of the late 1990s, is essential for skeletal homeostasis. Elucidation of this system has been one of the most important advances in bone biology (Anderson et al. 1997; Lacey et al. 1998; Simonet et al. 1997). RANKL (Receptor Activator of NFκB Ligand) is a type II transmembrane protein that is expressed within osteoblasts and bone marrow cells. RANKL binds to its receptor, RANK, located on the surface of osteoclast progenitors and mature osteoclasts to stimulate osteoclast formation and function. RANKL retains its biological activity in a soluble form and associates as a homotrimeric protein upon binding to RANK. Binding to RANK initiates downstream signalling through the NFκB pathway, which is essential for the differentiation, survival and activity of osteoclasts (Fig. 4.4). RANKL promotes osteoclast differentiation and function by inducing the expression of various receptors and enzymes including tartrate-resistant acid phosphatase (TRAP), cathepsin K and the $\alpha_V\beta_3$ integrin and calcitonin receptors (Zhu et al. 2005).

RANKL alone is not sufficient to stimulate osteoclast formation; M-CSF (macrophage colony stimulating factor) is also vital as it promotes precursor cell proliferation and osteoclast survival. M-CSF induces osteoclast survival via ERK (extracellular signal-regulated protein kinase) activation through its tyrosine kinase receptor c-fms. M-CSF also regulates osteoclast function by modifying MITF

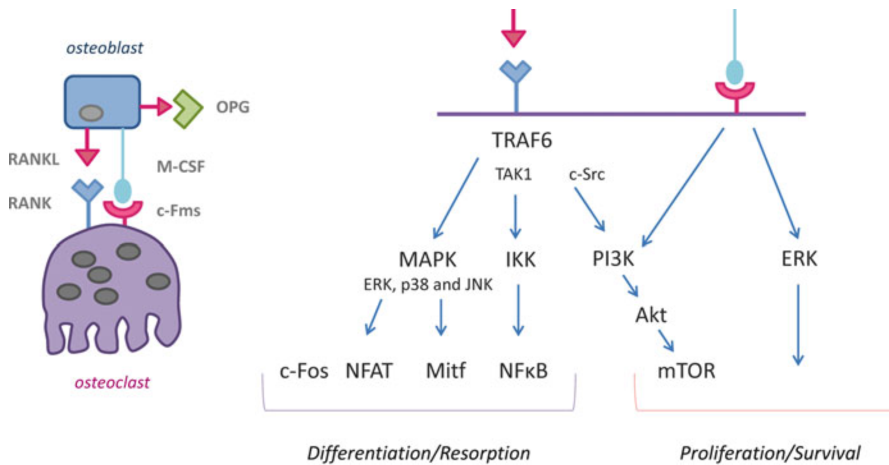

Fig. 4.4 RANK signalling in osteoclasts. Osteoclast differentiation and function requires RANKL and M-CSF which are produced by osteoblasts and bind to RANK and c-Fms receptors present on monocytic precursors and osteoclasts. Binding of RANKL to RANK signals monocytic precursors to stop proliferating and begin the process of differentiation to osteoclasts. Osteoblasts also produce OPG, a soluble decoy receptor for RANK that suppresses osteoclast number and activity. Activation of RANK by RANKL results in a series of downstream signalling events brought about by the recruitment of TRAFs (Tumour necrosis factor Receptor Associated Factors). All three MAPK (mitogen activated protein kinase) pathways are activated in addition to IKK (Inhibitor of Kappa B). These cascades transcend on osteoclastogenic transcription factors that mediate osteoclast terminal differentiation; NFATc1 (nuclear factor-activated T cells), c-Fos, Mitf (Microphthalmia-associated transcription factor) and NFκB (nuclear factor kappa B). Interaction of TRAF6 with c-Src plays a major role in osteoclast resorption through PI3K/Akt pathway. This pathway is also key for osteoclast survival and is activated by M-CSF through c-Fms receptor

expression (Weilbaecher et al. 2001), a transcription factor that activates cathepsin K, TRAP and CLCN7 (chloride channel-7) gene expression (Motyckova et al. 2001; Weilbaecher et al. 2001; Meadows et al. 2007; Luchin et al. 2000). M-CSF-deficient (*op*/*op*) mice develop osteopetrosis, characterised by the absence of osteoclasts, emphasising its importance (Yoshida et al. 1990); however, M-CSF alone is not sufficient to stimulate formation and resorption. A prerequisite of osteoclastogenesis in vivo is osteoblast/stromal cell-mediated production of *both* RANKL and M-CSF.

In addition to being an important source of RANKL and M-CSF, osteoblasts also produce and secrete osteoprotegerin (OPG), a soluble protein that acts as a decoy receptor for RANKL. OPG blocks the interaction between RANK and RANKL, leading to an inhibition of osteoclast differentiation and activation (Lacey et al. 1998).

Mutations in OPG, RANKL and RANK give rise to human diseases as demonstrated in animal models. $OPG^{-/-}$ mice develop osteoporosis due to the unopposed action of RANKL (Simonet et al. 1997), and $RANK^{-/-}$ or $RANKL^{-/-}$ mice develop osteopetrosis (Dougall et al. 1999; Odgren et al. 2003), highlighting the importance of these key components in a vitally important signalling pathway in bone.

4.4.1 Control of Osteoclasts

Osteoclasts are supported locally by cells of the osteoblast lineage through the production of RANKL and M-CSF, and are rapidly influenced, both directly and indirectly, by cytokines, hormones and central pathways emanating from the brain. As a result, in vivo, osteoclastogenesis is the sum of all activating and inhibitory signals (Ross 2000).

4.4.1.1 Immune Control

Osteoimmunology is a term used to account for the interplay between the adaptive immune system and bone metabolism (Arron and Choi 2000). Alterations in hormones and pro-inflammatory cytokine levels generally regulate osteoclasts, mostly by indirectly regulating the expression of RANKL and M-CSF by osteoblasts. Most cytokines that regulate osteoclast differentiation are produced by osteoblast/stromal cells within the bone microenvironment, further emphasising the key role of these cells in osteoclast differentiation. In addition, T cells and B cells produce a variety of cytokines that promote and inhibit osteoclast formation.

The pro-inflammatory cytokines interleukin-1 (IL-1), IL-6, tumour necrosis factor-α (TNFα) and prostaglandin E2 [PGE_2] increase bone resorption mainly by increasing the pool of osteoclast precursors in the bone marrow. TNFα, IL-1 and IL-6 increase RANKL and OPG expression by osteoblasts, with a dominant outcome being a net increase in RANKL. Other factors do the opposite and act to decrease RANKL and increase OPG levels, one example being transforming growth factor-β (TGFβ). Interferons (IFN) are potent inhibitors of osteoclast function but also stimulate osteoclast function. IFNγ secreted by T cells, negatively regulates RANK signalling through TNF receptor associated factor-6 (TRAF6) degradation thereby inhibiting osteoclastogenesis (Takayanagi et al. 2000) but has also been shown to stimulate osteoclast formation and bone loss through activation of T cells (Gao et al. 2007). IFNβ production by precursor cells is induced by RANKL and acts to inhibit osteoclastogenesis, a potential osteoclast suppressor through an autoregulatory loop (Del Fattore et al. 2008b).

4.4.1.2 Hormonal Control

In addition to autocrine/paracrine control, endocrine and systemic regulators also play a role in bone remodelling. One of the most important regulators of bone remodelling in women is estrogen. Estrogens have a negative impact on osteoclast differentiation and a positive impact on osteoblast differentiation. Estrogens act to inhibit osteoclast resorption indirectly via regulation of cytokines from monocytes, stromal cells and osteoblasts. Estrogen downregulates expression of potent stimulators of

osteoclast formation/activity such as IL-1, IL-6, TNFα and PGE_2 and upregulates the expression of the osteoclast inhibitor TGFβ (Riggs 2000). Estrogen also suppresses RANKL production by osteoblasts and increases OPG expression which gives rise to anabolic effects through decreased osteoclast activity (Hofbauer et al. 1999; Syed and Khosla 2005; Compston 2001). As such, estrogen deficiency after menopause leads to catabolic effects through increased osteoclast differentiation and activation.

Parathyroid hormone (PTH) indirectly increases bone resorption in order to increase circulating calcium levels (Martin and Udagawa 1998). PTH is a potent stimulator of RANKL expression, and an inhibitor of OPG expression (Lee and Lorenzo 1999), and increases IL-1 and IL-6 production by osteoblasts.

4.4.1.3 Central Control

The concept that the brain regulates bone mass was first brought to light when leptin, a hormone secreted by adipocytes, was shown to have an inhibitory role on bone formation, acting through its receptor in the hypothalamus. This led to a new field concerned with the central control of bone. Leptin is a small polypeptide released by adipocytes which signals through the hypothalamus to reduce food intake (Ducy et al. 2000). In addition to regulating food intake, leptin positively regulates sympathetic β adrenergic receptor-mediated inhibition of bone formation as demonstrated by the high bone mass phenotype of leptin-deficient ob/ob mice due to increased bone formation (Takeda et al. 2002). This phenotype is normalised by intracerebroventricular (ICV) infusion of leptin, showing that leptin regulates bone mass through a central relay (Ducy et al. 2000). This effect was later shown to be mediated through the sympathetic nervous system signalling though the β adrenergic receptor on osteoblasts as leptin ICV infusion in $\beta_2^{-/-}$ mice resulted in decreased fat mass but had no effect on bone (Takeda et al. 2002). Overall the results from studies so far have shown that the main cell type in bone that is responsive to sympathetic signalling is the osteoblast. Osteoblasts express the β_2 adrenergic receptor and are responsive to sympathetic tone, which results in inhibition of osteoblast proliferation and function (Takeda et al. 2002).

The discovery of leptin driving the central control of bone was followed by the observations that other neuropeptides also regulate bone; these include Neuropeptide Y (NPY), cocaine- and amphetamine-regulated transcript (CART) and Neuromedin U (NMU). Remarkably, these three neuropeptides are also involved in food intake and energy expenditure (Takeda 2008). Signals that emanate from the brain to bone could be attractive therapeutic targets, particularly those that act to increase bone formation as there is a current lack of anabolic therapies (Takeda 2008).

Defective leptin signalling is also associated with increased levels of endogenous cannabinoids (Di Marzo et al. 2001). When it was first discovered that leptin inhibits bone formation (Ducy et al. 2000), this brought to light the concept that bone remodelling is subject to central controls and implied that endocannabinoids, which are present in the hypothalamus along with the CB_1 receptor, may affect bone metabolism

via the CNS, analogous to leptin. Between 2005 and 2006, three research groups published the first papers studying such associations between the endocannabinoid system and bone (Idris et al. 2005; Ofek et al. 2006; Karsak et al. 2005).

4.5 Osteoporosis

With improvements in health care and nutrition, diseases of the elderly are becoming more common and produce a significant financial strain on the national health system. Osteoporosis is a bone disease that affects around three million people in the UK, with one in two women and one in five men over the age of 50 predicted to suffer an osteoporotic-related fracture in their lifetime.

Osteoporosis is a progressive bone disorder characterised by severe bone loss attributable to increased osteoclast activity. The rate of bone formation does not match that of bone loss, resulting in an overall decrease in bone mass which together with alterations in the bone architecture, the combination of which has deleterious effects on bone strength leading to increased fracture risk (Compston 2009). Osteoclasts derived from peripheral blood of osteoporosis patients show increased resorptive capacity and although osteoblast activity increases due to the coupling between these two cells, the osteoblasts cannot fully compensate for the increase in resorption producing a negative balance and subsequent bone loss (Reid 2008; as depicted in Fig. 4.5).

Osteoporosis is an asymptomatic condition that becomes symptomatic after one or more fractures and is a major cause of morbidity and mortality in the elderly. In humans, bone mass peaks around the beginning of the third decade of life. Up to this point, the overall accrual of bone mass is a very important determinant of bone health later in life (Specker and Schoenau 2005). Men and women begin to lose bone in their fifth decade (Compston 2009) and sex hormones play a key role in the bone health of both men and women. In particular for women, estrogen plays an important role in the suppression of osteoclast resorption by decreasing circulating levels of RANKL and decreasing IL-1, IL-6 and TNFα production by bone marrow stromal and mononuclear cells (Riggs 2000). Bone loss is exacerbated in women during menopause as a result of the dramatic drop in estrogen levels which removes the restraint on osteoclasts and results in excessive osteoclast activity (postmenopausal osteoporosis). Lifestyle factors such as physical activity and vitamin D status together with genetic factors also play a role in the pathogenesis of osteoporosis (Compston 2009).

With the current demographic changes, fracture rates are set to rise considerably in the next decade. Common fracture sites in the elderly are the hip and the spine. A number of treatment options exist for osteoporosis, with bisphosphonates being the main line, the so-called gold standard, treatment option. Second-line treatments for patients who are unable to tolerate or simply unresponsive to first line methods are also widely used. Regardless of cause, osteoporosis is associated with increased

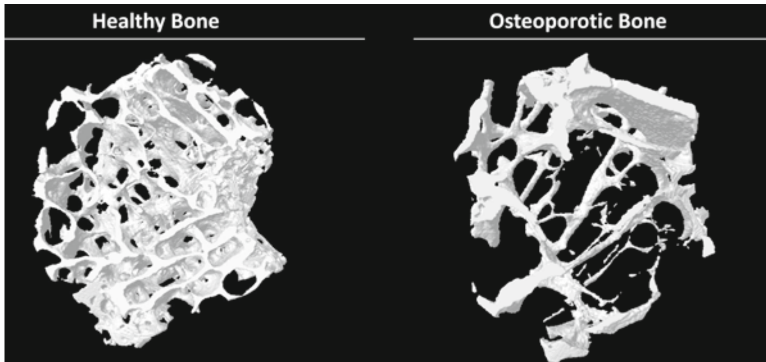

Fig. 4.5 Representative images of trabecular bone loss in osteoporosis. Microcomputed tomographic images of trabecular bone architecture (mouse femur) demonstrating phenotypic changes associated with an osteoporotic bone phenotype—overall decrease in percentage bone volume, decreased number of trabeculae, trabecular thinning and a transition from a plate to rod-like structure

osteoclast resorption, making pharmacological arrest of this unique cell the obvious therapeutic target for bone loss with anti-resorptive agents. In addition to osteoporosis there are a number of other bone diseases attributed to alterations in osteoclast formation or function, which are summarised in Table 4.1.

4.5.1 *Current Treatments for Osteoporosis*

Anti-resorptive agents such as bisphosphonates classically work in one of three ways: (1) inhibit osteoclastogenesis, (2) inhibit resorption or (3) induce apoptosis.

Bisphosphonates are the mainstay treatment for osteoporosis (Boonen et al. 2008). Bisphosphonates target specifically to bone and are internalised by the only cell that resorbs bone, the osteoclast, where they then inhibit bone resorption. There are two different types of bisphosphonates which inhibit osteoclasts in different ways (see Table 4.2) to produce the same overall effect. The reduction in resorption with bisphosphonate treatment restores the balance such that bone formation matches bone resorption. By restoring the balance this helps to preserve and improve not just bone density but also bone microarchitecture, which together increases bone strength. Bisphosphonates are the recommended first-line treatment for postmenopausal and glucocorticoid-induced osteoporosis, but it is important that in patients where bisphosphonates are not well tolerated or contraindicated, that other treatment options are available. Other treatment options include the SERM (selective estrogen receptor modulator) Raloxifene, Salmon Calcitonin, PTH (Teriparatide) and Strontium Ranelate (Protelos), the mechanism of action of these drugs is described in Table 4.2.

Table 4.2 Treatment options for osteoporosis

	Class	Mechanism	Outcome
SERM—Raloxifene	Anti-resorptive	Decreases IL6 and TNFα production by osteoblasts[a]	Inhibits osteoclast formation and function
Calcitonin	Anti-resorptive	Disrupts actin rings and osteoclast polarity through cAMP-PKA[b]	Inhibits osteoclast function *but not* formation
PTH—Teriparatide	Anabolic	Enhances BMP signalling[c]	Increases bone formation—when given intermittently
Strontium Ranelate	Dual acting bone antigen	Increases OPG production by osteoblasts[d]	Increases bone formation and decreases resorption
Nitrogen containing bisphosphonates	Anti-resorptive	Inhibits FDPS in osteoclast which decreases small GTPase prenylation[e]	Inhibits osteoclast function and induces apoptosis
Non-nitrogen containing bisphosphonates	Anti-resorptive	Interferes with ATP supply of osteoclast[e]	Induces osteoclast apoptosis

[a]Gianni et al. (2004)
[b]Yang and Kream (2008)
[c]Chan et al. (2003)
[d]Brennan et al. (2009)
[e]Coxon et al. (2006)

4.5.2 New Treatments

Most anti-resorptive agents have little effectiveness at actually increasing bone density; a 10% increase during long-term therapy is possibly the best that can be achieved due to the overall rate of remodelling upon inhibition of osteoclast function. Anabolic drugs may produce greater increases in bone density and strength (Grey 2007). Ideally, a new treatment that can increase osteoblast function whilst decreasing osteoclast function would provide the best of both worlds. Alternatively, combination therapies with two anti-resorptive drugs, each with different modes of action may provide supra-additive suppressive effects to increase bone mass, or combinations of anabolic therapies such as PTH together with an anti-resorptive agent, may prove beneficial compared to either alone (as a way to expand the anabolic window) (Bilezikian 2008; Epstein 2006). Such studies and comparisons are currently underway. The future holds much promise for the management of osteoporosis, with a plethora of agents currently under investigation that target specific pathways and proteins.

4.5.3 Osteoclast Targets

The discovery of the RANKL/RANK/OPG system has provided a target for the development of drugs to treat bone loss. Denosumab is a monoclonal antibody to RANKL. Results from phase I and II clinical trials show that the prospect looks bright for denosumab as this anti-RANKL therapy reduces markers of bone resorption by 90% (Lewiecki 2009). Other osteoclast targets include those involved in the secretion of acid (chloride channel inhibitors), cell polarisation (c-src inhibitors), attachment ($\alpha_v\beta_3$ antagonists) or proteolytic enzymes involved in the degradation of bone (cathepsin K inhibitors) (Epstein 2006). The advantage of c-src and cathepsin K inhibitors is that they appear to inhibit bone resorption without decreasing formation, hence uncoupling resorption from formation, similar to strontium, a dual acting bone agent, producing a lasting increase in bone density (Grey 2007). Agents targeting osteoblast pathways are also under development as novel anabolic agents. In particular, Wnt signalling through low density lipoprotein receptor-related protein-5 (LRP5) by sclerostin antagonism is of particular interest across the field (Hoeppner et al. 2009). Another interesting target still under development is the calcium sensing receptor in the parathyroid gland that controls PTH production. Low calcium levels increase endogenous PTH release hence antagonism of the receptor with short acting antagonists, the so-called calcilytics, might stimulate short-term endogenous PTH production similar to intermittent dosing with Teriparatide, the N-terminal peptide of PTH (Gowen et al. 2000). Anti-TNF therapies such as Infliximab, indicated for the treatment of rheumatoid arthritis, may hold promise as a new category of treatment for osteoporosis. Infliximab is a monoclonal antibody that binds to TNFα and neutralises its biological activity. Patients with inflammatory conditions taking anti-TNF therapy show decreased bone turnover markers of resorption and increased hip and spine bone mineral density. Raloxifene, an SERM, inhibits osteoclast activity by reducing TNFα synthesis (Gianni et al. 2004), further demonstrating the potential of targeting this inflammatory cytokine. In addition, animal studies found that TNFα knock-out mice did not lose bone after ovariectomy (Roggia et al. 2001).

Despite showing great potential in the treatment of osteoporosis, there is still a need for expansion and diversification of drug therapies to treat this disease. Anti-resorptive agents are limited in their efficacy in that even the most potent anti-resorptive can only be expected to produce a 50% reduction in fracture rate as these compounds have little ability to improve bone density (Grey 2007). The ideal agent to revolutionise the treatment of osteoporosis would be a safe, effective and affordable anabolic agent, but it seems realistic to assume that anti-resorptive agents used in combination or sequentially with anabolic agents is the greater likelihood for improving treatment in the near future. Anti-resorptive agents that inhibit osteoclast function but not formation would be ideal as this would result in a continually positive net effect. The coupling with osteoblasts would mean that bone formation is maintained as opposed to the scenario where drugs that induce osteoclast apoptosis result in an overall reduction in bone turnover and have limited effects on increasing bone density. One promising new target is the cannabinoid system.

The past two decades have seen a major surge in cannabinoid research. The initial characterisation of the cannabinoid receptors CB_1 and CB_2 in the early 1990s was a significant landmark in the cannabinoid field. This discovery aided in the characterisation of key components of the endocannabinoid system as well as the development of CB_1 and CB_2 selective compounds. Increased scientific interest within the cannabinoid field has resulted in rapid developments characterising therapeutic targets within different human tissues and functions, including bone. Clinically there are certain commonalities between the cannabinoid and bone fields. Increased appetite is one of the common side effects of cannabis use, and it is known that central factors that control food intake such as leptin regulate bone turnover (Elefteriou et al. 2005). The finding that bone can be controlled centrally by leptin suggested that bone mass may be regulated by a variety of neuromodulators such as cannabinoids. Indeed, several studies have now demonstrated a role for the endocannabinoid system in bone physiology, since mice lacking the cannabinoid receptors CB_1 or CB_2 have abnormal bone phenotypes (Idris et al. 2005; Ofek et al. 2006).

Regulation of bone mass is clinically important and treatments for diseases such as osteoporosis commonly target the cells responsible for bone resorption, the osteoclast. These cells, as well as others in the bone environment, have been shown to be modulated by cannabinoid ligands where both cannabinoid receptors have been implicated in the regulation of bone mass and ovariectomy-induced bone loss (Idris et al. 2005, 2008; Tam et al. 2008; Ofek et al. 2006). Modulation of CB_2 is thought to have direct effects on bone as CB_2 is expressed by bone cells. CB_1, however, is only lowly expressed by bone cells and although some studies, including a recent publication by Whyte et al. (2011) have shown that CB_1 ligands have direct effects on osteoclasts in vitro, previous studies found that CB_1 also mediates effects on bone through a central component (Tam et al. 2008). CB_1 is the most abundant GPCR in the brain and is thought to control osteoblast function by negatively regulating norepinephrine release from sympathetic nerve terminals in the bone and thus elevating the suppression induced on bone by norepinephrine at β_2 adrenergic receptors present on osteoblasts. It is therefore likely that a combination of local and central events work together to produce the overall bone phenotype observed in these mice.

These studies suggest a major role for the endocannabinoid system in bone; however, they also require further investigation in human bone cells with only two studies to date utilising human bone cells to study the effects of cannabinoids in vitro (Rossi et al. 2009; Whyte et al. 2011). The search for novel cannabinoid receptors in bone devoid of psychoactive effects, such as GPR55, would further launch this field as a new specific target for the treatment for bone loss.

Before 2009, no link had been established in any of the published literature or company patents between GPR55 and bone. In light of current literature reporting a role for both CB_1 and CB_2 cannabinoid receptors in the control of bone mass, this raised the possibility that along with CB_1 and CB_2, GPR55 may be expressed in osteoclasts, and ligands targeting this receptor may have direct effects on osteoclast activity in vitro.

4.6 GPR55

As part of a continuing search for novel genes encoding G protein-coupled receptors, GPR55 was first discovered in silico and cloned in 1999. GPR55 is a 319 amino acid protein with the characteristic seven transmembrane spanning domains ubiquitous to all GPCRs. GPR55 is located on chromosome 2q37 and is expressed in certain areas of the brain such as the hippocampus, hypothalamus, cerebellum and frontal cortex (Sawzdargo et al. 1999). Levels of GPR55 in the brain are significantly lower than that of CB_1 (Ryberg et al. 2007), the most widely expressed GPCR in the brain (Herkenham et al. 1991). GPR55 is also expressed in the adrenals, spleen, lung, liver, kidney, bladder, uterus, adipose tissue and throughout the gastrointestinal system, therefore similar to CB_1, GPR55 is thought to be widely expressed (Ryberg et al. 2007; Pertwee 2007). More recently GPR55 expression was confirmed in bone (Whyte et al. 2009).

GPR55 is only 13.5% similar to CB_1 and 14.4% similar to CB_2 (Joost and Methner 2002), which is rather surprising given that GPR55 has affinity for endogenous, natural and synthetic cannabinoid ligands (Brown 2007). Company patents were the first to establish a link between cannabinoids and GPR55 as they showed that GPR55 could bind a variety of established CB_1/CB_2 cannabinoid receptor ligands including 2-arachidonoylglycerol (2-AG), anandamide (AEA), Δ^9THC and CP 55,940, findings that were later supported by Ryberg et al. (2007). Together these studies pointed towards GPR55 as being, albeit not structurally but pharmacologically, a cannabinoid receptor. GPR55 is also activated by compounds with low affinity for CB_1 and CB_2 receptors (>30 μM), including the phytocannabinoid-like compound O-1602 ($EC_{50}=13$ nM) [agonist] and the non-psychoactive cannabis constituent cannabidiol (CBD, $IC_{50}=445$ nM) [antagonist] (Ryberg et al. 2007). These compounds are therefore useful pharmacological tools to elucidate the role of GPR55, distinct from CB_1 and CB_2.

In the last 5 years, the pharmacology of ligands at GPR55 has been studied in further depth and although GPR55 has been shown to bind the endogenous cannabinoids 2-AG and AEA, the ability of these compounds to activate GPR55 is controversial. Not all studies observe GPR55-mediated signalling events in response to 2-AG and AEA (Oka et al. 2007; Henstridge et al. 2009; Lauckner et al. 2008), and those studies that do show low potency by these compounds in the micromolar range (Ryberg et al. 2007; Lauckner et al. 2008; Waldeck-Weiermair et al. 2008) are occurring at concentrations well above that needed to activate the CB_1 receptor (Ross 2009). This introduced the possibility that perhaps 2-AG and AEA were not the true natural ligands for GPR55, posing the question—*what is the endogenous ligand for GPR55?*

In addition to O-1602 and CBD, GPR55 has also been shown to be activated by the bioactive lipid, L-α-lysophosphatidylinositol (LPI) (Oka et al. 2007; Henstridge et al. 2009). Lysophosphatidylinositol is a membrane-derived phospholipid that has often been described as the forgotten by-product of phospholipid metabolism—this is surprising given that over 25 years ago LPI was shown to have a potential

signalling role in its own right, stimulating insulin release from pancreatic cells (Metz 1986). Some years later, k-ras-transformed epithelial cells were shown to produce elevated levels of LPI, signifying a potential role in cancer (Falasca and Corda 1994). It took almost another decade before its role in cancer was further highlighted when it was found that levels of LPI were elevated in patients with ovarian cancer, suggesting that LPI may be a suitable biomarker for certain types of cancer (Sutphen et al. 2004; Xiao et al. 2001). At the time of these studies, the pharmacological target for LPI was not known, this perhaps halting any significant progression in determining the role of LPI in cancer. Characterisation of GPR55 as the receptor for LPI (Oka et al. 2007) has justifiably reignited interest in LPI as a potential driver/biomarker for cancer. Recent studies have now shown LPI increases cancer cell proliferation (Pineiro et al. 2011) and migration (Ford et al. 2010) via GPR55. It is important to note that non-GPR55-mediated effects of LPI have been reported whereby LPI increases prostate cancer migration through TRPV2 receptor activation (Monet et al. 2009). Similarly LPI induces effects in endothelial cells that are only partially GPR55 mediated (Bondarenko et al. 2010). For this reason the use of GPR55$^{-/-}$ mice is key to determining the pharmacological characteristics of GPR55.

The physiological role(s) of GPR55 remains largely unknown but due to the wide receptor distribution GPR55 is likely to have wide-ranging therapeutic potential. The current literature points towards effects at GPR55 that are both ligand and tissue specific. GPR55$^{-/-}$ mice have been used by four groups to investigate the role of this receptor. The first study aimed to shed light on a potential role for GPR55 in the vasculature, where the “atypical” cannabinoid O-1602 had previously been shown to induce a vasodilator response that was not mediated by either CB_1 or CB_2 (Jarai et al. 1999). However, the haemodynamic response (lowering of blood pressure) with O-1602 was not significantly different between wildtype and GPR55$^{-/-}$ mice, showing that the vasodilatory effect of this compound was not mediated through GPR55 (Johns et al. 2007). The second study demonstrated a physiological role for GPR55 is in the treatment of pain (Staton et al. 2008). GPR55$^{-/-}$ mice do not develop mechanical hyperalgesia in both inflammatory and neuropathic pain. It is thought that GPR55 agonists increase intracellular calcium in neurons which may serve to increase neuronal excitability and so targeting GPR55 may be a novel way to reduce pain which can be notoriously difficult to treat. In a third study a high bone phenotype was characterised in GPR55$^{-/-}$ mice due to an osteoclast defect (Whyte et al. 2009). This study will be discussed in greater depth in the remainder of this chapter. Most recently insulin secretion was found to be increased in the presence of O-1602, an effect that was blunted in GPR55$^{-/-}$ mice, suggesting that GPR55 may play a role in glucose homeostasis (Romero-Zerbo et al. 2011). Together these studies indicate that GPR55 may represent a therapeutic target in the management of neuropathic/inflammatory pain, osteoporosis and diabetes. The role for GPR55 in cancer has yet to be investigated in GPR55$^{-/-}$ mice; such studies are key in defining a role for GPR55-LPI in cancer.

4.6.1 GPR55 Downstream Signalling

GPR55 pharmacology remains a contentious issue, with little consensus among reports in the ability of the endocannabinoids 2-AG and anandamide to activate GPR55. However, the picture is less contentious for the endogenous lipid LPI. Unlike other cannabinoids, in all studies examining the effects of LPI at GPR55, LPI has demonstrated responses (for review, see Ross 2009, 2011). Current studies investigating the pharmacology of this endogenous lipid at GPR55 have shown responses that are absent or diminished in untransfected, siRNA knockdown or GPR55$^{-/-}$ experiments, signifying that LPI may be the endogenous ligand for GPR55 (Oka et al. 2007; Henstridge et al. 2009; Waldeck-Weiermair et al. 2008). The pharmacology of LPI at CB_1 and CB_2 receptors is, as of yet, unknown.

The downstream signalling mechanisms of GPR55 have been identified and are different to that of CB_1 and CB_2. A patent from GlaxoSmithKline initially suggested coupling of GPR55 with $G_{\alpha12}$ and $G_{\alpha13.}$ These G protein subunits are linked to Rho activation via p115Rho GEF, which facilitates GDP release and subsequent GTP binding (Hart et al. 1998). GPR55 coupling through $G_{\alpha13}$ to Rho was verified by Ryberg et al. (2007) where the GPR55 agonist O-1602 activated RhoA in GPR55 transfected cells, a response antagonised in the presence of CBD. Activation of GPR55 induces several downstream signalling events: increases phosphorylation of ERK, p38 and Akt, activates Rho and ROCK, increases levels of intracellular calcium via PLC activation and activates transcription factors NFAT and NFκB (Ryberg et al. 2007; Oka et al. 2009, 2010; Lauckner et al. 2008; Waldeck-Weiermair et al. 2008; Henstridge et al. 2009; Whyte et al. 2009; Pineiro et al. 2011; Sharir and Abood 2010). Most recently, GPR55-mediated changes in cell conductance and membrane potential in the presence of GPR55 ligands (Bondarenko et al. 2010), and GPR55 interactions with the GPCR-associated sorting protein 1 (GASP-1) (Kargl et al. 2012) and β-arrestin (Kapur et al. 2009), add to the emerging picture of GPR55 signalling. For a comprehensive review of GPR55 pharmacology, signalling and (patho)physiology in cell types other than bone, please refer to Henstridge et al. (2011). This chapter will now focus on the only study to investigate the role of GPR55 in bone.

4.7 GPR55 and Bone

The aim of a study by Whyte et al. (2009) was to examine GPR55 expression in osteoclasts and osteoblasts and its role in regulating osteoclast and osteoblast function in vitro. In addition, they also characterised the bones from GPR55$^{-/-}$ mice in order to determine whether the loss of GPR55 results in a bone phenotype in vivo.

4.7.1 Expression of GPR55 in Bone Cells

GPR55 has been shown previously to be expressed in brain, spleen, intestine, testis and breast adipose tissue (Baker et al. 2006). Using a combination of approaches including western blotting, immunocytochemistry and quantitative PCR, Whyte et al. (2009) demonstrate that both human and mouse osteoclasts and osteoblasts express GPR55. GPR55 mRNA is present in human monocytic osteoclast precursors and expression increases during the differentiation of monocytes into mature, multinucleated osteoclasts in the presence of RANKL. In line with an increase in GPR55 mRNA, levels of GPR55 protein were also significantly increased during osteoclast differentiation (Fig. 4.6). This increase in GPR55 mRNA suggests that GPR55 has a role in regulating the terminal differentiation and activation of osteoclasts but may not be as important during the initial stages of osteoclast differentiation. Staining for GPR55 was absent in GPR55$^{-/-}$ mouse osteoclasts.

The control of bone via CB_1 is thought to have a strong central component. Several studies suggest a role for sympathetic signalling via CB_1 in the control of bone remodelling and bone mass (Tam et al. 2006, 2008). GPR55 is expressed in the spinal cord, hippocampus, frontal cortex, cerebellum, striatum, brain stem and in the hypothalamus, although levels of GPR55 in these brain regions are more than tenfold lower than that of CB_1. In particular in the hypothalamus, levels of GPR55 are 0.3% of CB_1 (Ryberg et al. 2007) so it seems unlikely that a strong central component is involved in the regulation of bone mass by GPR55. However at present this remains to be further investigated.

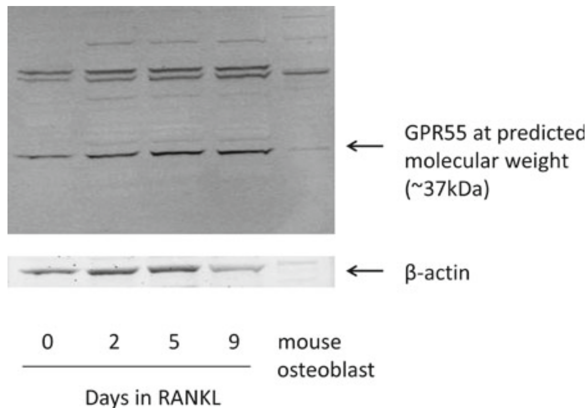

Fig. 4.6 GPR55 receptor protein expression in human monocytes, human osteoclasts and mouse osteoblasts. Cell lysates were prepared and equal amounts of protein were electrophoresed on polyacrylamide-SDS gels, immobilised to PVDF membranes by western blotting. Membranes were incubated with an antibody for human GPR55 and protein detected using the LI-COR Odyssey Infrared imaging system. GPR55 expression is shown in M-CSF-dependent monocytes (day 0) and osteoclasts formed in the presence of RANKL (isolated at days 2, 5 and 9). GPR55 antibody courtesy of Prof Ken Mackie, Indiana University, Bloomington

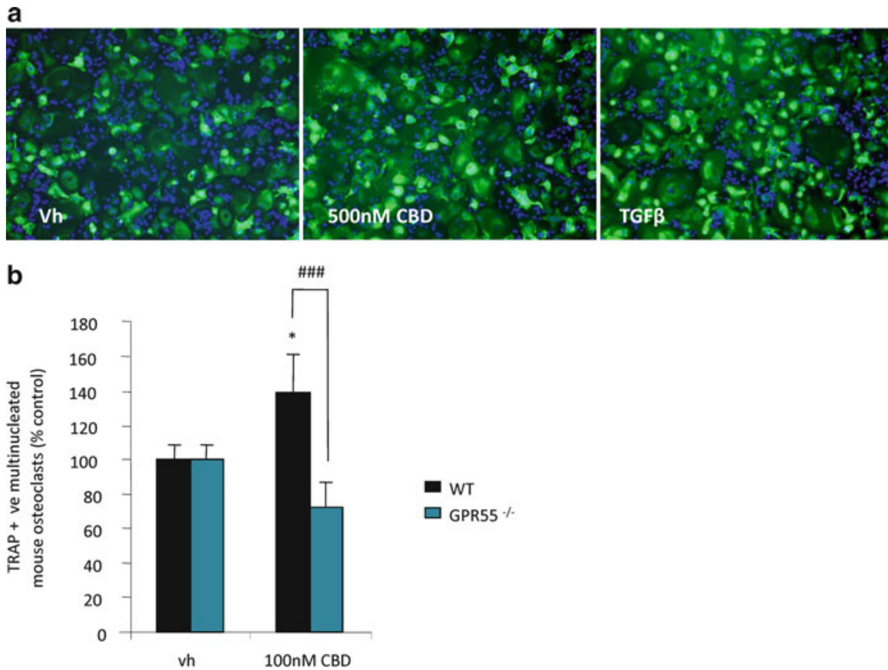

Fig. 4.7 CBD increases human and mouse osteoclast formation—GPR55 mediated. (**a**) Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of vehicle (control), 10 ng/mL TGFβ (positive control) or 1 μM cannabidiol (CBD) for 7 days. Representative images of vitronectin receptor (αvβ3 integrin highly expressed by osteoclasts) stained osteoclasts in *green* with nuclear staining in *blue* (×10 magnification). (**b**) M-CSF-dependent BMMs from wildtype (WT) and $GPR55^{-/-}$ mice were cultured with M-CSF and RANKL in the presence of vehicle or 100 nM CBD for 5 days then fixed and stained for TRAP. The number of TRAP positive, multinucleated osteoclasts were counted and expressed as a percentage of control. Mean ± SEM; $n=5$ WT and 3 $GPR55^{-/-}$ experiments—5 replicates for each. $^{*}P<0.05$—compared to vh control and $^{###}P<0.001$—compared to wildtype—one-way ANOVA with Bonferroni post test

4.7.2 The Effect of Endogenous and Synthetic GPR55 Ligands on Bone Cells In Vitro

Role of GPR55 in osteoclast formation: To investigate the role of GPR55 in osteoclast formation, Whyte et al. (2009) utilised the synthetic GPR55 agonist, O-1602 (Johns et al. 2007; Ryberg et al. 2007), the endogenous agonist LPI (Oka et al. 2009) and the GPR55 antagonist CBD (Ryberg et al. 2007) to study osteoclast formation in human and mouse cultures. Neither O-1602 nor LPI significantly affected the formation of human osteoclasts in vitro, although the GPR55 antagonist CBD significantly increased human osteoclast formation (Fig. 4.7). Interestingly, despite having no effects alone, LPI did significantly attenuate the increase in human osteoclast formation seen after treatment with TGFβ (Fig. 4.8). Likewise, O-1602 significantly decreased human osteoclast formation in the presence of CBD

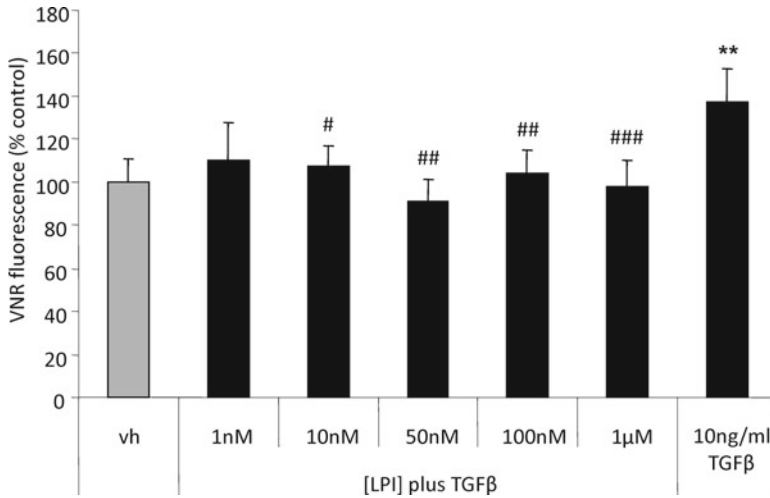

Fig. 4.8 LPI attenuates the stimulatory effect of TGFβ on osteoclast formation. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured in the presence of 1 nM to 1 μM LPI plus TGFβ or TGFβ alone for 7 days and then fixed and stained for VNR to quantify osteoclast number. Immunofluorescence intensity measurements shown are an indication of osteoclast number and expressed relative to control cultures. Mean ± SEM; $n = 3$ experiments—5 replicates for each. $^{**}P < 0.01$ compared to control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ LPI plus TGFβ compared to TGFβ alone—one way ANOVA with Bonferroni post test

compared to CBD alone. Together these data suggest that endogenous GPR55 agonists such as LPI may act to restrain human osteoclast formation in response to stimuli in vivo. Although GPR55 ligands had no effect on human osteoclast formation alone, both O-1602 and LPI significantly inhibited the late stages (including cell fusion) of osteoclastogenesis from mouse precursors as seen by the abundance of TRAP positive mononuclear cells.

As O-1602 has been shown to be involved in mediating a vascular response that is not attributed to actions at GPR55, it was important to show that the effects in osteoclasts were mediated through GPR55. Whilst there is evidence that O-1602 can act on additional targets (Johns et al. 2007), in osteoclasts the effects of O-1602 are mediated via GPR55 since the effect of O-1602 and LPI on mouse osteoclast formation was absent in bone marrow macrophages (BMMs) from $GPR55^{-/-}$ mice (Fig. 4.9 and Whyte et al. 2009) but retained in macrophages from $CB_1^{-/-}$ and $CB_2^{-/-}$ mice (Whyte et al. 2009).

It remains unclear why O-1602 and LPI inhibited the formation of mouse osteoclasts but not human osteoclast formation, although one possibility is that human osteoclasts already produce higher levels of an endogenous agonist such as LPI compared with mouse osteoclasts. Consistent with the hypothesis that GPR55 plays some inhibitory role in osteoclastogenesis, the antagonist CBD did significantly increase both mouse and human osteoclast formation, an effect that was absent in

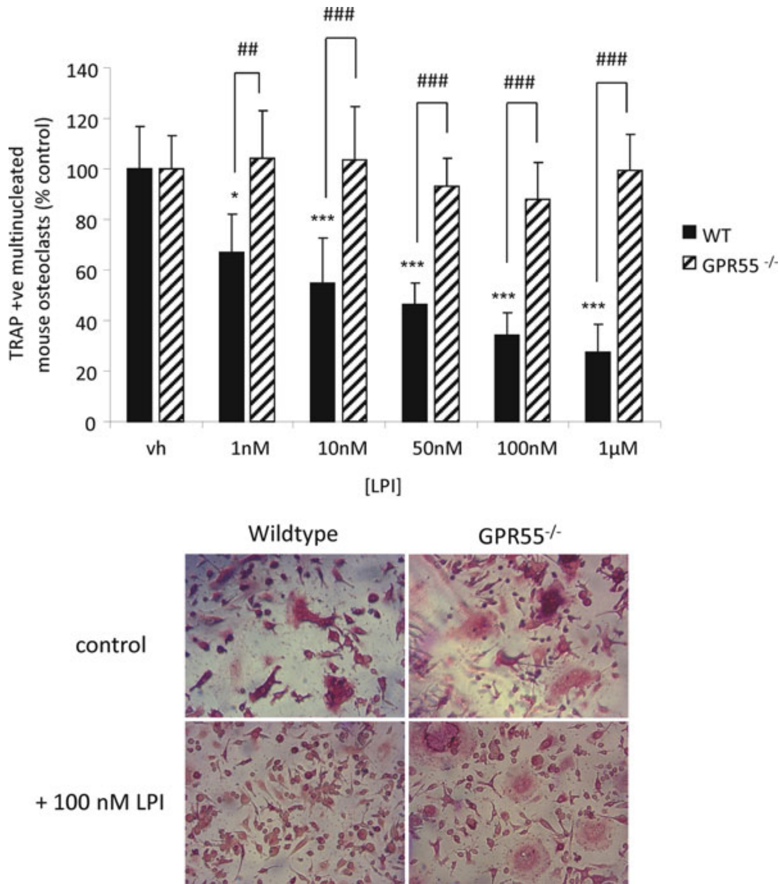

Fig. 4.9 LPI decreases mouse osteoclast formation—GPR55 mediated. M-CSF-dependent BMMs from wildtype (WT) and GPR55$^{-/-}$ mice were cultured with M-CSF and RANKL in the presence of vehicle (control) or 1 nM to 1 μM LPI for 5 days then fixed and stained for TRAP. The number of TRAP positive, multinucleated osteoclasts were counted and expressed as a percentage of control. Mean ± SEM; $n = 3$ experiments—5 replicates each. $^{*}P < 0.05$, $^{***}P < 0.001$—compared to control and $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$—compared to wildtype—one-way ANOVA with Bonferroni post test

osteoclasts generated from GPR55$^{-/-}$ BMMs (Fig. 4.7). Together, these data suggest that GPR55 plays some inhibitory role in osteoclastogenesis, particularly in mice.

Role of GPR55 in osteoclast function: To study osteoclast function independently of osteoclast formation, mature osteoclasts can be generated on dentine, a bone-like substrate consisting of mineral (hydroxyapatite), collagen and several non-collagenous matrix proteins, and then treated with compounds of interest—in this case GPR55 ligands. O-1602 had a stimulatory effect on actin polarisation and resorption in human and mouse osteoclasts, an effect attenuated in the presence of the GPR55 antagonist CBD (Fig. 4.10). Furthermore, treatment with 1 μM

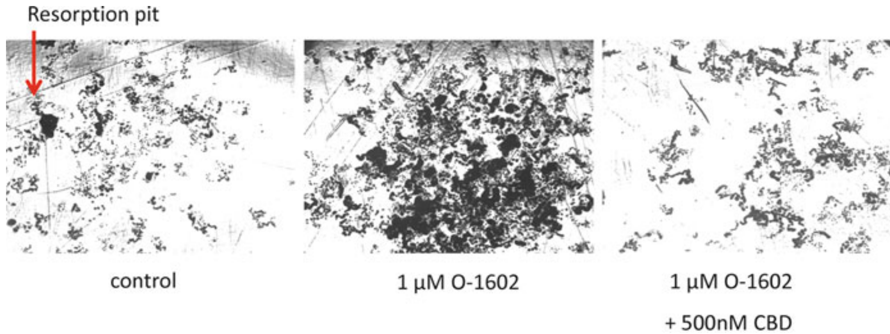

Fig. 4.10 O-1602 increases human osteoclast resorption—prevented by CBD. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured on dentine for 7 days and then treated with vehicle (control) or 1 μM O-1602 ± 500 nM cannabidiol (CBD) for 72 h. Representative images of dentine discs (cleared of cells) after treatment with resorption pits on the surface shown in *black*

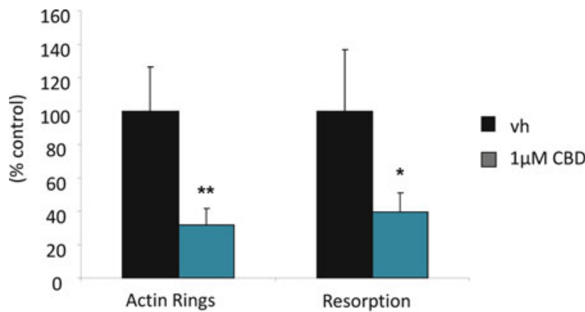

Fig. 4.11 CBD inhibits human osteoclast polarisation and resorption. Human osteoclasts derived from highly enriched M-CSF-dependent human mononuclear cells cultured on dentine for 7 days, then treated with vehicle (control) or 1 μM CBD for 72 h. Cultures were then analysed for the number of F-actin rings and resorption pit area and expressed as percentage of control. Mean ± SEM; $n = 5$ experiments (actin rings), 4 experiments (resorption) with 5 replicates for each. $^{*}P < 0.05$, $^{**}P < 0.01$—compared to control cultures—one-way ANOVA with Bonferroni post test

CBD alone significantly decreased cell polarisation and resorption, presumably by antagonising the effect of GPR55 ligands present in the culture medium or produced endogenously (such as LPI) (Fig. 4.11). Similar to the effects of O-1602, the endogenous GPR55 agonist LPI also stimulated human osteoclast polarisation and resorption (Fig. 4.12). This was the first demonstration that this endogenous bioactive lipid had an effect on osteoclasts. Both O-1602 and LPI elicit a bell-shaped concentration–response relationship for these effects on osteoclasts; this is a typical response of cultured cells to many mediators, including cannabinoids (Paton and Pertwee 1973).

In addition to its role in osteoclast function and migration, the β_3 integrin is also involved in osteoclast adhesion (Helfrich et al. 1996). Mice lacking the β_3 integrin

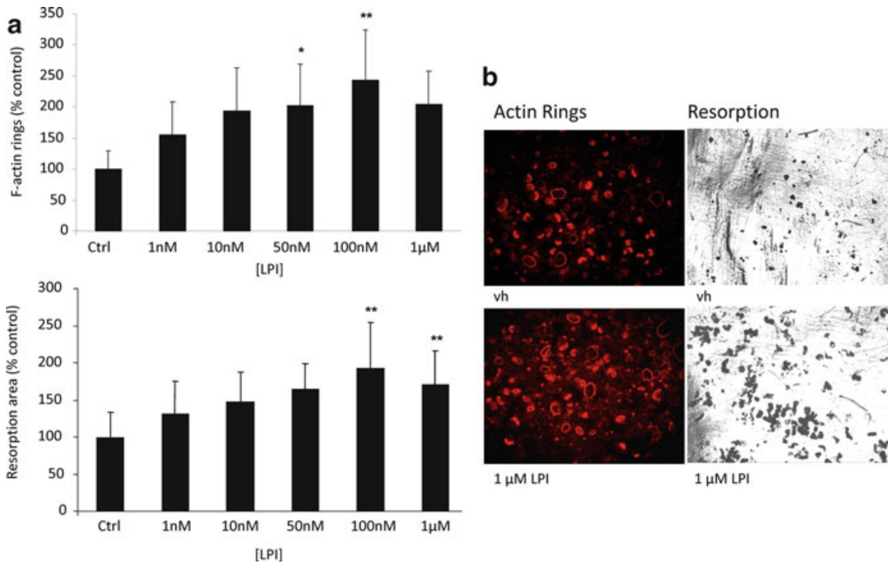

Fig. 4.12 LPI increases human osteoclast resorption. Human osteoclasts derived from highly enriched M-CSF-dependent mononuclear cells were cultured on dentine discs for 7 days and then treated with vehicle (control) or 1 nM to 1 μM LPI for 72 h. (**a**) The number of actin rings were counted and (**b**) resorption pit area measured and expressed as a percentage of control. Mean ± SEM; $n=4$ experiments—5 replicates each. $*P<0.05$, $**P<0.01$ compared to vh control—one-way ANOVA with Bonferroni post test. Representative images of actin rings and resorption pits after treatment with vehicle (control) or 1 μM LPI

have a high bone mass phenotype. BMMs from these mice differentiate, thus showing that the β_3 integrin is not essential for osteoclast formation, but $\beta_3^{-/-}$ osteoclasts fail to resorb on dentine (McHugh et al. 2000). One major limitation of the study by Whyte et al. (2009) was the lack of evidence for the effect of GPR55 ligands on human and mouse osteoclast resorption being solely GPR55 mediated. To validate that GPR55 is the receptor involved in mediating the effects of O-1602 and LPI on osteoclast function in vitro, osteoclasts derived from GPR55$^{-/-}$ BMM should be used to determine whether osteoclasts from GPR55$^{-/-}$ are capable of resorption, and if so, whether GPR55 agonists (O-1602 and LPI) are able to increase osteoclast function in the absence of GPR55. It seems likely that in vitro osteoclast resorption would still be present in the absence of GPR55 even if GPR55 has a major role to play in resorption. Studies of other mutations that affect the bone resorbing abilities of osteoclasts suggest that there is redundancy in proteins involved in bone resorption, i.e. a knockout lacking one particular enzyme or channel is not always enough to completely abolish resorption.

Downstream signalling of GPR55 in osteoclasts: GPR55 is coupled to $G_{\alpha 13}$ that signals downstream to Rho (Ryberg et al. 2007; Henstridge et al. 2009; Lauckner et al. 2008). The stimulatory effect of GPR55 agonists on osteoclasts is likely mediated, at least in part, by activation of the small GTPase Rho since Rho activation is

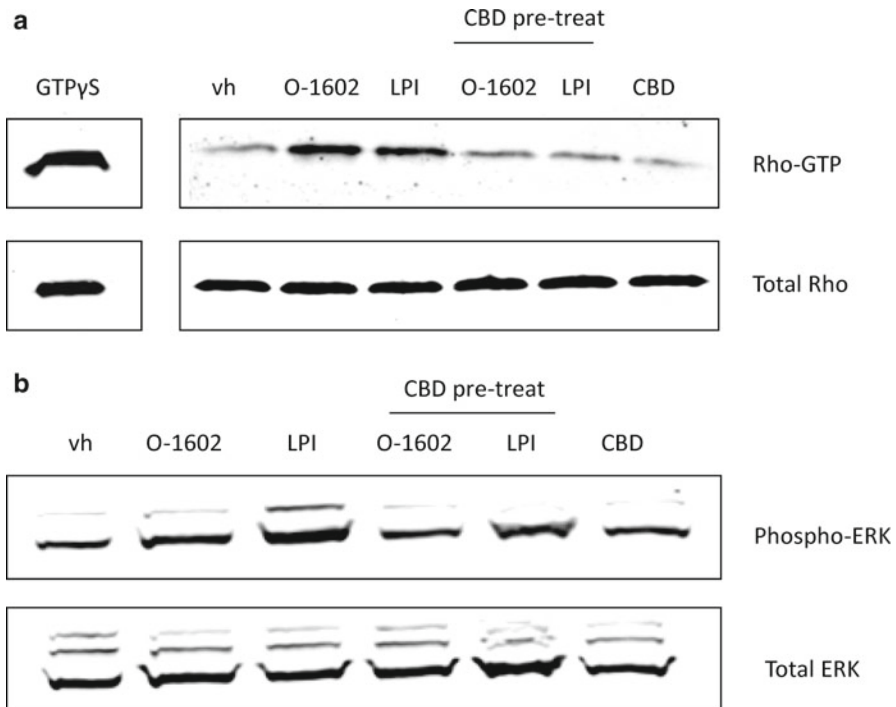

Fig. 4.13 O-1602 and LPI stimulate Rho and ERK activation in human osteoclasts. (**a**) Human osteoclasts were serum starved of FCS and cytokines for 18 h before treating with vh (0.1% DMSO), 1 μM O-1602, 1 μM LPI or 1 μM CBD for 10 min with or without a 10 min pre-incubation with 1 μM CBD. Cells were lysed, total fractions removed and GTP samples generated by subjecting the remaining lysates to a pull down assay using Rho assay reagent containing Rhotekin RBD bound agarose. Total and GTP-bound samples were subject to SDS-PAGE under reducing conditions, transferred to PVDF membranes and probed for Rho before visualising using a LI-COR Infrared Imager. Blot shown is representative of 3–7 independent experiments. (**b**) Human osteoclasts were serum starved of FCS and cytokines for 4 h before treating with vh (0.1% DMSO), 1 μM O-1602, 1 μM LPI or 1 μM CBD for 10 min with or without a 10 min pre-incubation with 1 μM CBD. Cells were lysed, subject to SDS-PAGE under reducing conditions, transferred to PVDF membranes before probing for both total p42/44 and phosphorylated (phospho) p42/44 and visualised using a LI-COR Infrared Imager. Blot shown is representative of 6 independent experiments

associated with cytoskeletal responses involved in osteoclast polarisation and resorption (Chellaiah 2005, 2006). Expression of constitutively active RhoA enhances cytoskeletal rearrangement, podosome formation and bone resorption (Chellaiah et al. 2000), and inhibition of Rho causes osteoclast depolarisation and inhibits resorption (Zhang et al. 1995). Treatment of human osteoclasts with O-1602 (and, to a lesser extent, LPI) activates Rho, a response antagonised by CBD (Fig. 4.13a). The 2.5-fold increase in Rho activation in human osteoclasts after

treatment for 10 min with LPI is in line with the fivefold increase in Rho activation in GPR55 transfected HEK cells observed by Henstridge et al. (2009). The stimulatory effect of O-1602 and LPI on Rho was mediated via GPR55 since this effect was absent in mouse osteoclasts derived from $GPR55^{-/-}$ bone marrow-derived macrophages (BMM) compared to osteoclasts from wildtype BMMs.

O-1602, and particularly LPI, also caused activation of ERK in osteoclasts, which is associated with increased osteoclast activity and survival (Fig. 4.13b) (Gingery et al. 2003; Lee et al. 2001; Miyazaki et al. 2000). All studies examining ERK activation downstream of GPR55 have been carried out using GPR55 transfected cells, and despite studies being performed in the same cellular background there are a number of inconsistencies regarding GPR55 pharmacology. Although some studies have failed to show that ERK lies downstream of GPR55 (Lauckner et al. 2008), other studies have demonstrated robust GPR55-mediated ERK activation (Oka et al. 2007; Waldeck-Weiermair et al. 2008). The contradictory nature of these findings may relate to the concept of functional selectivity (Mailman 2007; Bosier and Hermans 2007) whereby differential signalling pathways (e.g. Rho and ERK) are activated in an agonist- and tissue-specific manner. In the study by Whyte et al. (2009), they demonstrate that in osteoclasts, O-1602 predominantly stimulates Rho whereas LPI predominantly stimulates ERK. Together, our studies strongly indicate that Rho and ERK signalling lie downstream of GPR55 in osteoclasts and that GPR55 plays a role in regulating osteoclast function.

There are several key signalling pathways associated with osteoclast resorption that warrant further investigation. GPR55 interacts with the β_3 integrin and Rho is activated downstream of the β_3 integrin (i.e. $\beta_3^{-/-}$ mice have impaired Rho activation; McHugh et al. 2000). β_3 integrin binds the tyrosine kinases src and Pyk2, essential effecter proteins of podosomes assembly and disassembly (Ory et al. 2008); therefore, future studies examining GPR55-mediated activation of c-src and Pyk2 may also be worthwhile.

Activation of GPR55 via LPI activates Rho via $G_{\alpha13}$ which then stimulates intracellular calcium release and nuclear translocation of NFAT (Henstridge et al. 2009); however, it is not yet known whether LPI induces the same response in osteoclasts via Rho. NFATc1 (nuclear factor of activated T cells calcineurin dependant 1) is a major transcriptional regulator of osteoclast differentiation and also regulates osteoclast function (Song et al. 2009). It may be hypothesised that the pro-resorptive or anti-formation effects of O-1602 and LPI in osteoclasts are mediated through activation or inhibition of NFAT, respectively. NFAT regulates the transcriptional activity of a number of genes in osteoclasts. The ability of Rho to induce NFAT expression, uncovered by Henstridge et al. (2009), is novel with only one previous study in human T cells showing that RhoA inhibits rather than induces the transcriptional activity of NFAT (Helms et al. 2007). The signalling of LPI via Rho to induce NFATc1 may be cell type specific. As GPR55 plays a role in the activation of osteoclast activity, a process involving NFAT, this seems a likely pathway through which GPR55 agonists would act to stimulate osteoclast activity. The ability of O-1602 to activate NFAT is not known in any cell type but as O-1602 is more effective at activating Rho than LPI in osteoclasts, O-1602 may also induce a greater activation of

NFAT than LPI. The consequence of this functional selectivity does not appear to impinge on the degree to which these GPR55 ligands augment osteoclast resorption and so the relevance of a potential functional selectivity in osteoclasts is yet to be determined.

CBD has been shown to increase c-fos expression in a cell-specific manner (Guimarães et al. 2004). C-fos is an essential transcription factor for osteoclast differentiation, and c-fos-deficient mice have a severe osteopetrotic phenotype (Takada et al. 2009). Increased c-fos expression due to CBD may explain the increases observed in osteoclast formation in vitro and in osteoclast number in vivo in GPR55$^{-/-}$ mice.

Role of GPR55 in osteoblast function: In addition to studying the role of GPR55 on osteoclast formation and function, Whyte et al. (2009) studied the effects of GPR55 ligands on osteoblast function. Overall the GPR55 agonist O-1602 did not have any effect on human or mouse osteoblast mineralisation, and had only modest inhibitory effects on ALP activity. ALP is a marker of osteoblast activity. ALP is the enzyme that supplies the inorganic phosphate (Pi) by hydrolysing phosphate containing compounds such as ATP and β-glycerophosphate. Pi is essential for mineralisation, and inhibition of ALP gives rise to cultures that accumulate non-mineralised collagen matrix.

Mineralisation is considered a functional endpoint of osteoblast cultures in vitro that reflects advanced cell differentiation. Mineral is deposited as calcium-phosphate substituted hydroxyapatite similar to the mineral found in bone. O-1602 did not significantly affect osteoblast mineralisation in vitro when added either during the entire culture, when added midway through the culture or when cultures were terminated early. The rationale for adding compounds at different times throughout the culture and terminating cultures early was that confluent cultures of primary calvarial osteoblasts follow a two-stage developmental process under osteogenic conditions. During the first 1–2 weeks osteoblasts proliferate slowly and express ALP whilst assembling a collagen matrix. During the second phase, mineralisation of the collagen matrix occurs (Nefussi et al. 1985).

Overall these results suggest that GPR55 ligands do not directly affect osteoblast function in vitro but it is still possible that these compounds may have effects on the ratio of RANKL/OPG production by osteoblasts and thus indirectly affect osteoclast formation and function. Therefore future studies aimed at determining the expression of RANKL/OPG in osteoblasts after treatment with O-1602 may shed more light on the role of the GPR55 receptor in bone.

Osteoblasts are derived from a mesenchymal lineage, capable under the appropriate stimuli to differentiate into not only osteoblasts, but also myoblasts, chondrocytes and adipocytes. Osteoblasts express GPR55 and it is also known that adipocytes express GPR55 (Ryberg et al. 2007; Obara et al. 2011). The effect of GPR55 ligands on the differentiation of precursors along either the adipogenic or osteogenic lineage has not been examined but would be an interesting avenue to pursue.

Conclusion: The ability of O-1602 to maintain a haemodynamic response in the GPR55$^{-/-}$ mice points towards O-1602 acting at another, as of yet unknown target.

Whyte et al. (2009) clearly demonstrate effects of O-1602 and LPI on mouse osteoclast formation and Rho activation through GPR55 as effects were absent in GPR55$^{-/-}$ mice. Therefore, in osteoclasts O-1602 restrains osteoclastogenesis and stimulates osteoclast function via GPR55 but it remains to be determined whether this other receptor that binds O-1602 in the vasculature is also expressed in osteoclasts. It will be interesting to determine whether the effects of O-1602 are tissue specific, and if perhaps the effects of O-1602 in osteoclasts are mediated specifically through GPR55 as opposed to the other as of yet unidentified target of O-1602 in the vasculature.

The advantage of GPR55 selective compounds that lack any pharmacological activity at CB_1 is that such compounds could be exploited clinically. For example, CBD is a phytocannabinoid not associated with psychotropic side effects but has many beneficial therapeutic effects and has been shown to act at GPR55. This work significantly expands on the earlier discovery demonstrating a role for CB_1 and CB_2 receptors in bone and demonstrates a role for the novel cannabinoid receptor GPR55 in vitro.

4.7.3 Phenotype of GPR55$^{-/-}$ Mice

Local, systemic and central effects all contribute to bone mass, in particular the coupling between osteoclasts and osteoblasts is very important in vivo. The combined actions of the osteoclasts and osteoblasts regulate bone turnover, and in addition to the regulation of RANKL and M-CSF production by osteoblasts, many local and systemic factors present within the bone microenvironment are known to influence both osteoblasts and osteoclasts; these include a cocktail of interleukins, IFNs, growth factors and chemokines. The benefit of studying in vivo models is that it takes into account all of the effects that may have an impact on the overall bone phenotype, providing an as close as possible representation of effects that may be produced by pharmacological intervention with drugs targeting this receptor. Studying the effects of GPR55 ligands on bone cells in vitro is important, particularly as this provides the opportunity to examine the effects of ligands on human bone cells. Conversely, the control of bone remodelling in vivo is complex. Thus studying the effects of GPR55 ligands in vitro provides valuable data on the pharmacology of a particular receptor in a particular cell type, but it does not provide a comprehensive impression of the likely effects these ligands will have on other cells and pathways that can also control bone cells in vivo. This brings to light the advantage of studying targets such as GPR55 in a whole body knockout.

GPR55 knockout mice have no gross abnormal signs at birth (Whyte et al. 2009). GPR55$^{-/-}$ mice have been used in two previous studies, one of which demonstrating a phenotype associated with loss of GPR55 in the control of inflammatory and neuropathic pain (Staton et al. 2008). GPR55 is known to be expressed in many tissues including the brain and adipose tissue, both of which have links to the regulation of bone turnover. In order to determine whether the effects of GPR55 ligands seen

in vitro are translated to a bone phenotype in vivo, GPR55$^{-/-}$ and wildtype littermate control mice were subject to phenotypic analysis as described below.

In order to study bone phenotypes in vivo, two specific techniques can be utilised—micro-computed tomography (μCT) and histomorphometry. μCT, first introduced by Feldkamp et al. (1989), is a non-destructive precise means of reconstructing complex bone architecture in 3D, with a resolution on the order of a few micrometres. As discussed at the beginning of this chapter, cortical bone gives long bones their hard outer surface, and trabecular bone is found mainly at the interior ends of long bones arranged in a network of plates and rods. The trabeculae orientate to provide structural support. Trabecular bone is more rapidly turned over than cortical bone and, therefore, more subject to changes induced by ageing, disease or therapeutic agents, which ultimately affect bone strength. Trabecular microarchitecture is strongly related to biomechanical bone strength (Ito 2005) and its importance is now recognised in the pathogenesis of bone fragility. Both trabecular and cortical bone measurements can be made using μCT.

Before the advances in technology that led us to use μCT, 3D parameters such as bone volume were inferred indirectly from 2D histological sections of bone. Histomorphometry has not been made completely redundant by techniques such as μCT as histomorphometric analysis provides information on bone that cannot be obtained from μCT or biochemical markers of bone turnover such as osteoblast and osteoclast number. As such, histomorphometry continues to play an important role in identifying the mechanisms underlying bone mass changes at the tissue and cellular level, and is therefore used in parallel with μCT.

Consistent with the stimulatory effects of a GPR55 agonist (O-1602) on osteoclast function in vitro, male GPR55$^{-/-}$ mice have a high bone mass phenotype. In addition to the increase in trabecular number and thickness together with a decrease in separation, 12-week-old male GPR55$^{-/-}$ mice showed a significant decrease in trabecular bone pattern factor (Tb.Pf), signifying an increase in trabecular connectivity, and a decrease in structural model index (SMI), signifying a transition towards a more plate like structure. Together such changes may infer increased strength. As a consequence of ageing, there is a change from a plate-like structure to a more rod-like structure that overall confers decreased bone strength and increased fracture risk (Jiang et al. 2005). Paired biopsies from the same pre and postmenopausal women show changes in 3D structure such as reduced number of trabeculae with pronounced trabecular thinning and loss of connectivity (Jiang et al. 2007). As the disease continues the increased remodelling rate leads to deterioration of the trabecular structure with deep resorption cavities causing the plates to become perforated and the connecting rods to dissolve. As osteoporosis is characterised by bone loss and a transition from plate to rod-like architecture, this highlights the relevance of the opposite changes seen in male GPR55$^{-/-}$ mice and how they relate to the mild osteopetrotic phenotype. Osteopetrosis is a congenital bone disorder of which there are several forms, each of differing severity, characterised by excessive accumulation of bone and mineralised cartilage throughout the skeletal system. Abnormal osteoclast function is the pathogenic mechanism for the increased bone density.

As a result of decreased bone resorption, bones become very dense; however, they are brittle and more prone to fracture.

Although the changes in trabecular SMI and Tb.Pf in male GPR55$^{-/-}$ mice infer increased bone strength, mechanical testing such as three-point bending experiments are necessary to confirm this. Studies in mice containing a mutation in LRP5 (G171V) resulting in a high bone mass phenotype have shown that despite significant gender differences in some trabecular bone structural parameters there were no gender-specific differences in strength (Dubrow et al. 2007). A series of mechanical tests such as three-point bending experiments should be performed in GPR55$^{-/-}$ mice to determine whether female GPR55$^{-/-}$ mice show increased strength despite the non-significant change in bone volume. In addition such mechanical tests should determine whether the increase in trabecular bone volume relates to an increase in strength or if the osteopetrotic phenotype does in fact cause bones to fracture more readily as is seen in patients with the disease.

In agreement with μCT analysis, histomorphometric analysis of male GPR55$^{-/-}$ mice revealed evidence of decreased bone resorption and a mild osteopetrotic phenotype. Consistent with the inhibitory effect of O-1602 on mouse osteoclast formation in vitro, osteoclast number was significantly higher in the long bones from GPR55$^{-/-}$ mice compared to wildtype mice. While osteoclast numbers were significantly increased, impaired function of osteoclasts was indicated by increased cartilage remnants within the trabecular bone. An impairment of osteoclast function in GPR55$^{-/-}$ mice is consistent with the effects of O-1602 on osteoclasts in vitro and points towards a role for GPR55 in stimulating osteoclast activity. The high bone mass phenotype of the GPR55$^{-/-}$ mice appears to be predominantly the result of a defect in osteoclast function rather than an increase in osteoblast-mediated bone formation, since O-1602 had little effect on the differentiation or mineralisation of osteoblasts cultured in vitro, and osteoblast number and osteoblast surface were not altered in the GPR55$^{-/-}$ mice compared to wildtype controls in vivo. The increase in osteoclast number is not likely due to an indirect increase in monocyte number as the proportions of monocytes in GPR55$^{-/-}$ mice are not different to wildtype mice (Staton et al. 2008). An increase in osteoclast number in the presence of impaired function is often observed in osteopetrotic phenotypes and appears to be a homeostatic response—perhaps as a compensatory mechanism.

Gender disparity in bone phenotypes is not uncommon, for example 12-week-old $CB_1^{-/-}$ mice have a high bone mass phenotype present only in male mice (Tam et al. 2006). The reason for this is unknown. Similarly the reason for the apparent sex-specific difference in bone phenotype in the GPR55$^{-/-}$ mice is unknown. There were no noticeable differences in GPR55 expression, response to GPR55 ligands or osteoclast formation in cells from males or females (mouse or human) in vitro. However a recent study demonstrating a role for GPR55 in pain responses, whereby the absence of GPR55 results in reduced neuropathic and inflammatory pain, has identified a potential gender difference between male and female GPR55$^{-/-}$ mice that may add to the evidence for differential roles of GPR55 between sexes (Staton et al. 2008).

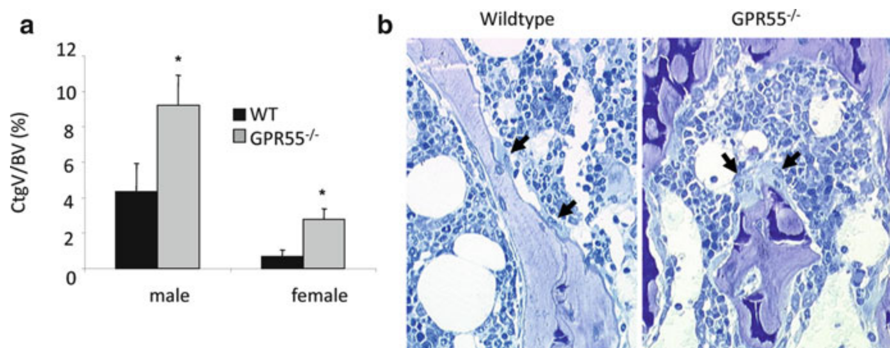

Fig. 4.14 Increased cartilage remnants in GPR55$^{-/-}$ mice. (**a**) Quantification of cartilage (Safranin O staining) within male and female femora from wildtype and GPR55$^{-/-}$ mice. (**b**) Representative sections from WT and GPR55$^{-/-}$ mice—*Note the appearance of plump osteoclasts in GPR55$^{-/-}$ mice. Sections stained using Toluidine Blue*

The most obvious cause for any gender difference in vivo would most likely be of hormonal origin. The CB_1 cannabinoid receptor is classed as an estrogen responsive gene, due to the estrogenic upregulation of *CNR1 gene* expression in colon cancer cells (Notarnicola et al. 2008). The estrogen receptor positive breast cancer cell line MCF-7 expresses 30-fold less GPR55 compared with the highly metastatic estrogen receptor negative MDA cell line, suggesting there may be some association between GPR55 and estrogen (Ford et al. 2010). Interestingly estrogen receptor positive cells are more likely to metastasize to bone (Wei et al. 2008), whether GPR55 plays a role in metastasis is also yet to be determined.

The modulation of CB_1 receptor activity by androgens has also been suggested and requires further research. However, it is known that $CB_1^{-/-}$ mice have decreased levels of testosterone in the testis and serum (Wenger et al. 2001), and that testosterone itself decreases CB_1 expression after castration, an effect that is restored by testosterone treatment (Busch et al. 2006). The effect of sex hormones on the expression or function of GPR55 remains unknown, but hormonally driven differences in GPR55 may result in male mice being more susceptible to loss of GPR55-mediated signalling.

The high bone mass phenotype of GPR55$^{-/-}$ male mice in the face of increased osteoclast number suggested an osteoclast defect. Upon further evaluation it was found that the amount of unresorbed cartilage was significantly higher in GPR55$^{-/-}$ mice when compared to wildtype mice, suggesting an impairment of osteoclast function (Fig. 4.14a). GPR55 has been shown to interact via integrins such as β_3 (Waldeck-Weiermair et al. 2008). Similar to GPR55 knockout mice, $\beta_3^{-/-}$ mice have a high bone mass phenotype despite the 3.5-fold increase in osteoclast number (McHugh et al. 2000). These osteoclasts were shown to be dysfunctional as they failed to form normal ruffled borders, showing that the β_3 integrin is associated with ruffled border formation.

Osteopetroses are a rare, but severe group of genetic disorders characterised by increased bone density (Stark and Savarirayan 2009). Some underlying osteoclast defects have been identified as sole factors driving the disease. Mutations in at least ten genes including V-ATPase (Ochotny et al. 2006), CLCN7 (Besbas et al. 2009; Waguespack et al. 2003), RANK (Guerrini et al. 2008), RANKL (Sobacchi et al. 2007), the lysosome-associated protein gene, PLEKHM1 (Del Fattore et al. 2008a) and carbonic anhydrase II (CAII) (Borthwick et al. 2003) can now account for the pathology seen in ~70% of cases. The genetic basis for the remaining 30% of sufferers remains to be determined. Osteopetrosis can result from either impaired osteoclast formation or lack of function. The osteopetrotic phenotype of male GPR55$^{-/-}$ mice observed in this study is due to a lack of function. Akin to osteopetrosis being a congenital condition, GPR55$^{-/-}$ mice have developed an osteopetrotic-like phenotype due to the absence of GPR55 from birth; however, it is important to realise that often targets identified in osteopetrotic mice, which would appear detrimental, can actually be developed for the treatment of osteoporosis where the aim is to inhibit osteoclast function.

With the identification of target genes, described above, future studies taking these mutations in account may lead us to determine the mechanism of action of ligands acting at GPR55 in osteoclasts through identification of potentially novel downstream signalling pathways. Likewise, information provided from GPR55$^{-/-}$ mice might also be a means to better understanding osteoclast biology and function. Genetic polymorphisms of GPR55 in patients with osteopetrosis should be investigated, particularly since genetic polymorphisms of GPR55 (Gly195Val) transcending at the level of decreased ERK phosphorylation have already been identified and shown to have functional relevance in anorexia nervosa (Ishiguro et al. 2011). Additionally, polymorphisms in CB_2 have been reported in a cohort of postmenopausal osteoporotic women (Karsak et al. 2005). At present hundreds of SNPs in GPR55 have been identified but whether any of these will prove relevant in disease remains to be determined.

4.7.4 Effect of the GPR55 Antagonist Cannabidiol (CBD) on Bone Turnover In Vivo

Having shown that GPR55 may be a potential therapeutic target for bone loss, the effect of pharmacological antagonism of GPR55 was examined. CBD was once considered an inactive compound present in cannabis due to its reported low affinity for both of the CB_1 and CB_2 receptors (McPartland et al. 2007). It is exactly this property that has now become its own unique selling point. CBD lacks the psychotropic effects of the better known active agent in cannabis, Δ^9-tetrahydrocannabinol (THC) and is therefore now recognised as an attractive therapeutic agent. Only recently has interest in CBD escalated in line with that of cannabis in general (Zuardi 2008). Many of the pharmacological effects of CBD have shown immense therapeutic potential, for example studies with CBD have demonstrated anti-epileptic, anti-psychotic,

anxiolytic, anti-cancer, anti-emetic, anti-inflammatory and anti-oxidative (neuroprotective) effects, to name but a few (Brown 2007; Zuardi 2008). At present a CBD-based medicine called Sativex (GW Pharmaceuticals) is licensed in the UK, Canada and New Zealand to treat spasticity associated with multiple sclerosis (MS). Sativex is also available in Canada for the treatment of neuropathic and cancer pain.

There are numerous animal studies demonstrating beneficial effects of CBD. Most relevant in terms of bone is the effect of CBD in the collagen-induced arthritis (CIA) model of arthritis. CBD, when given either intraperitoneally or orally after the onset of clinical symptoms, blocked the progression of arthritis (Malfait et al. 2000). It was concluded that both the anti-inflammatory and immunosuppressive properties of CBD attributed to its anti-arthritic effect. Inflammatory conditions such as rheumatoid arthritis (RA) are often associated with bone loss. Peridontitis is another such chronic inflammatory disease associated with bone destruction. Peridontitis can be simulated by experimentally induced ligature placement. Treatment of male mice with 5 mg/kg CBD daily for 30 days significantly inhibited the volume of bone loss after ligature placement and also lowered the expression of RANK/RANKL (Napimoga et al. 2009).

Having established that activation of GPR55 in vitro increases osteoclast activity and that male GPR55$^{-/-}$ mice have a high bone mass phenotype, this indicated that antagonists of GPR55 may protect against bone loss. In an attempt to determine whether pharmacological antagonism of GPR55 in vivo could produce the same outcome as that observed in GPR55$^{-/-}$ mice (*high bone mass phenotype*), healthy young male C57Bl6 mice were treated for 8 weeks with the GPR55 antagonist CBD (10 mg/kg) (Whyte et al. 2011). Previously CBD was found to protect against CIA in DBA/1 mice treated with 5 or 10 mg/kg/day CBD for 10 days after the onset of symptoms of CIA (Malfait et al. 2000). Lower concentrations of 2.5 mg/kg and higher concentrations of 20 mg/kg were inactive at protecting against CIA. CBD did not significantly affect any pharmacological parameter of trabecular bone volume or structure in male mice after treatment for 8 weeks in this study as assessed by μCT analysis. There was however a trend towards increased bone volume that may have required longer treatment or different doses/regimens in order to reach significance. Changes in bone volume in healthy mice may be hard to identify and would require larger numbers of mice than those used in this study. Potential beneficial effects of CBD would be better investigated in animal models of bone loss induced by ovariectomy or gonadectomy. These results may help to decipher the apparent gender disparity in trabecular bone phenotype or, alternatively, such studies may reveal a phenotype in female mice. Short-term markers of bone turnover were measured in the serum from both vehicle and CBD-treated mice. Treatment with CBD was found to significantly decrease serum levels of C-terminal telopeptide of type I collagen (CTX) by 18%, signifying a reduction in bone resorption. Inhibition of osteoclast resorption in vivo with CBD is most likely the result of an inhibition of endogenously released ligands such as LPI.

In summary, treatment of mice with CBD significantly decreased serum levels of CTX, indicative of decreased osteoclast resorption. These studies in bone further add to the potential benefits of CBD in the treatment of multiple disease states and show that antagonism of GPR55, with a CBD-based therapeutic, may be of value in

the treatment of bone diseases. CBD acts as an antagonist at GPR55, at a concentration well below any concentration needed to displace agonists from CB_1 or CB_2, therefore as highlighted earlier, effects seen with CBD are likely attributed to GPR55. However, this also brings to light one major limitation of the study by Whyte et al. (2009). Studies examining the effects of CBD in $GPR55^{-/-}$ and wildtype mice are required in order to truly show that the effects of CBD on bone in vivo are GPR55 mediated—*at present the effects of CBD* in vivo *have not been attributed to any specific receptor.*

CBD clearly has many beneficial therapeutic effects but the receptor mechanisms underlying these therapeutic effects are unknown. Several modes of action for CBD other than GPR55 have been proposed and accordingly more than one non-CB_1, non-CB_2 target may exist other than GPR55. For example, CBD binds to the abnormal cannabidiol receptor and TRPV2 as well as GPR55 (Ross 2009); therefore, specific GPR55 antagonists are perhaps of greater interest than CBD and as such specific small molecule inhibitors of GPR55 are already under development.

4.8 Concluding Remarks

Observations clearly indicate a novel and necessary role for GPR55 in bone physiology by regulating osteoclast formation and more particularly osteoclast function (Fig. 4.15). The role of GPR55 in bone is one of the first studies to characterise a physiological role for GPR55 in vivo. These studies will have major implications

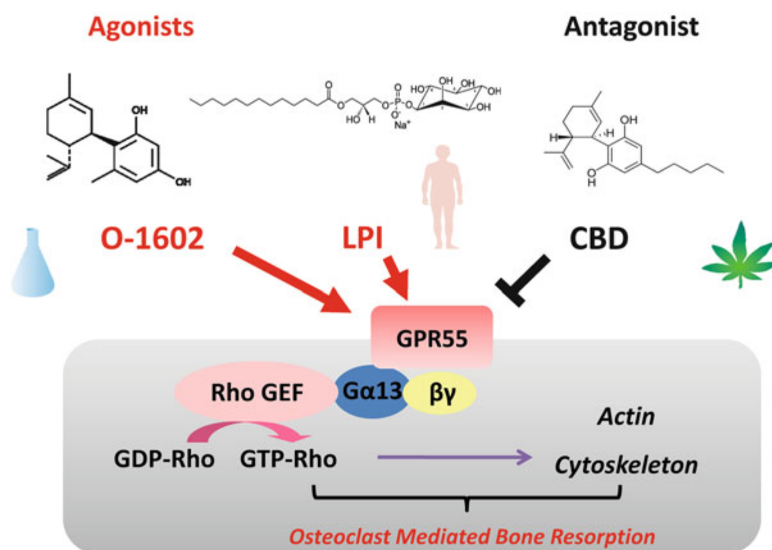

Fig. 4.15 Schematic overview of GPR55 pharmacology in osteoclasts

for the development of novel GPR55- and CBD-related therapeutics for the treatment of diseases associated with osteoclast-mediated bone loss such as osteoporosis. With GPR55-based therapeutics already under development, the next few years will be crucial in defining the physiological/pathophysiological role of LPI-GPR55 in bone and beyond. Whether this orphan receptor will be classified as a cannabinoid receptor remains to be seen, but with such potential in the treatment of pain, bone loss and cancer it seems guaranteed that GPR55-based therapeutics look set for a “high” future.

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Chapter 5
The Role of GPR55 in Cancer

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Abbreviations

AEA	Arachidonoylethanolamide (anandamide)
CBD	Cannabidiol
COX-2	Cyclooxygenase-2
cPLA2	Cytosolic phospholipase A2
ERK	Extracellular signal-regulated kinase
GPCR	G protein-coupled receptor
HMVEC	Human microvascular endothelial cells
HUVEC	Human umbilical vein endothelial cells
LOX	Lipoxygenase
LPA	Lysophosphatidic acid
LPI	Lysophosphatidylinositol
NFAT	Nuclear factor of activate T-cells
NGF	Nerve growth factor
PI3K	Phosphoinositide 3-kinase
PPAR	Peroxisome proliferator-activated receptor
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
shRNA	Short hairpin RNA
siRNA	Small interference RNA
TRP	Transient receptor potential
VEGF	Vascular endothelial growth factor

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The orphan G protein-coupled receptor (GPCR) GPR55 has been recently proposed to be engaged by lipids, specifically by lysophosphatidylinositol (LPI) and cannabinoids (Pertwee et al. 2010). Since it is well established that both cannabinoids (Guzmán 2003; Sarfaraz et al. 2008; Velasco et al. 2007) and lysophospholipids (Choi et al. 2010; Murph et al. 2006) modulate cancer progression, an obvious question arises: is GPR55 involved in the physiopathology of cancer? This chapter intends to address this issue.

5.1 GPR55 Expression in Cancer Cells

GPR55 is widely expressed throughout the body and its mRNA and/or protein have been found in many different organs, tissues, and cell types (Table 5.1) (Balenga et al. 2011; Daly et al. 2010; Fonseca et al. 2011; Ford et al. 2010; Huang et al. 2011; Jenkin et al. 2010; Lauckner et al. 2008; Pietr et al. 2009; Ryberg et al. 2007; Sawzdargo et al. 1999; Waldeck-Weiermair et al. 2008; Whyte et al. 2009; Zhang et al. 2010). Recent data have demonstrated that GPR55 is also expressed by human tumors. Thus, this receptor was detected in a vast collection of human cancer cell lines obtained from breast (Andradas et al. 2011; Ford et al. 2010), brain, cervix, skin, pancreas, liver (Andradas et al. 2011), ovaries, prostate (Piñeiro et al. 2011), bile ducts (Huang et al. 2011), and hematological tumors (Andradas et al. 2011; Cantarella et al. 2011; Oka et al. 2010) (Fig. 5.1). Moreover, GPR55 was found in biopsies from breast, brain, and pancreatic cancer patients (Andradas et al. 2011).

Table 5.1 Expression of GPR55 in mammalian organs, tissues, and cells

System	Organ/cell type/structure	References
Nervous system	Brain	Sawzdargo et al. (1999) and Ryberg et al. (2007)
	Dorsal root ganglia neurons	Lauckner et al. (2008)
Immune system	Spleen	Sawzdargo et al. (1999) and Ryberg et al. (2007)
	Microglia	Pietr et al. (2009)
	Neutrophils	Balenga et al. (2011)
Digestive system	Gastrointestinal tract	Sawzdargo et al. (1999) and Ryberg et al. (2007)
	Liver	Huang et al. (2011)
Endocrine system	Adrenal glands	Sawzdargo et al. (1999) and Ryberg et al. (2007)
Skeletal system	Bone	Whyte et al. (2009)
Urinary system	Kidney	Jenkin et al. (2010)
Vascular system	Endothelial cells	Daly et al. (2010), Waldeck-Weiermair et al. (2008), and Zhang et al. (2010)
	Vascular smooth muscle cells	Daly et al. (2010) and Fonseca et al. (2011)
Reproductive system	Decidual cells	Fonseca et al. (2011)

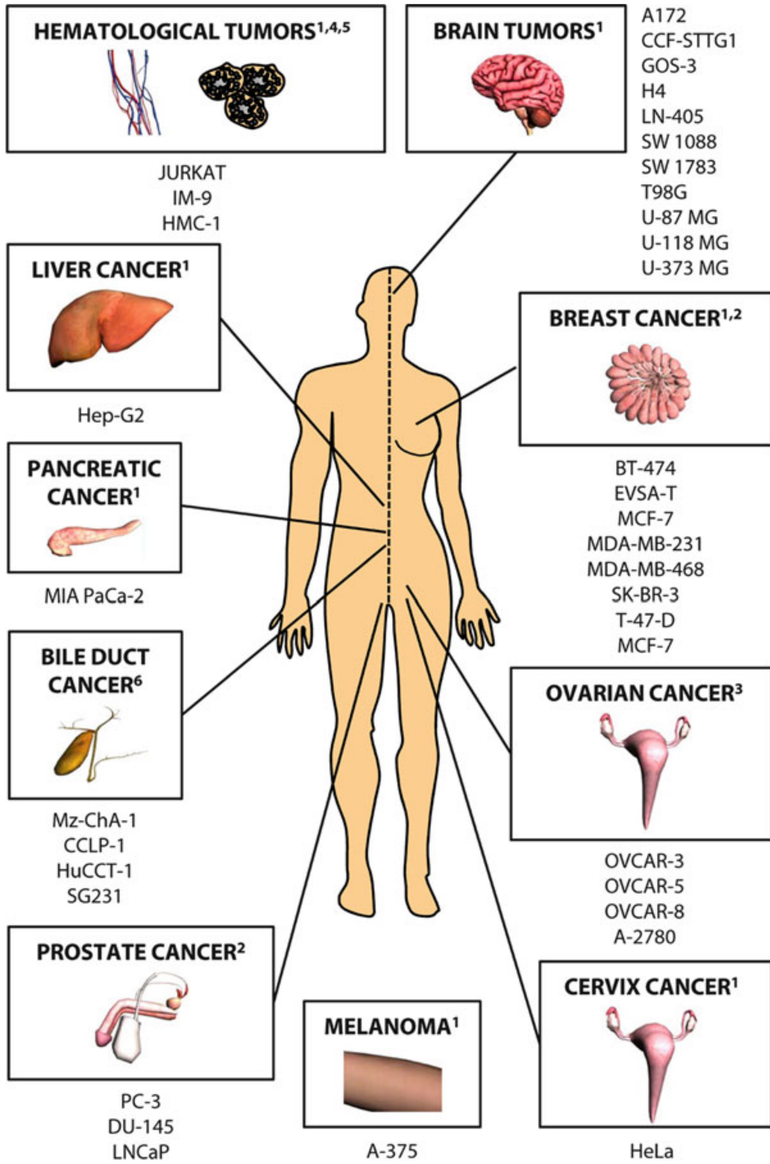

Fig. 5.1 GPR55 expression in human cancer cell lines. GPR55 mRNA and/or protein have been detected in a wide spectrum of human cancer cell lines established from very different tumor tissues and organs. *1* Andradas et al. (2011); *2* Ford et al. (2010); *3* Piñeiro et al. (2011); *4* Oka et al. 2010; *5* Cantarella et al. (2011); *6* Huang et al. (2011)

Interestingly, GPR55 expression in these tumors correlates with their aggressiveness. Consequently, elevated GPR55 levels were found in those tumors with higher histological grades, as well as in those gliomas from patients with lower survival rates

(Andradas et al. 2011). Although the membrane receptors typically associated to malignant growth are those with intrinsic tyrosine kinase activity (RTKs), there is ample evidence showing that GPCRs are overexpressed in human cancers and that their aberrant expression favors tumor progression (Dorsam and Gutkind 2007; Lappano and Maggiolini 2011). Of interest, there are two families of GPCRs associated to cancer that are intimately related to GPR55: lysophospholipid receptors and cannabinoid receptors. GPR55 is engaged by both lysophospholipids (specifically LPI) and cannabinoids (Pertwee et al. 2010). It is well described that lysophospholipid and cannabinoid signaling are deregulated in cancer. Thus, the endogenous agonists of lysophosphatidic acid (LPA) receptors (Choi et al. 2010; Murph et al. 2006) and cannabinoid receptors (Malfitano et al. 2011) are frequently elevated in samples from cancer patients compared to those of healthy volunteers. Interestingly, the same seems true for a putative GPR55 endogenous agonist (LPI) (see below) (Sutphen et al. 2004; Xiao et al. 2001). Additionally, LPA receptors (primarily LPA_2) (Choi et al. 2010; Murph et al. 2006) and cannabinoid receptors (CB_2 in particular) (Malfitano et al. 2011) are upregulated in many human tumors, as it is the case for GPR55 (Andradas et al. 2011).

There is an additional interesting parallelism between LPA receptors, the classical cannabinoid receptors (CB_1 and CB_2), and the proposed LPI receptor GPR55: LPA binds to six known GPCRs termed LPA_1/EDG2, LPA_2/EDG4, LPA_3/EDG7, LPA_4, LPA_5, and LPA_6 (Choi et al. 2010; Houben and Moolenaar 2011). The first three cluster very close to each other in a phylogenetic analysis of human Class A GPCRs, and they are very proximal to cannabinoid receptors CB_1 and CB_2 (Brown 2007). LPA_4 (Noguchi et al. 2003) and LPA_5 (Lee et al. 2006) (formerly GPR23 and GPR92, respectively) were deorphanized more recently and cluster in a very phylogenetically distal group (Brown 2007), which makes them somewhat "atypical" LPA receptors in a sense. Fascinatingly, the "atypical" cannabinoid receptor GPR55 is located in this same group (Brown 2007).

5.2 GPR55 and Cancer Cell Proliferation

It is well established that normal cells only proliferate in response to growth signals and that they remain in a quiescent, nonproliferative state when those signals are not present. These growth signals bind to specific membrane receptors that turn on the proliferative machinery through the activation of different intracellular signaling cascades. This stringent control of cell proliferation (no growth signals, no proliferation) is a pivotal strategy to regulate tissue homeostasis, architecture, and function. Cancer cells are not subjected to this type of control because they have acquired the capability to generate, one way or another, their own growth signals (Hanahan and Weinberg 2000, 2011). For example, some cancer cells synthesize growth factors to which they are responsive. In other cases, they overexpress growth factor receptors, making them responsive to very low levels of surrounding growth factors or eliciting ligand-independent signaling. They can also express anomalous forms of the receptors with constitutive activity. In addition, cancer cells often present

alterations in the main elements of the intracellular signaling cascades that drive cell proliferation. By these and other strategies, cancer cells become self-sufficient in growth signals and therefore acquire an increased and uncontrolled proliferative potential (Hanahan and Weinberg 2000, 2011).

5.2.1 Effects on Cancer Cell Proliferation

Recent work suggests that overexpression of GPR55 may be one of the strategies followed by cancer cells to increase their proliferation rates (Fig. 5.2). By modulating the expression levels of GPR55, Andradas et al. demonstrated that this receptor confers a proliferative advantage on human embryonic kidney HEK293 cells and different cancer cell lines (Andradas et al. 2011). Thus, selective GPR55 knockdown by small interference RNA (siRNA) significantly reduced EVSA-T (human breast cancer cell) proliferation (Andradas et al. 2011). The opposite experimental approach (overexpression of the receptor) had the opposite consequence (exacerbated cell proliferation) (Andradas et al. 2011). These effects were also observed in T98G (human glioblastoma) and MIA PaCa-2 (human pancreatic adenocarcinoma) cells (Andradas et al. 2011). Simultaneously, Piñeiro et al. demonstrated that GPR55 downregulation blocked proliferation of OVCAR3 (human ovarian adenocarcinoma) and PC3 (human prostate adenocarcinoma) cells (Piñeiro et al. 2011), suggesting that the GPR55-associated proliferative advantage may have a general (and not tumor type-specific) nature. Importantly, the capability of GPR55 to promote cancer cell proliferation was also demonstrated in vivo. Selective receptor knockdown in glioblastoma xenografts efficiently diminished tumor growth, an effect that was mostly produced by the decrease in cancer cell proliferation potential (Andradas et al. 2011).

It is important to highlight that the experiments mentioned above were carried out in the absence of exogenously supplied GPR55 agonists. Therefore, the enhanced proliferation rates observed upon GPR55 overexpression may be the consequence of a ligand-independent constitutive activation of the receptor and/or the activation of the receptor by endogenously produced agonists. It is well accepted that numerous GPCRs exhibit spontaneous activity in the absence of agonists (Bond and Ijzerman 2006; Smit et al. 2007; Tao 2008). According to the two-state model of receptor activation, GPCRs exist in an equilibrium between an active state (R*) and an inactive state (R). R*couples to G proteins, activating the corresponding downstream signaling pathways, and shows a higher affinity for agonists than R. Thus, agonists activate the receptor by stabilizing the R* state. Some receptors spontaneously adopt the R* state in the absence of agonists, acquiring constitutive activity, a phenomenon with important physiopathological consequences (Bond and Ijzerman 2006; Smit et al. 2007; Tao 2008). To date, no information is available on the degree of spontaneous activity of GPR55, but it is interesting to point out that a prominent example of GPCRs that exhibit high constitutive activity are two very close relatives of GPR55: the cannabinoid receptors CB_1 and CB_2 (Bouaboula et al. 1997; Howlett et al. 2011; Portier et al. 1999).

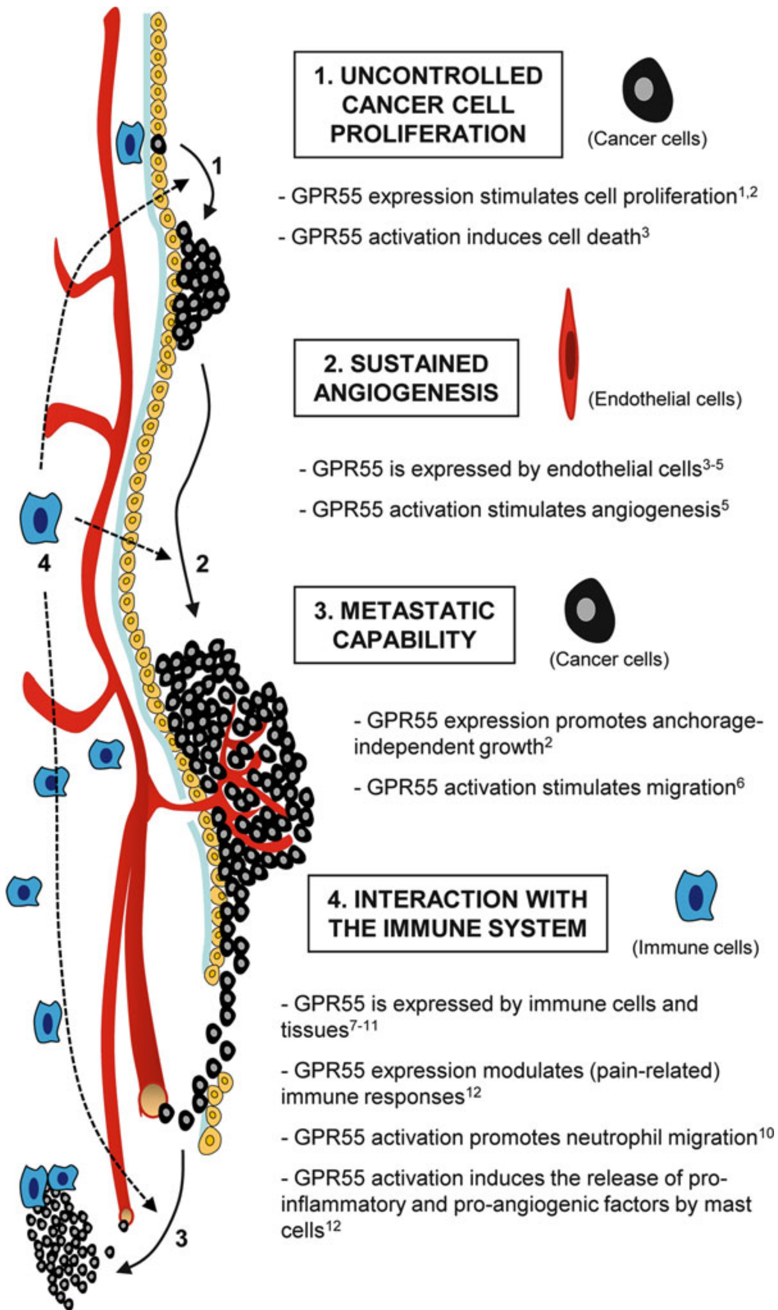

Fig. 5.2 The potential role of GPR55 in different steps of cancer. Diagram summarizes our current knowledge on the involvement of GPR55 in tumor physiopathology. GPR55 seems to modulate the basic driving forces of malignant growth, including uncontrolled cancer cell proliferation, sustained angiogenesis, metastatic capability, and the interaction with the immune system. The respective cellular targets are indicated in the figure. *1* Andradas et al. (2011); *2* Piñeiro et al. (2011); *3* Waldeck-Weiemair et al. (2008); *4* Daly et al. (2010); *5* Zhang et al. (2010); *6* Ford et al. (2010); *7* Ryberg et al. (2007); *8* Sawzdargo et al. (1999); *9* Pietr et al. (2009); *10* Balenga et al. (2011); *11* Cantarella et al. (2011); *12* Stanton et al. (2008)

An alternative (or complementary) explanation for the proliferation-inducing effects observed after GPR55 overexpression is the presence of GPR55 ligands in the experimental setting. Works by Andradas et al. (2011) and Piñeiro et al. (2011) demonstrate the existence of an endogenous tone of LPI, a proposed endogenous GPR55 agonist, in cell culture. Using different methodological approaches, the two groups showed that HEK293 and cancer cells generate LPI from metabolic precursors through the action of a cytosolic phospholipase A2 (cPLA2). Pharmacological blockade of this enzyme in HEK293 cells blocked the increased proliferation induced by GPR55 overexpression (Andradas et al. 2011). Genetic ablation of cPLA2 by using selective siRNA had the same effect on PC3 cells (Piñeiro et al. 2011). Interestingly, the decrease in cell proliferation induced by cPLA2 silencing was rescued by the addition of exogenous LPI (Piñeiro et al. 2011), consistent with the hypothesis that cancer cells endogenously generate a mitogenic GPR55 agonist (LPI) through the action of cPLA2. There is evidence that at least human gynecological tumors may use this strategy to stimulate cancer cell proliferation. Elevated levels of LPI have been detected in ascites (Xiao et al. 2001) and plasma (Sutphen et al. 2004) from patients with ovarian cancer compared with samples from healthy controls or patients with non-tumor diseases. The most prominent LPI species in these patients' plasma was arachidonoyl-LPI (Sutphen et al. 2004), the LPI species that engages GPR55 most potently (Oka et al. 2009). Whether patients with other types of tumors present elevated LPI plasma levels deserves further investigation.

Another possibility that could explain the effect of GPR55 on cancer cell proliferation in the absence of exogenously applied ligands is that GPR55 may interfere with the signaling of other receptors. It has been recently proposed that GPR55 modulates CB_2 receptor-mediated responses (Balenga et al. 2011). In this study, Balenga et al. showed that LPI, via GPR55, induces the rearrangement of the actin cytoskeleton in HEK293 cells. This effect was attenuated by the presence of nonactivated CB_2 receptors. Moreover, when both GPR55 and CB_2 receptors were activated (with LPI and 2-AG, respectively), LPI-induced effects in HEK293 cells (filamentous actin formation, nuclear factor of activated T-cells [NFAT] activation and dynamic mass redistribution) were dramatically enhanced, while the 2-AG effects in neutrophils and HL60 cells (increase in reactive oxygen species [ROS] production) were significantly inhibited (Balenga et al. 2011). Together, these data suggest that GPR55 modulates the activity of CB_2 receptors, and vice versa, in HEK293 and neutrophils. It is tempting to speculate that this may also be the case of cancer cells, in which CB_2 receptors play an important role in controlling proliferation (Caffarel et al. 2006, 2010; Carracedo et al. 2006; Cianchi et al. 2008; Gustafsson et al. 2008; Qamri et al. 2009; Sánchez et al. 2001).

5.2.2 *Signaling Mechanisms*

Regarding the signaling pathways activated by GPR55 in cancer cells, both Andradas et al. and Piñeiro et al. demonstrated that GPR55 controls the activity of one of the master regulators of cell proliferation: the extracellular signal-regulated kinase

(ERK) cascade (Andradas et al. 2011; Piñeiro et al. 2011). Activation of this cascade upon GPR55 engagement has been previously observed in different cell systems, including HEK293 cells (Henstridge et al. 2010; Oka et al. 2007, 2009), osteoclasts (Whyte et al. 2009), human dermal microvascular endothelial cells (Zhang et al. 2010), and U2OS cells (Kapur et al. 2009; Kotsikorou et al. 2011). Andradas et al. (2011) showed that modulation of GPR55 levels in HEK293, EVSA-T, and T98G cells produced parallel changes in the activity of the cascade. Thus, the levels of phosphorylated ERK and the ERK-regulated transcription factor c-Fos were increased in response to GPR55 overexpression and reduced upon receptor knockdown in cell cultures (Andradas et al. 2011). The effect on phospho-ERK and c-Fos levels following GPR55 overexpression was prevented by pharmacological inhibition of the ERK kinase MEK (Andradas et al. 2011). Importantly, decreased ERK activity upon GPR55 silencing was also observed in vivo (Andradas et al. 2011). Piñeiro et al. (2011) did not observe changes in the basal levels of phospho-ERK after GPR55 silencing, but clearly showed that activation of the receptor by adding LPI to PC3 and OVCAR3 cell cultures stimulated ERK activity (Piñeiro et al. 2011). Genetic (by selective siRNA) and pharmacological (with cannabidiol, CBD) blockade of GPR55 prevented this effect (Piñeiro et al. 2011), consistent with the hypothesis that GPR55 controls cancer cell proliferation by modulating the ERK cascade.

Piñeiro et al. also observed that GPR55 silencing diminished the basal levels of Akt (Piñeiro et al. 2011), a key component of the pro-tumorigenic phosphoinositide 3-kinase (PI3K)/Akt signaling pathway (Engelman 2009; Vivanco and Sawyers 2002). Whether this change is a further cause of the effects on proliferation or a mere bystander alteration is still unknown. Oka et al. (2010) demonstrated that LPI induces the phosphorylation of p38 MAPK in IM-9 B-lymphoblastoid cells, an action that was prevented by GPR55 silencing (Oka et al. 2010). Interestingly, this effect on p38 was not observed in other human leukemia (Jurkat) or lymphoma (Raji and Daudi) cells which express GPR55 at low level (Oka et al. 2010). It would be interesting to analyze whether the described changes in p38 phosphorylation have any consequence on IM-9 cell proliferation, or whether LPI also affects the ERK cascade or the PI3K/Akt pathway.

5.3 GPR55 and Cancer Cell Death

The uncontrolled proliferation that characterizes cancer cells is the result of not only acquisition of an increased proliferative potential but also a reduced responsiveness to antiproliferative/death signals (Hanahan and Weinberg 2000). As mentioned in the previous section, GPR55 favors cancer cell proliferation. Accordingly, we can expect reduced (or at least unchanged) death rates resulting from GPR55 activation. Indeed, pharmacologic activation of this receptor reduces cancer cell proliferation by inducing cancer cell death (Huang et al. 2011) (Fig. 5.2). Huang et al. (2011) showed that the GPR55 agonist O-1602 and anandamide (AEA), an endocannabinoid that has been shown to activate GPR55 in some experimental

settings (Lauckner et al. 2008; Ryberg et al. 2007; Waldeck-Weiermair et al. 2008), reduce cholangiocarcinoma cell viability by inducing apoptosis both in cell cultures and in a xenograft-based model of cancer. The authors also generated a cholangiocarcinoma cell line in which GPR55 expression was stably silenced by selective short hairpin RNA (shRNA) and observed that these cells were insensitive to O-1602 and AEA in vitro and in vivo (Huang et al. 2011), consistent with the idea that both compounds were, at least partially, acting through GPR55. The study of the molecular mechanisms underlying AEA- and O-1602-induced cancer cell death revealed that it involves the recruitment of the Fas death receptor complex into lipid rafts (together with GPR55 itself) and the activation of the c-Jun N-terminal kinase (JNK) cascade (Huang et al. 2011). However, these findings deserve further investigation as the pharmacological actions of AEA and O-1602 have not been fully established. For example, AEA may act through a large variety of metabotropic and ionotropic receptors either directly or by binding to biomembranes, or may generate a wide array of lipid metabolites with potential cytotoxic capacity (Pertwee et al. 2010). In addition, the precise pharmacological profile of O-1602 remains to be clearly established.

Evidence shows that cannabinoids from different origins (plant-derived, endogenous, and synthetic) induce antitumor effects (Guzmán 2003; Sarfaraz et al. 2008; Velasco et al. 2007). Most of these actions are mediated by the activation of the classical cannabinoid receptors CB_1 and/or CB_2, but the involvement of additional receptors cannot be ruled out. In most cases, the participation of CB_1 and/or CB_2 receptors has been demonstrated by using selective antagonists (see original articles reviewed in Guzmán 2003; Sarfaraz et al. 2008; Velasco et al. 2007). However, these antagonists did not always exert a full prevention of the agonists' actions. Moreover, for some cannabinoid compounds, a specific receptor target has not yet been defined. One such compound is CBD. It is well described that CBD exerts antitumor effects on different types of cancer cells (Izzo et al. 2009; McAllister et al. 2011), but its precise mechanism of action remains obscure (Izzo et al. 2009). Just to mention a few examples, CBD inhibits AEA inactivation, and modulates transient receptor potential (TRP) channels, the peroxisome proliferator-activated receptor γ (PPARγ), lipoxygenase (LOX), and cyclooxygenase-2 (COX-2) (Izzo et al. 2009). CBD has very low affinity for cannabinoid CB_1 and CB_2 receptors. However, it has been shown to act as a GPR55 antagonist (Ford et al. 2010; Piñeiro et al. 2011; Ryberg et al. 2007; Whyte et al. 2009). It would be therefore interesting to analyze whether the antitumor responses elicited by CBD are mediated by acting on GPR55 receptors.

The apparently conflicting notion that a GPCR may be associated with cancer cell proliferation or death responses is not new in the field of lipid-sensing GPCRs in general, or cannabinoid receptors in particular. For example, it is well described that CB_2 receptor activation produces antitumor responses in different models of breast (Caffarel et al. 2006, 2010; Ligresti et al. 2006; Qamri et al. 2009), prostate (Olea-Herrero et al. 2009), endometrial (Guida et al. 2010), lung (Preet et al. 2011), colon (Cianchi et al. 2008), thyroid (Shi et al. 2008), skin (Blazquez et al. 2006), and pancreatic cancer (Carracedo et al. 2006), as well as in models of glioma

(Aguado et al. 2007; Blazquez et al. 2008; Massi et al. 2004; Sánchez et al. 2001) and tumors of immune origin (Herrera et al. 2005; McKallip et al. 2002, 2006). At the same time, it is known that CB_2 (as is GPR55) is overexpressed in many types of tumors compared to healthy matching tissue (Caffarel et al. 2006, 2010; Calatozzolo et al. 2007; Carracedo et al. 2006; Cianchi et al. 2008; De Jesus et al. 2010; Guida et al. 2010; Gustafsson et al. 2008; Qamri et al. 2009; Rayman et al. 2007; Xu et al. 2006), and in high histological grade tumors (highly aggressive) compared to the respective low histological grade tumors (less aggressive) (Caffarel et al. 2006, 2010; Calatozzolo et al. 2007; Ellert-Miklaszewska et al. 2007; Sánchez et al. 2001). Moreover, CB_2 might share with GPR55 some proto-oncogenic properties. For instance, a series of reports by Delwel's group suggests that CB_2 receptor aberrant expression due to retroviral integration may play a tumorigenic role in some virus-induced leukemias. These authors showed that the gene encoding the CB_2 receptor (*cnr2*) is located near a common virus integration site in retrovirally induced murine myeloid leukemia cell lines (Valk et al. 1997), and that *cnr2* is in fact a frequent target for insertion of leukemia virus in primary tumors in mice (Valk et al. 1999). In these tumors, CB_2 receptor overexpression blocks the differentiation of myeloid precursor cells into neutrophils and enhances precursors' migration capability (Jorda et al. 2003a, b; Valk and Delwel 1998). Additional pro-oncogenic properties were attributed to CB_2 by Zheng et al. (2008) who showed that animals lacking CB_1 and CB_2 receptors were more resistant than their corresponding wild-type littermates to UV-induced skin carcinogenesis. The percentage of animals with papillomas after irradiation was dramatically reduced in the $CB_1/CB_2^{-/-}$ population, and $CB_1/CB_2^{-/-}$-derived papillomas were significantly fewer and smaller. Similar results have been recently obtained in our laboratory using $CB_2^{-/-}$ mice in a genetic model of breast cancer (Pérez-Gómez et al., unpublished data). In summary, further efforts should be made to understand the precise biological role of cannabinoid receptors (including GPR55) in tumor generation and progression.

5.4 GPR55 and Angiogenesis

A mass of rapidly growing cells within a tissue has extra metabolic needs that cannot be supported by normal tissue vasculature. Therefore, to grow beyond a certain size, incipient tumors have to develop the ability to generate a new vascular network to assure nutrient and oxygen supply and the removal of metabolic wastes (Hanahan and Weinberg 2000, 2011). The growth of new blood vessels (angiogenesis) not only allows the tumors to progress in terms of size but also provides an escape route for cancer cells to enter the circulation and reach remote organs to generate new metastases (Hanahan and Weinberg 2000, 2011). Angiogenesis in adult healthy tissues is a transitory process that is carefully regulated by positive and negative signals. Tumors are able to alter this equilibrium and switch on a sustained angiogenic program by increasing the expression of angiogenesis inducers and/or by down-regulating angiogenesis inhibitors (Hanahan and Weinberg 2000, 2011).

GPR55 is expressed by different populations of endothelial cells (Fig. 5.2). In particular, the receptor has been found in endothelial cells from human umbilical vein (HUVEC) (Waldeck-Weiermair et al. 2008), rat tail artery (Daly et al. 2010), and human microvasculature (HMVEC) (Zhang et al. 2010). In HUVEC, AEA induces an increase in intracellular Ca^{2+} that is not prevented by the CB_1 antagonist AM251 and not produced by the cannabinoid receptor agonist HU210, but that is mimicked by the GPR55 agonist O-1602, attenuated by GPR55 silencing, and increased upon GPR55 overexpression (Waldeck-Weiermair et al. 2008). These data indicate that HUVEC express GPR55 receptors that can be activated by AEA, thereby producing a raise in intracellular Ca^{2+} levels. Interestingly, activation of GPR55 in HMVEC stimulates tube formation in an in vitro angiogenesis assay, cell migration in transwell-based experiments, and the upregulation of the vascular endothelial growth factor (VEGF) (Zhang et al. 2010), one of the main angiogenesis-inducing signals (Hanahan and Weinberg 2000, 2011). Interestingly, downregulation of GPR55 by siRNA diminished per se both cell migration and tube formation (Zhang et al. 2010). Together, these results show that GPR55 modulates endothelial cell physiology and suggest that it could also play a role in tumor-related neoangiogenesis (Fig. 5.2). Further research should be carried out to confirm this attractive hypothesis.

5.5 GPR55 and Metastasis

The advanced steps of cancer are associated to the spread of cancer cells from the primary tumor into distant locations, generating new tumors in additional organs (i.e., metastases). In fact, metastases, rather than primary tumors, are responsible for most cancer-related deaths (Hanahan and Weinberg 2000, 2011). For cancer cells to form a metastasis, they have to acquire a series of functional capabilities which allows them to leave the tumor and enter the blood and lymph streams (intravasation), to survive in the circulatory system until they reach a new niche to colonize and, once there, to get out of the circulation (extravasation), seed in a foreign tissue, and generate a new tumor (Chambers et al. 2002). These capabilities are intimately related to changes in cell shape as well as in the cell–cell and cell–extracellular matrix interactions that keep normal cells tethered in the appropriate tissue context (Hanahan and Weinberg 2000, 2011). In general, adhesion molecules and other proteins favoring cytostasis are usually downregulated in cancer, while those associated to migratory phenotypes are typically upregulated (Hanahan and Weinberg 2000, 2011).

Anchorage-independent growth, a crucial feature of cancer cells during the metastatic spreading, is regulated by GPR55. Piñeiro et al. (2011) showed that the growth of PC-3 cells in soft agar was clearly inhibited by GPR55 downregulation. PC-3-derived colonies in the absence of GPR55 were not only less numerous but less organized and more spread than those formed by GPR55-expressing cells. These data suggest that GPR55 facilitates the growth of those cells that have lost attachment to the original tissue (Fig. 5.2). Recently, Ford et al. (2010) demonstrated that LPI

induces migration and elongation of the highly metastatic MDA-MB-231 human breast adenocarcinoma cell line. Unfortunately, these authors were unable to knockdown GPR55 in these cells and, therefore, it is still unclear if such effects were mediated by GPR55. This issue is especially relevant considering that LPI has been reported to induce cancer cell migration via additional targets, specifically via TRPV2 channels (Monet et al. 2009). Ford et al. (2010) also observed that MCF-7 cells (poorly metastatic human breast adenocarcinoma cells) express low levels of GPR55 compared with MDA-MB-231 cells. When GPR55 was overexpressed in MCF-7 cells, they acquired the ability to migrate towards a chemoattractant. Moreover, exogenous addition of LPI further increased this effect. Finally, GPR55 knockdown by selective siRNA completely blocked the LPI-enhanced migratory response in GPR55-overexpressing MCF-7 cells (Ford et al. 2010). In summary, these data suggest that GPR55 modulates migration of breast cancer cells (Fig. 5.2).

It is also important to point out that GPR55 does not couple to $G_{i/o}$ proteins (as its close relatives the cannabinoid receptors CB_1 and CB_2 usually do) but to $G_{12/13}$ (Balenga et al. 2011; Brown et al. 2011; Henstridge et al. 2009; Huang et al. 2011; Lauckner et al. 2008; Ryberg et al. 2007) and G_q (Lauckner et al. 2008) proteins (as its other close relatives, the lysophospholipid receptors (Choi et al. 2010)). This is especially relevant in the context of cancer because these heterotrimeric G proteins signal oncogenic effects (Dorsam and Gutkind 2007; Worzfeld et al. 2008). For example, their overexpression induces fibroblast transformation, and their activation (by binding of ligands to their corresponding GPCRs) enhances invasive potential and induces angiogenic responses (Kelly et al. 2006; Lappano and Maggiolini 2011).

An additional piece of data pointing to a functional link between GPR55 signaling and metastasis is the fact that GPR55 activates Rho GTPases (Balenga et al. 2011; Henstridge et al. 2009; Lauckner et al. 2008; Oka et al. 2010; Ryberg et al. 2007; Whyte et al. 2009). These proteins are well-established regulators of cell dynamics, an attribute that is based on their ability to modulate cytoskeleton organization, motility, and cell adhesion amongst other cellular functions (Jaffe and Hall 2005). Not surprisingly, in the context of cancer they have been extensively related to the promotion of tumor cell migration, invasion, and metastasis (Vega and Ridley 2008). In fact, the expression and/or activity of several Rho GTPases, including RhoA, Rac1, and Cdc42, is frequently increased in human tumors (Vega and Ridley 2008). By different methodological approaches, several groups have shown that pharmacological activation of GPR55 induces the activation of Rho GTPases. Pull-down assays have demonstrated that O-1602 (Ryberg et al. 2007) and LPI (Henstridge et al. 2009) enhance the levels of GTP-bound (i.e., active) RhoA in HEK293 cells via GPR55. The same effect (although in this case the precise member of the Rho family activated was not identified) was observed in human osteoclasts (Whyte et al. 2009). By using this technique, Ryberg et al. (2007) demonstrated that not only RhoA but also Cdc42 and Rac1 are activated by GPR55. The use of dominant-negative RhoA mutants further supported that this particular GTPase mediates some GPR55-triggered effects in HEK293 cells, including the increase in intracellular Ca^{2+} induced by THC, JWH-015 (Lauckner et al. 2008) or LPI (Henstridge et al. 2009), the LPI-induced stimulation of NFAT transcriptional activity (Henstridge et al. 2009), and the formation of F-fibers in the cytoskeleton in response to LPI

(Balenga et al. 2011). The involvement of Rho GTPases was further confirmed by blockade of some of these effects with inhibitors of Rho-associated protein kinase (ROCK, the main downstream target of RhoA) (Balenga et al. 2011; Henstridge et al. 2009; Oka et al. 2010).

Considering all the issues mentioned in this section, it is tempting to speculate that GPR55, by favoring anchorage-independent cancer cell growth, enhancing cancer cell migration, coupling to G_q and $G_{12/13}$ oncogenic signals, and activating Rho GTPases, may overall induce pro-metastatic effects.

5.6 GPR55 and Inflammation

It is increasingly apparent that malignant progression is not a cancer cell-autonomous process. The cross talk between tumor cells and their environment seems to be essential in every stage of cancer growth (Hanahan and Weinberg 2011). Among those cells modulating transformed cell behavior, attention has focused on inflammatory cells surrounding or infiltrating tumors. Immune cells are present, to a greater or lesser extent, in virtually all tumor masses, and their presence has been classically interpreted as an attempt by the immune system to halt tumor growth. Although this is true in some cases, recent evidence suggests that immune cells within the tumors can favor tumor formation and progression (Hanahan and Weinberg 2011). Thus, tumor-related inflammatory responses can be the source of growth factors that stimulate cancer cell proliferation, pro-angiogenic and pro-invasive signals, and mutagenic compounds (such as ROS) that increase cancer cell genomic instability (Hanahan and Weinberg 2011).

Evidence suggests the involvement of GPR55 in the modulation of immune responses (Fig. 5.2). First, this receptor is highly expressed in the spleen (Ryberg et al. 2007; Sawzdargo et al. 1999), microglia (Pietr et al. 2009), neutrophils (Balenga et al. 2011), and mast cells (Cantarella et al. 2011). Second, GPR55-deficient mice present a hyperalgesic phenotype in response to inflammatory and neuropathic pain that is probably due to an altered immune reaction (Staton et al. 2008). These animals have elevated basal levels of IL-2 in the paws and produce larger increases in IL-4, IL-10, IFNγ, and GM-CSF after injection with an inflammatory agent (Staton et al. 2008). Moreover, Balenga et al. (2011) have recently reported that activation of GPR55 promotes neutrophil chemotaxis. This group also observed that some CB_2 receptor-mediated effects on neutrophils, in particular the enhanced ROS production in response to receptor activation, are blocked by GPR55 activation (Balenga et al. 2011). Collectively, their data suggest that GPR55 and CB_2 cooperate in regulating neutrophil migration towards inflammatory foci, an effect that may have important implications in cancer physiopathology. More recently, Cantarella et al. (2011) reported that activation of GPR55 blocks the pro-inflammatory-induced release of nerve growth factor (NGF) by mast cells. They showed that conditioned medium of these activated mast cells induced HUVEC proliferation (due to the elevated levels of released NGF) (Cantarella et al. 2011). Based on these observations and previous results from the group showing

that NGF induces HUVEC proliferation and angiogenesis in vivo (Cantarella et al. 2002), these authors proposed that activation of GPR55 in mast cells may have anti-inflammatory effects by preventing NGF-induced angiogenic responses (Cantarella et al. 2011). Although this hypothesis has to be further tested, it is of potential interest for the field of cancer, in which inflammation and angiogenesis are critical processes that determine the aggressiveness of tumors.

5.7 Concluding Remarks

As summarized herein, several lines of evidence point to the involvement of GPR55 in the control of the different aspects governing malignant growth, including cancer cell proliferation, angiogenesis, and metastasis-related processes such as cancer cell migration. The experimental evidence supporting these ideas is, in most cases, still pretty limited and further research is needed to firmly establish the connection between GPR55 and cancer. Regarding cancer cell proliferation, it seems clear that GPR55 expression confers a proliferative advantage on cancer cells, although pharmacological activation of the receptor under other settings appears to elicit just the opposite effect. It would be important to determine the context-dependent features of this apparent inconsistency and whether this is a general feature of GPR55 signaling in other cancer-related processes. In addition, it will be necessary to analyze whether the pro-angiogenic properties of GPR55 are also present in tumor-associated vasculature and whether the migration-inducing effects of the receptor have a functional relevance in the metastatic process. To address these issues and improve our general knowledge on the GPR55-cancer link, appropriate in vivo models of angiogenesis, metastasis, and cancer in general should be extensively used. The development of a good GPR55 pharmacological armamentarium (selective agonists, antagonists, inverse agonists) is also essential to help expand this field of research. To date, the pharmacology of GPR55 is still quite controversial and most compounds with activity on this receptor bind to additional targets. It would be also very interesting to widen the studies on GPR55 expression in human tumor samples to determine whether this receptor (alone or in combination with other cannabinoid receptors, lysophospholipid receptors, and/or other genetic signatures) has any predictive or prognostic value.

In summary, we believe that there are promising data to look into the interesting possibility that GPR55 is a new therapeutic target in oncology, and that the study of the GPR55-cancer connection fully deserves the intense research efforts that are currently being made.

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Chapter 6
GPR18 and NAGly Signaling: New Members of the Endocannabinoid Family or Distant Cousins?

Douglas McHugh and Heather B. Bradshaw

Mammalian G protein-coupled receptors (GPCRs) constitute a superfamily of diverse proteins with hundreds of members (Bockaert and Philippe Pin 1999). All members have seven transmembrane domains but, on the basis of shared sequence motifs, they are grouped into four classes: A, B, C, and F/S (Horn et al. 1998). Cannabinoid receptors, CB_1 and CB_2, belong to the class A (i.e., rhodopsin-like) family of GPCRs and share considerable structural and phylogenetic homology (Elphick rev. 2010). We have recently identified another GPCR that is activated by the phytocannabinoid, Δ^9-THC, and by an oxygenated metabolite of the endogenous cannabinoid anandamide (*N*-arachidonoyl glycine; NAGly) (Bradshaw et al. 2009; McHugh et al. 2010, 2011). This GPCR, GPR18, likewise belongs to the class A family of GPCRs; however, its structural and phylogenic background are dissimilar to CB_1 and CB_2 (Pertwee et al. 2010). In the past few years the international community of researchers who study the cannabinoid system, and especially those involved with the pharmacological nomenclature of receptor/ligand interactions, have begun to debate how to identify and name recently de-orphanized GPCRs such as GPR18. Here, we will outline what is known about this receptor, the ligands that activate and block the receptor, and how these interactions fit within cannabinoid/endocannabinoid signaling.

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6.1 GPR18 Expression and Distribution

Until very recently, reports describing GPR18 arose unanticipated from various broad expression studies of GPCRs. In 1997, while exploiting relaxed stringency PCR to identify a receptor for gastrin-releasing hormone, Gantz et al. inadvertently isolated fragments of a novel 7TM GPCR, 331 amino acids long, from canine gastric mucosa and the human colonic cancer Colo 320DM cell line. Subsequent cloning and genomic library screening identified this gene as GPR18 (designated according to HGMW (Human Gene Mapping Workshop) nomenclature). Notably the human and canine clones were highly conserved (Gantz et al. 1997). Fluorescence in situ hybridization was used to localize GPR18 to human chromosome 13q32, where it clusters with Epstein–Barr virus-induced receptor 2 (EBI2) and the lipid receptors, cysteinyl leukotriene receptor 1 and 2 (CysL1 and CysL2) (Gantz et al. 1997; Rosenkilde et al. 2006). Northern blot analysis conducted by Gantz et al. in multiple human tissues reported GPR18 transcripts in spleen, thymus, peripheral blood leukocytes, small intestine, appendix, and lymph node—suggesting a possible role for GPR18 in immune system regulation. However, the most abundant expression observed was in the testis, where transcripts were found in several cell types. GPR18 mRNA was detected in gametes of all levels of differentiation, with the highest in the most terminally differentiated cells. Tissues lacking any apparent GPR18 mRNA included brain, heart, lung, liver, kidney, pancreas, colon, skeletal muscle, ovary, placenta, prostate, adrenal medulla, and adrenal cortex.

In a similar vein, Vassilatis et al. (2003) conducted a study examining the GPCR repertoires of humans and mice via RT-PCR tissue profiling. They reported the following four different expression levels for GPR18: *no expression*—amygdala, frontal cortex, hippocampus, liver, and muscle; *low expression*—cortex, thalamus, adrenal tissue, colon, intestine, kidney, prostate, skin, spleen, stomach, and uterus; *moderate expression*—lung, ovary, testis, thymus, and striatum; and *strong expression*—hypothalamus, thyroid, peripheral blood leukocytes, cerebellum, and brain stem (Vassilatis et al. 2003).

Kohno et al. (2006) found GPR18 while searching for chemokine receptors and GPCRs expressed in adult T-cell leukemia (ATL) cells. After cloning and further analysis, the study reported that GPR18 was more highly expressed in lymphocytes ($CD4^+$, $CD4^+CD45RA^+$, $CD4^+CD45RO^+$, $CD8^+$, and $CD19^+$) in comparison to monocytes. Stably transfected GPR18-expressing cell lines were created and used in conjunction with the Bioactive Lipid Library, Ca^{2+} mobilization, and cAMP assays to subsequently identify *N*-arachidonoyl glycine (NAGly) as an endogenous ligand for GPR18. Kohno et al. observed concentration-dependent inhibition of forskolin-stimulated cAMP production in GPR18-transfected CHO cells from 1 nM to 10 μM NAGly, with an IC_{50} value of 20 ± 8 nM. The NAGly-mediated inhibition was abolished by pertussis toxin (PTX), indicating GPR18 coupling to $G\alpha_i$ (Kohno et al. 2006).

The most current of the PCR-based GPCR screening investigations to involve GPR18 was published by Qin et al. (2011), who performed a comprehensive array-based, quantitative PCR analysis of the expression profile of 130 genes in three typical sites of melanoma metastases. A comparison between metastases and benign nevi revealed 16 genes that were significantly differentially expressed. Of these, GPR18 and the chemokine receptor, CCL4, had the greatest changes in expression levels, which were 24.1- and 27.4-fold higher, respectively, in metastatic cells. Subsequently, functional experiments in yeast and melanoma were designed to test the hypothesis that GPR18 mediates proliferative or anti-apoptotic signaling (Qin et al. 2011). They found that the GPR18 sequence deviated from other GPCRs at position 3.35, where an alanine is present in place of a normally highly conserved asparagine. Asparagine to alanine mutations at 3.35 have been previously shown to result in constitutive activity in the chemokine receptors, CXCR3 and CXCR4, precluding the requirement of an agonist ligand to activate them (Ballesteros et al. 1995). Qin et al. (2011) report that mutating the alanine back to asparagine at 3.35 resulted in the loss of constitutive activity of GPR18. This is of interest given that malignant cells are dependent on the constitutive or overexpression of driver genes for maintenance of cell survival or inhibition of apoptosis. Qin et al. (2011) found that in vitro siRNA-mediated knockdown of GPR18 in human melanoma cells enhanced death via apoptosis, in further support of their hypothesis that GPR18 mediates proliferative or anti-apoptotic signaling.

6.2 Pharmacology of GPR18

We have recently published two studies describing the pharmacology of NAGly, various cannabinoids, and other ligands at GPR18. The first (McHugh et al. 2010) investigated the relationship between GPR18 and the abnormal cannabidiol (Abn-CBD) receptor in BV-2 microglia. The Abn-CBD receptor is a prominent non-CB_1/non-CB_2 cannabinoid receptor, discriminated by means of various pharmacological and genetic tools, and implicated in the modulation of microglial, endothelial, and glioma cell migration, and a selection of cardiovascular responses (Franklin and Stella 2003; Walter et al. 2003; Begg et al. 2005; Mackie and Stella 2006; Járai et al. 1999; Offertáler et al. 2003; Mo et al. 2004; Vaccani et al. 2005). Using Boyden chamber migration experiments, yellow tetrazolium (MTT) conversion, in-cell Western, qPCR, and immunocytochemistry, we found that NAGly at sub-nanomolar, and Abn-CBD and O-1602 at low nanomolar, stimulated directed cell migration in both BV-2 microglia and HEK293-GPR18 transfected cells in a concentration-dependent manner, and that these compounds had no effect on non-transfected HEK293 cells; the migration effects were blocked or attenuated in both systems by the Abn-CBD receptor antagonist O-1918, and its low efficacy agonist, cannabidiol; NAGly promoted proliferation and activation of MAP kinases in BV-2 microglia and HEK293-GPR18

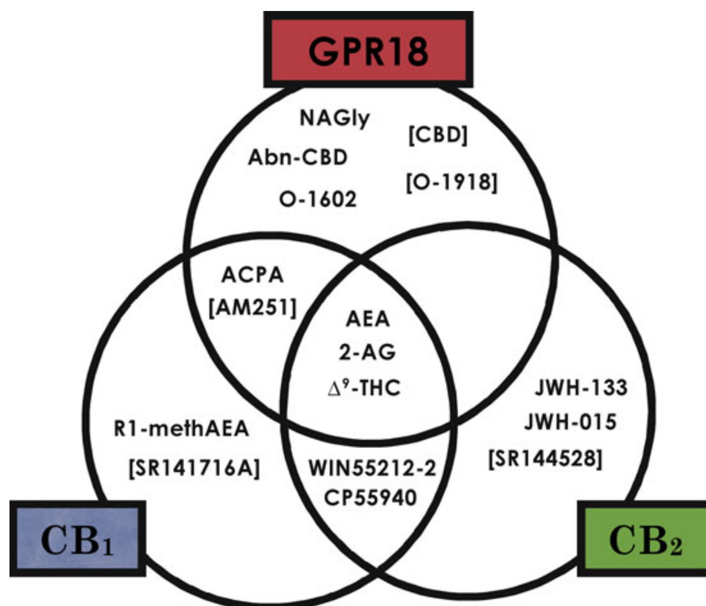

Fig. 6.1 Venn diagram indicating the agonist and antagonist activity that is selective for or shared between CB_1, CB_2, and GPR18 receptors with regard to various cannabinoid or related compounds. Compounds in *square parentheses* act as antagonists

cells at low nanomolar concentrations—cellular responses correlated with microglial migration; and BV-2 microglia displayed GPR18 immunocytochemical staining and abundant GPR18 mRNA, while qPCR demonstrated that primary microglia, likewise, express abundant amounts of GPR18 mRNA. At the 2011 symposium of the International Cannabinoid Research Society (ICRS), we presented data from siRNA GPR18 knockdown studies showing that migration induced by NAGly, O-1602, and Abn-CBD was significantly attenuated in GPR18 knockdown BV-2 cells compared to control, whereas migration to vehicle and fMLP (a formylated tripeptide chemoattractant ligand known to stimulate migration through its own distinct GPCRs) remained unchanged (McHugh et al. 2012). Collectively, these data provide definitive evidence that these compounds, characteristic of Abn-CBD receptor pharmacology, are acting via GPR18 in BV-2 microglia.

In the second of our two recent publications (McHugh et al. 2011), we presented evidence that the endocannabinoid system plays a regulatory role in human endometrial HEC-1B cell migration. Endogenous and phytocannabinoid effects implied a signaling mechanism mediated through CB_2 receptors and, to a greater extent, GPR18. The most effective activator of endometrial cell migration was

NAGly, and RT-qPCR revealed that HEC-1B endometrial cells express GPR18 mRNA. In addition, we screened for the specificity of cannabinoid ligands, including traditional CB_1 and CB_2 receptor agonists and antagonists, at GPR18 via p44/42 MAPK activation in stably transfected HEK293-GPR18 cells. In order of potency, NAGly, O-1602, Abn-CBD, Δ^9-THC, anandamide (AEA), and the selective CB_1 receptor agonist, ACPA, are full agonists at GPR18; cannabidiol (CBD) and the selective CB_1 receptor antagonist, AM251, are weak GPR18 partial agonists/antagonists; and the nonselective cannabinoid agonists, WIN55212-2, CP55940, R1-methAEA, JWH-133, and JWH-015, had no effect (for EC_{50} values refer to McHugh et al. 2011). Figure 6.1 shows a Venn diagram of our findings demonstrating the overlap of AEA, 2-AG, and Δ^9-THC for all three receptors as well as the specificity of agonists and antagonists at each receptor.

6.3 GPR18 and *N*-Arachidonoyl Glycine: Additional Members of the Endogenous Cannabinoid System?

Twenty-five years ago the endocannabinoid system was undiscovered and unimagined. Dr. Raphael Mechoulam and Dr. Yehiel Gaoni's key breakthrough in isolating Δ^9-tetrahydrocannabinol (Δ^9-THC) consequently led to the molecular cloning of CB_1 and CB_2 in the early 1990s (Mechoulam and Gaoni 1965). The identification of these two distinct GPCRs, in turn, triggered a search for an endogenous cannabinoid, which culminated in the identification of the lipid molecule *N*-arachidonoyl ethanolamide or AEA (Devane 1992). The relationship between AEA and NAGly has been similarly instrumental in uncovering cannabinoid signaling via GPR18 receptors, adding a missing piece to the endocannabinoid system puzzle. Our reports (McHugh et al. 2011, 2012) on GPR18 pharmacology and the activity of phyto- and synthetic cannabinoids certainly lend support to the hypothesis that GPR18 is a member of the endogenous cannabinoid system. Our data further support the hypothesis that NAGly is a potent endogenous ligand for GPR18, suggesting that the GPR18-NAGly signaling system is akin to the CB1-AEA signaling system. That AEA is the precursor of NAGly further illustrates how these systems intertwine. It is important to consider that many signaling molecules are often also intermediates for other, distinct signaling molecules, having sometimes opposing or counterbalancing effects. One of the most established examples would be the precursor, and primarily excitatory neurotransmitter, glutamate for the, primarily inhibitory, neurotransmitter GABA (Petroff 2002). The AEA-to-NAGly conversion has been shown to be through a pathway analogous to glutamate-to-GABA conversion, and fatty acid amide hydrolase-dependent (Fig. 6.2; Aneetha et al. 2009; Bradshaw et al. 2009, further emphasizing the close relationship of these two signaling molecules).

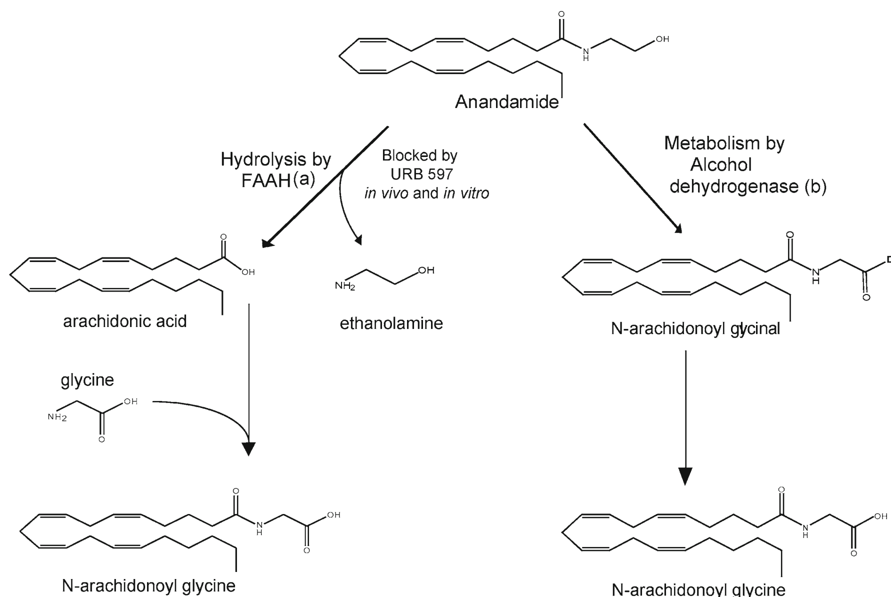

Fig. 6.2 Proposed biosynthetic pathways of *N*-arachidonoyl glycine (NAGly) from the precursor molecule, *N*-arachidonoyl (anandamide). (a) Bradshaw et al. (2009), (b) Aneetha et al. (2009), and Bradshaw et al. (2009)

6.4 Conclusions

We are now in the midst of major advances in the biochemistry and physiology associated with therapeutic actions of the endocannabinoids, including fertility, neurodegeneration and neuroprotection, learning and memory, anxiety, pain-relief, treatment of cancer, anti-nausea, appetite and obesity, and drug abuse (for review, see the *British Journal of Pharmacology*'s themed issue on the cannabinoids 2010). The discovery of a new endogenous ligand (NAGly) and novel, atypical cannabinoid receptor (GPR18) is an incredibly important contribution to our understanding of the molecular mechanisms responsible for the effects of both endogenous and phytocannabinoids.

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Chapter 7
Cannabinoid Signaling Through Non-CB_1R/Non-CB_2R Targets in Microglia

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7.1 Microglia

7.1.1 Introduction

Microglia are the resident macrophage-like cells of the central nervous system (CNS). They exhibit a ramified resting phenotype and perform continuous surveillance in order to maintain CNS homeostasis. However, under pathological conditions, they undergo a series of morphological changes leading to retraction of their ramifications and reaching the state of activated microglia (Davoust et al. 2008). Microglia play an important role in the brain's innate immunity and in the neuroinflammatory responses developed in response to various pathologies (Hanisch and Kettenmann 2007; Graeber and Streit 2010). They represent ca. 20% of the total non-neuronal cells in the CNS, and are activated by infections, injuries, or endogenously released neurotoxic factors (Soulet and Rivest 2008). Microglial activation is associated with production and secretion of a variety of mediators such as

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cytokines, reactive oxygen species (ROS), reactive nitrogen species (RNS), matrix metalloproteinases, and prostaglandins. Although microglial activation is considered a protective mechanism involved in regulating tissue repair and recovery, excessive or chronic activation can lead to harmful effects (Hanisch and Kettenmann 2007). The mechanisms that give rise to either the protective or the damaging microglial phenotypes are not fully elucidated. According to the "in vitro model of multi-step activation," microglia can be driven sequentially in response to multiple signals from resting ramified phenotype (quiescence state) to responsive (involved in chemotaxis and phagocytosis), primed (antigen presentation), and fully activated (cytotoxic) phenotypes. Each of these states is characterized by differential gene expression and acquisition of distinctive functional capabilities (Cabral et al. 2008). Enhancing the microglial-mediated innate immunity in the CNS while preventing the harmful effects associated with their chronic activation may offer new therapeutic approaches for the treatment of brain injury and neurodegenerative diseases.

Postnatal microglia in rodents and humans arise from two different pools of myeloid cell progenitors that colonize the developing CNS (for reviews, see Prinz and Mildner 2011; Prinz et al. 2011). The first pool of microglial progenitors invades the embryonic and fetal CNS, and derives from extramedullary sources of hematopoiesis, including the yolk sac (Chan et al. 2007). The second pool is formed by bone marrow-derived monocytic cells that colonize the CNS during the early postnatal period (P0–P15) in rodents, or before birth in humans (Davoust et al. 2008; Soulet and Rivest 2008). Bone marrow-derived microglial progenitors have been reported to penetrate the brain even in adult mice to replace senescent microglial cells. Moreover, in neurodegenerative diseases, bone marrow-derived progenitors, or circulating monocytes can engraft into the brain and become an integral part of the cellular network of the CNS. However, recent data suggest that resident microglia may be functionally distinct from bone marrow- or blood-derived phagocytes, which infiltrate the CNS under pathological conditions (Prinz et al. 2011). Thus, the differential distribution and differentiation of these specific subsets of myeloid cells may eventually allow the design of new therapeutic strategies aimed to induce or suppress CNS recruitment and/or differentiation of bone marrow-derived phagocytes in a large array of neurological and chronic progressive neurodegenerative disorders. Although the myelo-monocytic origin of microglia has now been widely accepted, some researchers believe that microglia are derived from neuroectodermal matrix cells that differentiate locally into microglia (Chan et al. 2007).

7.1.2 BV-2 Cells as a Microglial Cell Model

One of the microglial cell lines frequently used as a substitute for primary microglia is the BV-2 cell line. BV-2 cells are derived from raf/myc-immortalized murine neonatal microglia and exhibit morphological, phenotypic, and functional properties comparable to those of primary microglial cells (Blasi et al. 1990; Bocchini et al. 1992). These include expression of nonspecific esterase activity, phagocytic

ability, and the absence of peroxidase activity (Blasi et al. 1990). BV-2 cells release lysozyme and, when stimulated, also release interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) (Blasi et al. 1990; Bocchini et al. 1992). Similar mechanisms mediating microglial activation by lipopolysaccharide (LPS), β-amyloid or S100B were reported between BV-2 and microglia in primary cultures (Kim et al. 2004). Although BV-2 cells were extensively used as an in vitro model of microglia, this cell line does not always reliably model the activation and changes in phenotype measured in primary microglia in culture. For example, BV-2 cells do not reliably mimic the response of primary cultured microglial cells to LPS or to the cytokine, interferon-γ (IFNγ) (Häusler et al. 2002; de Jong et al. 2008; Horvath et al. 2008; Pietr et al. 2009). Accordingly, whenever possible it is important to compare results obtained in BV-2 cells to other microglial models.

BV-2 cells and microglia in primary culture express the components forming the cannabinoid signaling system, including endocannabinoids, endocannabinoid-like ligands, receptors, and enzymes that produce and inactivate these ligands (Pietr et al. 2009; Stella 2009; McHugh et al. 2010; Muccioli and Stella 2008; Tham et al. 2007; Stella 2004; Muccioli et al. 2007; Tsuboi et al. 2007; Marrs et al. 2010; Blankman et al. 2007; Fiskerstrand et al. 2010; Kreutz et al. 2009; Rimmerman et al. 2011, 2012). The specifics of the endocannabinoid signaling system in microglia are reviewed in the following sections.

7.2 Characterization of the Endocannabinoid System in Microglia

7.2.1 *CB_1R and CB_2R*

The diversity of physiological effects caused by marijuana and its bioactive constituents, the cannabinoids, suggests that different cannabinoid receptors/targets may be responsible for mediating their biological activities (Mechoulam 1986). To date, two types of cannabinoid receptors, CB_1R and CB_2R, have been identified at the molecular level. CB_1R is primarily, but not exclusively, expressed in the CNS and mediates many of the neurobehavioral and psychotropic effects of Δ^9-tetrahydrocannabinol (THC), the main psychoactive constituent of *Cannabis*. CB_1R is also expressed, but at lower levels, in testis, heart, vascular tissue and immune cells (Howlett et al. 2002). Our laboratory has shown that both primary microglia and BV-2 cells express low amounts of CB_1R mRNA and protein (Pietr et al. 2009; Rimmerman et al. 2011, 2012), in agreement with the studies showing that CB_1R is expressed by neonatal rat and mouse microglia at low levels (Carlisle et al. 2002; Facchinetti et al. 2003; Walter et al. 2003; Cabral and Marciano-Cabral 2005).

The CB_2R is mainly expressed by hematopoietic cells, with particularly high levels in B cells and natural killer cells (Howlett et al. 2002; Cabral and Staab 2005; Pertwee et al. 2010). CB_2R is also expressed by brain stem neuronal cells

(Van Sickle et al. 2005; Pertwee et al. 2010 and references therein). Many laboratories have shown that microglia in primary culture (prepared from human, rat, or mouse brain tissue), as well as BV-2 cells, express relatively high levels of CB_2R (Carlisle et al. 2002; Facchinetti et al. 2003; Walter et al. 2003; Núñez et al. 2004; Cabral and Marciano-Cabral 2005; Pietr et al. 2009; Stella 2009, 2010; Rimmerman et al. 2011, 2012) and that CB_2R ligands regulate inflammatory reactions and immune responses (Cabral and Staab 2005; Benito et al. 2007; Cabral et al. 2008; Cabral and Griffin-Thomas 2009; Romero-Sandoval et al. 2009; Stella 2010). Several reviews have highlighted the ligands, pharmacology and functions of CB_1R and CB_2R (Howlett et al. 2002; Pertwee and Ross 2002; Howlett 2005; Pertwee 2008; Cabral and Griffin-Thomas 2009; Stella 2009, 2010; Pertwee et al. 2010; Pacher and Mechoulam 2011; Stadel et al. 2011).

Another compound produced by the *Cannabis* plant is cannabidiol (CBD). Unlike THC, CBD has a low affinity for both CB_1R and CB_2R and is devoid of the typical psychotropic effects produced by *Cannabis* or THC mediated via CB_1R (Pertwee 2005; Mechoulam et al. 2007; Izzo et al. 2009). CBD produces diverse actions, including anticonvulsive, sedative, hypnotic, antipsychotic, anti-inflammatory, and neuroprotective properties (Mechoulam et al. 2002, 2007; Scuderi et al. 2009; Liu et al. 2010; Kozela et al. 2010, 2011; Juknat et al. 2011, 2012). As CBD is not a potent CB_1R or CB_2R ligand, these effects are probably mediated through other receptors/targets.

7.2.2 Endocannabinoid and Endocannabinoid-Like Ligands in Microglia

The two classical endocannabinoids, *N*-arachidonoyl ethanolamine (anandamide; AEA; Devane et al. 1992) and 2-arachidonoyl glycerol (2-AG; Vogel et al. 1994; Mechoulam et al. 1995; Sugiura et al. 1995) are produced by microglia (Walter et al. 2003; Carrier et al. 2004; Rimmerman et al. 2012). AEA is a member of a larger family of *N*-acyl ethanolamines (NAEs) that are produced in the body. The levels of AEA and of other NAEs increase following cell stimulation or in response to pathological conditions such as ischemia, neuronal damage, and stroke (Hansen et al. 1995, 2001; Ueda et al. 2005; Natarajan et al. 1986; Moesgaard et al. 1999; Di Marzo et al. 1994; Cadas et al. 1996; Walter et al. 2003). AEA acts as a partial agonist at both CB_1R and CB_2R, and activates other targets including the transient receptor potential vanilloid 1 (TRPV1) (Pertwee et al. 2010; Zygmunt et al. 1999; Di Marzo et al. 2001). A second NAE synthesized by microglia is *N*-palmitoyl ethanolamine (PEA), a saturated analog of AEA containing a 16:0 acyl chain (Muccioli and Stella 2008; Rimmerman et al. 2012; Franklin et al. 2003; Muccioli et al. 2009). PEA has anti-inflammatory and anti-nociceptive effects (Kuehl et al. 1957; Calignano et al. 1998; Lambert et al. 2002), and interacts with the nuclear receptor peroxisome proliferator-activated receptor alpha (PPARα), GPR119, and GPR55 (Lo Verme et al. 2005a, b; Fu et al. 2005; Overton et al. 2008; O'Sullivan 2007;

Godlewski et al. 2009). Our group recently demonstrated the presence of two additional NAEs in BV-2 cells, *N*-oleoyl ethanolamine (OEA) and *N*-stearoyl ethanolamine (SEA) (Rimmerman et al. 2012). OEA interacts with non-CB_1, non-CB_2 targets such as PPARα (Fu et al. 2003, 2005), and the orphan G protein-coupled receptor, GPR119 (Overton et al. 2008), while SEA interacts with a yet unidentified target to modulate cellular signaling (Maccarrone et al. 2001; Hansen 2010). Microglia also produce homo-γ-linolenyl ethanolamine and docosatetraenyl ethanolamine, lipids containing 20:3 and 22:4 acyl chains, respectively (Walter et al. 2003).

When examining the compartmentalization of NAEs into lipid rafts in BV-2 cells, we found that their compartmentalization deviates from that found in neurons. This is probably due to the lack of caveolin-1, and caveolae-type lipid rafts, as well as differential cholesterol trafficking in these cells (Rimmerman et al. 2011, 2012). In BV-2 cells, the levels of the saturated NAEs (PEA and SEA) increased within lipid rafts following CBD treatment (Rimmerman et al. 2012), whereas the levels of the non-saturated NAEs (AEA and OEA) were significantly increased in whole cells with growth media, but not in lipid rafts (Rimmerman et al. 2012). Thus, following CBD treatment and depending on the level of acyl chain saturation, NAEs increase in different cellular/membrane compartments. Our hypothesis is that CBD is enhancing NAEs accumulation/synthesis through specific NAEs metabolic pathways (Liu et al. 2006; Rimmerman et al. 2011), or via CBD-induced inhibition of NAEs enzymatic degradation or uptake (Watanabe et al. 1996; Rakhshan et al. 2000; Bisogno et al. 2001; Rimmerman et al. 2011, 2012).

7.2.3 Synthesis of Endocannabinoids and Endocannabinoid Like Lipids by Microglia

The major pathway for the production of NAEs is through *N*-acyl phosphatidyl ethanolamine (NAPE) via the enzyme *N*-acyl phosphatidyl ethanolamine-hydrolyzing phospholipase D (NAPE-PLD) (Schmid et al. 2000; Di Marzo et al. 1994; Hansen et al. 2000; Morishita et al. 2005). NAPE species containing saturated and monounsaturated acyl chains are generally more abundant than polyunsaturated acyl chains (Schmid and Berdyshev 2002; Okamoto et al. 2004). Following the production of different NAPE species, the NAPE-PLD enzyme (a member of the zinc-metallo-hydrolase family of the β-lactamase fold) catalyzes the hydrolysis of NAPE to NAEs (Okamoto et al. 2004; Ueda et al. 2005; Daiyasu et al. 2001). NAPE-PLD was found to be expressed in human microglia/macrophages from multiple sclerosis (MS) patient autopsies (Zhang et al. 2011). Results from our BV-2 gene array studies revealed no significant changes in the expression of NAPE-PLD following incubation with plant cannabinoids (THC or CBD) and/or LPS stimulation (Table 7.1; Rimmerman et al. 2011; Juknat et al. in preparation). Additional pathways involved in AEA synthesis have been reported (Sun et al. 2004; Liu et al. 2006; Kurahashi et al. 1997), among them a two-step pathway following LPS stimulation in macrophages consisting of: (1) conversion of NAPE via a phospholipase C

Table 7.1 Effects of treatment with LPS, CBD, CBD + LPS, THC or THC + LPS on the expression of endocannabinoid-related genes in BV-2 microglial cells

Accession	Gene	mRNA expression level (values obtained vs. CTR = 1)				
		LPS vs. CTR	CBD vs. CTR	CBD + LPS vs. CTR	THC vs. CTR	THC + LPS vs. CTR
NM_182806.1	GPR18	2.9	0.9	2.0	1.4	2.6
NM_183031.1	EB12	0.1	0.4	0.1	0.7	0.1
NM_009924	CB2	0.4	1.0	0.4	1.0	0.5
NM_007726.1	CB1	1.0	0.9	1.2	1.0	1.0
NM_178728.3	NAPE-PLD	1.2	0.9	0.8	0.9	0.8
NM_008699.1	NKX2-3	0.9	0.9	1.0	0.9	0.9
NM_025341.2	ABHD6	1.2	1.0	1.0	1.0	1.1
NM_024465	ABHD12	0.7	0.7	0.5	0.8	0.7

Cells were treated for 2 h with 10 μM THC or CBD. LPS (100 ng/mL) was then added and 4 h later the cells were harvested and analyzed using the MouseRef-8 v1.1 Expression BeadChip Illumina Array (Juknat et al. 2012)

(PLC) to phospho-AEA and (2) activation of a specific phosphatase (protein tyrosine phosphatase nonreceptor 22; ptpn22) to produce AEA (Liu et al. 2006). In this regard, we found that in BV-2 cells treatment with CBD (but not THC) upregulated ptpn22 mRNA by 90%, possibly increasing AEA levels via this pathway (Rimmerman et al. 2011).

Hydrolysis of NAEs to arachidonic acid and ethanolamine is mediated through several enzymatic pathways: (1) Fatty acid amide hydrolase (FAAH), a membrane bound serine-hydrolase, and (2) *N*-acyl ethanolamine hydrolyzing acid amidase (NAAA), a soluble protein with optimal activity at acidic pH (Giang and Cravatt 1997; Tsuboi et al. 2005). In addition, a third enzymatic pathway present in microglia that is sensitive to the monoacylglycerol lipase (MGL) inhibitor, URB602, has been proposed (Muccioli and Stella 2008).

Cultured rat cortical microglia express FAAH mRNA and protein, and display FAAH activity (Tham et al. 2007; Stella 2004; Muccioli et al. 2007). In fact, FAAH seems to be the major hydrolytic enzyme catalyzing AEA hydrolysis in these cells (Muccioli and Stella 2008). In line with this finding, NAAA concentrations are below the detection limit in primary microglial cells (Tsuboi et al. 2007).

PEA hydrolysis in BV-2 cell homogenates proceeds through FAAH-like enzymatic activity; however, experiments using intact cells suggest a more complex pathway (Muccioli and Stella 2008). While the involvement of NAAA was ruled out, a URB602-sensitive enzyme may be involved (Muccioli and Stella 2008). In this regard, it has been proposed that the differences between intact cell and homogenate studies may be due to differential subcellular localization of enzymes and substrates. This idea is in line with our findings on the differential compartmentalization of NAEs in BV-2 microglial cells, which is dependent on the level of acyl chain saturation (Rimmerman et al. 2012).

The second classical endocannabinoid, 2-AG, belongs to the family of 2-acyl glycerols. 2-AG displays binding affinities similar to that of AEA at CB_1R and CB_2R but exhibits greater efficacy at these receptors compared to AEA (Vogel et al. 1994; Mechoulam et al. 1995). We found that 2-AG is tonically produced in BV-2 microglial cells and that its levels do not change following CBD treatment in lipid rafts or whole cells + media fraction (Rimmerman et al. 2012). BV-2 cells efficiently hydrolyze 2-AG but do not express MGL mRNA (which is considered the main 2-AG metabolic enzyme) (Muccioli et al. 2007; Savinainen et al. 2012). Muccioli et al. (2007) reported that 2-AG hydrolysis in BV-2 cells is attributed to the serine hydrolase enzyme alpha/beta hydrolase 6 (ABHD6); however, this group showed that this enzyme is not involved in 2-AG hydrolysis in primary microglia (Marrs et al. 2010). Another serine hydrolase that may be involved in 2-AG hydrolysis is alpha/beta hydrolase 12 (ABHD12) which is expressed in microglia (Blankman et al. 2007; Fiskerstrand et al. 2010). Our gene array experiments on BV-2 cells reveal that ABHD6 and ABHD12 mRNA expression are not changed following incubation with CBD or THC (Rimmerman et al. 2011). However, pretreatment with CBD + LPS leads to a 50% reduction in ABHD12 mRNA levels. Additional research may shed light on the role of ABHD12 in controlling 2-AG hydrolysis in microglia.

The endogenous NAEs and 2-AG have multiple actions on microglia, some mediated by CB_1R/CB_2R while others mediated via non-CB_1/non-CB_2 targets (Stella 2010; Carrier et al. 2004; Correa et al. 2010; Kreutz et al. 2009). In the next section, we review these targets with emphasis on non-CB_1/non-CB_2 (G-protein-coupled receptors) GPCRs expressed by microglial cells.

7.3 Non-CB_1/Non-CB_2 GPCR Targets

7.3.1 The GPR55 Receptor

7.3.1.1 Introduction

The complex pharmacological properties of cannabinoids and endocannabinoids are not fully explained by CB_1R and CB_2R signal transduction. Increasing evidence suggests that some cannabinoids mediate their effects independently of these targets as non-CB_1R/CB_2R effects have been observed with a range of cannabinoid ligands, including CBD, AEA, virodhamine, the CBD-analog known as abnormal-CBD (abn-CBD), and its analog O-1602 (Mackie and Stella 2006; Kozela et al. 2010).

Two "deorphanized" GPCRs have been reported as potential non-CB_1R/non-CB_2R targets for cannabinoids, the GPR55 and the GPR18 (the latter is discussed in a following section of this chapter). GPR55 is activated by several plant and synthetic cannabinoids and the possibility that GPR55 constitutes the missing candidate cannabinoid receptor subtype has attracted attention (Ryberg et al. 2007).

In this regard, two patents from GlaxoSmithKline and AstraZeneca (Brown and Wise 2001; Drmota et al. 2004) reported that several cannabinoid receptor ligands bind to the orphan receptor GPR55 (see also Baker et al. 2006). GPR55 was originally identified in silico from the expressed sequence tags database and was shown to share some sequence homology with the purinoceptor-like orphan receptors GPR23 (30%) and GPR35 (27%), as well as with the purinoceptor $P2Y_5$ (29%) and the chemokine receptor CCR4 (23%) (Sawzdargo et al. 1999). GPR55 has a low level of amino acid sequence homology with the CB_1R (13.5%) and the CB_2R (14.4%) (Baker et al. 2006). Human GPR55 is a classical intron-less GPCR that maps to chromosome 2q37, and consists of 319 amino acids (http://www.uniprot.org/uniprot/Q9Y2T6).

GPR55 is expressed in specific brain areas, including the caudate nucleus, putamen, hippocampus, thalamus, pons, cerebellum, frontal cortex, and hypothalamus. It is also expressed in the adrenal glands, dorsal root ganglia, endothelial cells, and gastrointestinal tract (Sawzdargo et al. 1999; Ryberg et al. 2007; Lauckner et al. 2008). Recent data demonstrate that GPR55 is expressed in many human cancer cell lines, including glioblastoma, astrocytoma, B-cell myeloma, and lymphoblastoid cells (Oka et al. 2010; Andradas et al. 2011). Moreover, GPR55 expression in human tumors correlates with their malignancy/aggressiveness, as levels of this receptor are significantly increased in poorly differentiated tumors compared to less aggressive

tumors (Ross 2011). GPR55 initiates a number of signaling cascades and couples to $G_{\alpha12/13}$ and $G_{\alpha q11}$ proteins (Ryberg et al. 2007; Lauckner et al. 2008; Henstridge et al. 2009, 2010; for reviews, see Ross 2008; Nevalainen and Irving 2010; Sharir and Abood 2010; Balenga et al. 2011a). Accordingly, $G_{\alpha12/13}$ signaling promotes cancer cell proliferation, invasion, and metastatic spread (Worzfeld et al. 2008).

7.3.1.2 GPR55 Agonists/Antagonists: Downstream Signaling Pathways

The effect of agonists/antagonists on GPR55 activity has been demonstrated by using various reporter assays. These include the measurement of intracellular calcium, phosphorylation of the extracellular signal-regulated kinase (ERK), activation of small GTPase proteins (rho, rac, and cdc42) and [^{35}S]GTPγS binding assay. Several groups have also used β-arrestin and internalization assays to assess the properties of ligands on GPR55 (Henstridge et al. 2009; Kapur et al. 2009; Yin et al. 2009).

GPR55 is activated by cannabinoid ligands including THC, JWH015, AEA and its stable analog meth-anandamide. These ligands increase intracellular calcium release from IP_3-sensitive ER stores in mice dorsal root ganglion neurons and in hGPR55-HEK293 transiently transfected cells (Lauckner et al. 2008). This GPR55-mediated calcium increase is pertussis toxin (PTX)-insensitive and is transduced via $G_{\alpha q/11}$, PLC, $G_{\alpha12}$, rhoA and an intact actin cytoskeleton (Lauckner et al. 2008).

Other reports showed that a number of endocannabinoids and endocannabinoid-like compounds, including AEA, 2-AG, virodhamine, noladin ether, PEA and OEA, as well as other cannabinoids such as THC, abn-CBD, O-1602 (an abn-CBD analog), and the synthetic ligands CP55,940 and HU-210, stimulate GTPγS binding in GPR55-transfected cells with different efficacies (Johns et al. 2007; Ryberg et al. 2007). Moreover, the CB_1R antagonist AM251 acts as a GPR55 agonist in this GTPγS assay (Ryberg et al. 2007). CBD did not stimulate GTPγS binding, however, it did antagonize the agonistic effect of CP55,940. No functional activity was found for WIN55,212-2 (potent agonist at CB_1R and CB_2R), or AM281 (a CB_1R antagonist) (Johns et al. 2007; Ryberg et al. 2007; for reviews see Ross 2008; De Petrocellis and Di Marzo 2010; Sharir and Abood 2010).

Conversely, Oka et al. (2007) suggested that GPR55 is not a cannabinoid receptor as typical ligands such as AEA, 2-AG, PEA, OEA, virodhamine, CP55,940, HU-210, WIN55212-2, THC, abn-CBD and the CB_1R antagonist SR141716A had no effect on GPR55 activity, as measured by ERK phosphorylation activity in GPR55-expressing HEK293 cells. However, Oka et al. (2007) did find that the endogenous lipid L-α-lysophosphatidylinositol (LPI) induced rapid ERK phosphorylation and increased intracellular calcium in GPR55-expressing cells. Furthermore, LPI stimulated the binding of [^{35}S]GTPγS to the GPR55-expressing cells in a dose-dependent manner (Oka et al. 2007). A more recent study found that LPI induced the rapid phosphorylation of p38 mitogen-activated protein kinase in IM-9, a human lymphoblastoid cell line that naturally expresses GPR55 (Oka et al. 2010). LPI induces oscillatory calcium release from intracellular stores via activation of PLC in HEK293 cells overexpressing recombinant hGPR55. This LPI-mediated calcium

signaling involves $G_{\alpha 13}$ and activation of the nuclear factor of activated T cells (NFAT) via an RhoA-dependent signaling cascade (Henstridge et al. 2009).

Studies reported by Lauckner et al. (2008) reveal that GPR55-mediated signaling pathway requires G_{12}, RhoA and an intact actin cytoskeleton in order to release calcium from intracellular stores following activation of the receptor. In agreement with these results, Oka et al. (2010) showed that LPI stimulates p38 MAPK activity in HEK293 cells expressing GPR55, via the key molecules Rho and ROCK, suggesting that the $G_{12/13}$-RhoA-ROCK signaling pathway is mediating the LPI-GPR55-induced cellular events. A recent study used the β-arrestin PathHunter assay system to examine the pharmacological interactions of various lipids with a range of recently deorphanized GPCRs (Yin et al. 2009). AM251, SR141716A, and LPI were shown to have comparable efficacies in inducing β-arrestin trafficking and stimulating G-protein-depending activation of PKCβII as opposed to the synthetic cannabinoid agonist CP55,940 that blocked GPR55 internalization as well as formation of β-arrestin GPR55 complexes and ERK1/2 phosphorylation. CP55,940 induces little recruitment of PKCβII to membranes and did not stimulate membrane remodeling (Kapur et al. 2009). Agonist-induced GPR55 receptor internalization was shown by Henstridge et al. (2010). Analysis of HA-immunoreactivity using confocal microscopy showed that GPR55 was predominantly located on the cell surface; however, a pronounced redistribution of GPR55 into intracellular vesicles was observed after treatment with LPI, AM251, or SR141716A. These data show the coupling of GPR55 to the $G_{12/13}$-RhoA-ROCK signaling pathway and the involvement of GPR55 ligands (LPI, AM251, and SR141716A) in GPR55 internalization and remodeling of the cytoskeleton. Kotsikorou et al. (2011) identified a series of GPR55 agonists, using a high-throughput β-arrestin screen and a cell line that stably expresses β-arrestin-GFP biosensor. This approach led to the discovery of three novel GPR55 agonists, CID11792197, CID1172084 (ML185) and CID2440433 (ML184) that lack CB_1R or CB_2R activity and yet activate GPR55 with potency similar to LPI. The physiological relevance of GPR55 has been investigated in GPR55 knock-out mice (Staton et al. 2008; Whyte et al. 2009). These $GPR55^{-/-}$ mice are resistant to mechanical hyperalgesia and have increased levels of anti-inflammatory cytokines (IL-4 and IL-10) as compared with wild type animals. These data suggest that manipulation of GPR55 may have therapeutic potential for the treatment of inflammatory and neuropathic pain (Staton et al. 2008). Other studies have revealed a role for GPR55 in bone development and remodeling (Whyte et al. 2009; see Chap. 4), and showed that GPR55 is highly expressed in various cancer cell types and may regulate cancer cell proliferation (Andradas et al. 2011; Piñeiro et al. 2011; see Chap. 5). Moreover, an LPI/GPR55 interaction was demonstrated to be pivotal in the maintenance of an autocrine loop, involved in prostate cancer cell proliferation. Indeed, LPI synthesized intracellularly by cPLA2 can be released into the extracellular media by the transporter ABCC1 and thus, activate GPR55 and promote cell proliferation (Piñeiro et al. 2011). These results provide a strong evidence for the mechanism of LPI action in cancer cells and suggest that LPI has a role in cancer progression.

7.3.1.3 GPR55 in Microglial Cells

Our laboratory showed that GPR55 mRNA is expressed by both mouse microglia in primary culture and by BV-2 cells (Pietr et al. 2009). We also confirmed the presence of CB_2R mRNA in these cells, and demonstrated that the pattern of regulation of these mRNAs following microglial activation is very similar. Specifically, the level of GPR55 mRNA in mouse microglia was downregulated by LPS treatment (by 87%) and by IFNγ treatment (by 45%). Similarly, treatment of BV-2 cells with LPS induced a dose- and time-dependent downregulation of GPR55 mRNA. In contrast, IFNγ (at 50–200 U/mL) resulted in a concentration- and time-dependent upregulation of GPR55 mRNA in BV-2 cells. This pattern of modulation of GPR55 mRNA by LPS and IFNγ parallels that of CB_2R mRNA in mouse microglia and BV-2 cells.

Furthermore, we showed that the IFNγ-stimulated upregulation of GPR55 mRNA leads to increased GPR55 functionality as IFNγ induced an increase in LPI-dependent ERK1/2 phosphorylation (Pietr et al. 2009). The similarities in the modulatory pattern of GPR55 and CB_2R in both primary mouse and BV-2 microglia suggest that in addition to CB_2R, GPR55 could also have a role in CNS immunity and inflammatory signaling.

7.3.1.4 Potential Reasons for the Discrepancy in Reported GPR55 Pharmacology

As described above, GPR55 induces a range of downstream signaling events, and the activity of GPR55 ligands depends on the functional assay employed. In this regard, De Petrocellis and Di Marzo (2010) reported a hypothetical mechanism explaining this differential activation of GPR55. It was proposed that these putative cannabinoid GPR55 ligands can act either at cannabinoid receptors or at other targets, such as phospholipase A_1 (PLA_1) or PLA_2. It is through the later that the ligands would induce the formation of LPIs that consequently activate GPR55. In support of this possibility, endocannabinoids and THC, as well as synthetic cannabinoids, have been found to directly activate PLA_2 (Evans et al. 1987; Nabemoto et al. 2008). This proposed pathway through which cannabinoids could regulate GPR55 function would also explain why cannabinoids activate GPR55 in some cells but not in others.

Alternatively, differences in ligand effectiveness may be a consequence of the assay conditions. Many of the in vitro studies were performed using transfected cells overexpressing GPR55 (Johns et al. 2007; Ryberg et al. 2007; Lauckner et al. 2008; Henstridge et al. 2009; Kapur et al. 2009) and it has been shown that overexpressed receptors can induce constitutive activity that leads to alterations in the affinity/binding of the ligands and/or to changes in the efficacy of the allosteric response (Kenakin 2001, 2009).

A third possibility that may explain the variations in GPR55 response to cannabinoids could be as a consequence of its interaction with other GPCRs (e.g., by dimerization) such as has been observed for the heteromeric regulation of CB_1R

signaling (Hudson et al. 2010). Indeed, a crosstalk between GPR55 and CB_1R/CB_2R has been reported in human endothelial cells (Waldeck-Weiermair et al. 2008; Balenga et al. 2011b). Waldeck-Weiermair et al. (2008) showed that GPR55 signaling induced by AEA depends on the activation status of integrins, and that this response is negatively regulated by CB_1R. They demonstrated that in the absence of integrin clustering, CB_1R interacts with β1 integrin and AEA induces CB_1R signaling that couples to spleen tyrosine kinase Syk activation. Syk activation subsequently inhibits PI3K, an enzyme within the GPR55-mediated signaling cascade, thereby preventing GPR55 signal transduction. Once αvβ3 and α5β1 integrins' clustering occurs, AEA induces the release of CB_1R from β1 integrin, and deactivates CB_1R-induced activation of Syk, thus blocking GPR55-triggered signaling (Waldeck-Weiermair et al. 2008). Balenga et al. (2011a, b) reported that GPR55 synergizes with CB_2R to augment the directional migration of neutrophils toward sites of inflammation. This crosstalk between GPR55 and CB_2R leads to synergistic recruitment and efficient migration of neutrophils as well as prevents tissue injury mediated by myeloperoxidase release and ROS production.

Phosphorylation of ERK1/2 has been reported as one of the main signaling pathways initiated by stimulation of the GPR55 receptor. Using the high throughput system AlphaScreen® SureFire® phosphor-ERK assay, Anavi-Goffer et al. (2012) demonstrated that the CB_1 receptor antagonists AM251 and SR141716A can act both as GPR55 agonists and as inhibitors of LPI signaling. These compounds significantly decrease the LPI-maximal stimulation of ERK1/2 phosphorylation, suggesting kinetics of a noncompetitive inhibition and an allosteric behavior. This study provides the first evidence that certain cannabinoids can display both activation of GPR55 and inhibition of the LPI-mediated pERK stimulation under the same conditions, partially explaining the controversy surrounding the pharmacology of GPR55.

7.3.2 GPR18

7.3.2.1 Introduction

GPR18 is a GPCR whose gene is localized to human chromosome 13q32.3. The gene encodes an open reading frame of 993 bp (Samuelson et al. 1996; Gantz et al. 1997). GPR18 mRNA is expressed in testis, spleen, thymus, peripheral blood leukocytes, small intestine, appendiceal, and lymph node tissues (Gantz et al. 1997). GPR18 mRNA is highly expressed in peripheral lymphocyte subsets (CD4+, CD4+ CD45+ RA+, CD4+ CD45+ RO+, CD8+, CD19+). It is also detected in monocytes, various lymphoid cell lines (i.e., Jurkat, Molt-4, Hut78) and HTLV-1 transformed cell lines (Hut108, MT-2, MT-4), but not in several nonlymphoid hematopoietic cell lines, including U937, HL60, K562 (Kohno et al. 2006). Using quantitative qPCR, we demonstrated that GPR18 is abundantly expressed by primary microglia isolated from newborn mice, as well as by the murine microglial cell line BV-2 (McHugh et al. 2010).

7.3.2.2 GPR18 Gene Expression and Transcriptional Regulation

GPR18 lies in close chromosomal proximity to the orphan GPCR Epstein–Barr virus-induced G-protein-coupled receptor 2 (EBI2), which directs B-cell migration (Rosenkilde et al. 2006). Our gene array analysis of BV-2 cells following LPS and cannabinoid treatment revealed differential gene expression patterns for these two genes. EBI2 expression is strongly downregulated in cells stimulated with LPS even in the presence of the plant cannabinoids THC and CBD, while GPR18 mRNA expression was upregulated by LPS (Table 7.1). With regard to human immune cells, Kapitein et al. (2008) compared the gene expression profiles of peripheral CD4+ T cells of 6–year-old infants when classified as transient wheezers or persistent wheezers. They found that GPR18 mRNA expression was 1.9-fold higher in CD4+ T cells from infant persistent wheezers compared with healthy controls. A recent study on human melanoma metastasis revealed that *GPR18* mRNA was expressed at 24-fold higher levels in melanoma metastasis compared to benign nevi (Qin et al. 2011).

The transcriptional regulation of GPR18 is not well understood; however, a few recent reports connected GPR18 gene expression with immune regulatory pathways in T cells and B cells. Benita et al. (2010) addressed the question of transcriptional networks in T-cell development and differentiation. They identified ZBTB25, a transcription factor highly expressed in T cells, as a negative regulator of the transcription factor, NFAT. Using shRNA, they knocked down ZBTB25 in the Jurkat E-6 cell line. This knockdown of ZBTB25 significantly enhanced NFAT activity and resulted in induction of IL-2, CD25, and GPR18 gene transcripts (Benita et al. 2010). Yu et al. (2010) investigated the effects linked to the knockdown of the transcription factor NKX2-3, which is highly expressed by B cells and in intestinal tissues from Crohn's disease patients. When NKX2-3 was knocked down in B cells from these patients, GPR18 was downregulated by 4.1-fold compared with controls. The later finding suggests a negative relation between this transcription factor and GPR18 mRNA levels. Using gene array analysis of BV-2 cells, we found that NKX2-3 expression was not influenced by cannabinoids (CBD or THC), inflammatory stimuli (100 ng/mL LPS), and their combination (Table 7.1).

7.3.2.3 GPR18 Agonists/Antagonists

In 2006, Kohno and colleagues reported that the endocannabinoid-like compound *N*-arachidonoyl glycine (NAGly) is an endogenous ligand of GPR18. Later, McHugh et al. (2010, 2012) provided pharmacological evidence showing that NAGly and THC act as full agonists at GPR18. NAGly is a member of a family of glycine conjugates of long fatty acids (*N*-acyl glycines; NAGs) which have anti-nociceptive, anti-inflammatory, anti-proliferative, and migratory activities depending on the fatty acid species (Huang et al. 2001; Bradshaw et al. 2009a, b; Rimmerman et al. 2008; Burstein et al. 2002; Burstein and Salmonsen 2008; McHugh et al. 2010, 2012). NAGs differ from NAEs by the oxidation state of the carbon beta to the

amido nitrogen (Sheskin et al. 1997; Bradshaw et al. 2009a, b). Despite its structural similarity to AEA, NAGly lacks binding affinity to the CB_1R (Sheskin et al. 1997), and its effects are not blocked by the CB_2R antagonist SR144528 (Succar et al. 2007). Apart from GPR18, NAGly also activates GPR92 (Oh et al. 2008), potentiates α(1) and inhibits α(2) and α(3) glycine receptors (Yevenes and Zeilhofer 2011a, b). In addition, it inhibits the $Na^{(+)}/Cl^{(-)}$-dependent glycine transporter 2 (Wiles et al. 2006; Edington et al. 2009), and inhibits T-type calcium channels (Ross et al. 2009; Barbara et al. 2009). Thus, this compound has numerous biological effects.

Several metabolic pathways responsible for NAGs production have been proposed (for a full review see Bradshaw et al. 2009b); briefly, (1) oxidation of AEA via sequential enzymatic activity of alcohol dehydrogenase and aldehyde dehydrogenase (Sheskin et al. 1997; Burstein et al. 2000; Bradshaw et al. 2009a) through an *N*-arachidonoyl glycinal intermediate (Aneetha et al. 2009), (2) oxidation via cytochrome c (McCue et al. 2008; Mueller and Driscoll 2007), (3) conjugation of glycine to arachidonic acid, the latter was found to be derived from AEA hydrolysis (Bradshaw et al. 2009a), and (4) conjugation of acyl CoAs to glycine via various acyl transferases such as the human bile acid *N*-acyl transferase (O'Byrne et al. 2003), glycine *N*-acyl transferase, and glycine *N*-acyltransferase-like enzymes (Schachter and Taggart 1954; Nandi et al. 1979; Merkler et al. 1996; Waluk et al. 2010). NAGly metabolism was reported to proceed through FAAH and COX-2 (Bradshaw et al. 2009b; Prusakiewicz et al. 2002).

7.3.2.4 Signaling Cascades Through GPR18

Kohno et al. (2006) were the first to identify and characterize NAGly as an endogenous ligand for GPR18. Using a calcium mobilization assay, they screened a library of bioactive lipids and found that NAGly increased intracellular calcium in GPR18-expressing L929, CHO, and K562 cells. In addition, NAGly inhibited forskolin-stimulated cAMP production in GPR18-CHO cells with an IC_{50} of ~20 nM. This effect was blocked by PTX pretreatment, suggesting a $G\alpha_{i/o}$-coupled pathway (Kohno et al. 2006). McHugh et al. (2010) compared the effects of NAGly and abn-CBD on GPR18-expressing cells and concluded that GPR18 exhibits the same pharmacological profile as the putative abn-CBD receptor. They found that NAGly, O-1602, abn-CBD, and THC induce cellular migration and activate ERK phosphorylation in GPR18-expressing HEK293 cells (McHugh et al. 2010, 2012). In this regard the following EC_{50} values were provided for the activation of ERK phosphorylation in GPR18-expressing HEK293 cells; full agonists: NAGly (~44 nM), O-1602 (~65 nM), abn-CBD (~836 nM), THC (~960 nM), AEA (~3.8 μM); partial agonists/antagonists: CBD (~51 μM), AM251 (~96 μM). Similar results were obtained using the human endometrial cell line, HEC-1B, which endogenously expresses GPR18 (McHugh et al. 2012). In HEC-1B cells, NAGly and THC induced cell migration that was blocked by PTX, CBD, and AM251. Finally, mouse microglia and BV-2 cells endogenously express GPR18 mRNA at high levels (McHugh

et al. 2010). NAGly induced robust BV-2 cell migration that is inhibited by O-1918, and the low efficacy agonists, *N*-arachidonoyl serine or CBD. In addition, NAGly promoted cell proliferation (in concentrations up to ~10 nM), and activated ERK and JNK in the range of 10 nM to 10 μM. Interestingly, while the CB_1R antagonist SR141716 had no effect, the CB_2R antagonist SR144528 (at 100 nM) inhibited NAGly-induced BV-2 cell migration by ~63%. Since this effect did not occur in GPR18-expressing HEK293 cells, the authors suggested that this response may be due to: (1) the inverse agonist-mediated interference of constitutively active CB_2R or (2) another interaction between these receptors (McHugh et al. 2010). To summarize, NAGly is the most potent and efficacious endogenous ligand known to date for GPR18. Its signaling proceeds through a $G\alpha_{i/o}$-coupled G protein, it inhibits forskolin-stimulated cAMP, modulates intracellular calcium mobilization, activates ERK phosphorylation, and induces cellular migration in different cell lines.

7.4 Microglial Migration and Cannabinoid-Responsive Receptors

Cannabinoids are potent regulators of microglial cell migration through different pathways (Franklin et al. 2003; Franklin and Stella 2003; Walter et al. 2003; McHugh et al. 2010). Microglia exhibit spontaneous and random migratory capabilities (chemokinesis), or migration along a chemical gradient (chemotaxis) (for review see Kettenmann et al. 2011). A long list of cannabinoid and cannabinoid-like ligands has been shown to induce chemotaxis, through the activation of CB_2R and the abn-CBD receptor (the putative GPR18; McHugh et al. 2010). Walter et al. (2003) reported that 2-AG-induced migration of BV-2 cells is mediated through CB_2R and the abn-CBD receptor (with subsequent activation of ERK). Involvement at these targets was supported by their findings that 2-AG-induced migration was inhibited by PTX, CBD and O-1918 (abn-CBD receptor antagonists), as well as by SR144528 and cannabinol (CB_2R antagonists). Similarly, the AEA analog, arachidonyl cyclopropylamide, induced microglial cell migration that was blocked by PTX, SR144528, cannabinol, O1918, and CBD, but not by SR141617. Additional materials that were tested in this system included THC or PEA (no effect on BV-2 migration up to 3 μM), CBD (slightly increased migration), abn-CBD (significantly increased migration), AEA (dose-dependently increased migration), and HEA or DEA (increased migration). Interestingly, while PEA itself did not induce migration, it potentiated AEA-induced microglial migration (but not 2-AG-induced migration) (Franklin et al. 2003). PEA was suggested to act through a non-CB_1R/CB_2R/abn-CBD receptor $G_{i/o}$-coupled pathway (Walter et al. 2003; Franklin et al. 2003; Walter and Stella 2004). Furthermore, McHugh et al. (2010) reported similar results for BV-2 cell migration with the following rank order of potency: 2-AG > abn-CBD > AEA. They reported that NAGly and O-1602, which are described above as GPR18 agonists, were the most potent activators of BV-2 cell migration. Recent experiments by Fraga et al. (2011) show that BV-2 cells also migrate toward the

Trans-Activator of Transcription (Tat) protein, an inflammatory factor secreted following infection with the human immunodeficiency virus. Several cannabinoids, including THC, 2-AG, and CP55,940 but not arachidonyl-2-chloroethylamide, inhibited microglial cell migration toward Tat. This effect was mediated through CB_2R since it was blocked by SR144528 and by CB_2R siRNA knockdown. However, the involvement of GPR18 was not investigated.

Finally, little is known about how cannabinoids and cannabinoid-like compounds affect migration in vivo. Some insights come from experiments performed using brain slice cultures. Kreutz et al. (2009) reported that 2-AG and abn-CBD reduced microglial accumulation and neuronal damage following excitotoxic lesions in organotypic hippocampal slice cultures. They showed that abn-CBD receptor antagonists (i.e., CBD and O-1918), but not CB_1R or CB_2R antagonists (i.e., AM251 and AM630, respectively) reversed this effect. Additional studies are needed to shed more light on the in vivo migratory effects mediated through these receptors and the role of non-CB_1R/non-CB_2R targets.

7.5 Transcriptional Regulation by Plant Cannabinoids in Microglia

Our group has studied the effects of plant cannabinoids on immune cell function, mainly of microglia and T cells (Juknat et al. 2012; Kozela et al. 2011) . We characterized the effects of the two major cannabinoids present in marijuana, THC and CBD. As described above, whereas THC has high affinity for CB_1R and CB_2R, CBD has very low affinity for these targets and is devoid of psychotropic effects. However, CBD antagonizes some of the undesirable effects of THC, including sedation, intoxication and tachycardia, while sharing neuroprotective, antioxidative, antiemetic, and anticarcinogenic properties (Izzo et al. 2009).

To characterize the transcriptional effects of CBD and THC, we treated BV-2 cells with each cannabinoid and performed comparative microarray analysis using the Illumina MouseRef-8 BeadChip platform (Juknat et al. 2012). Microarray analysis based on a threshold of $p \leq 0.005$ revealed that 1,298 transcripts out of the 24,000 targets of the Illumina gene set were differentially regulated by the two cannabinoids. Of these changes, 680 transcripts were upregulated after 6 h treatment with CBD, whereas 58 transcripts were increased by THC. However, 22 of these transcripts were upregulated by either CBD or THC indicating that 36 genes were upregulated by THC and not by CBD. Moreover, CBD had a much larger effect compared with THC on the number of gene products that were downregulated; 524 gene products were downregulated by CBD, 36 by THC and only 7 by either THC or CBD ($p \leq 0.005$). Additional analyses further showed that CBD and THC had a greater effect on the number of gene products that were upregulated than on the number of genes whose expression was repressed, and that the changes in gene expression after THC treatment were more modest compared to those observed following exposure to CBD (Juknat et al. 2012).

Ingenuity Pathway Analysis (IPA; Ingenuity® Systems, www.ingenuity.com) was performed to identify the functional subsets of genes and networks regulated by CBD and/or THC. IPA and gene-by-gene inspection revealed that genes known to contain the amino acid response elements (AAREs) as well as the antioxidant response element/electrophile response element (ARE/EpRE) dominate the list of the CBD-upregulated transcripts, which were much less affected by THC. Genes containing the AAREs are known to respond to amino acid deprivation, and genes with the ARE/EpRE are reported to be regulated in response to oxidative stress. CBD-specific gene expression profile showed changes normally occurring under nutrient limiting conditions or proteasome inhibition, involving the general control nonderepressible 2 (GCN2)/eukaryotic initiation factor-2α (eIF2α)/nuclear protein 1 (p8)/activating transcription factor 4 (ATF4)/DNA-damage inducible transcript 3 (CHOP)-tribbles homolog 3 (TRIB3) pathway. Lastly, CBD, but to a lesser extent THC, regulated genes involved in the nuclear factor-erythroid 2-related factor 2/heme oxygenase 1 (Nrf2/Hmox1) axis, a pathway relevant for the restoration of the redox homeostasis and for the modulation of inflammatory responses (Juknat et al. 2012). The anti-inflammatory effects of CBD seem to correlate with upregulation of *Hmox1* and *IFNb1* mRNA expression, and downregulation of chemokine 2 *(Ccl2) mRNA* expression (via the IFNβ-STAT pathway, as described by Kozela et al. 2010). In summary, CBD decreases the activation of proinflammatory signaling by interfering with the TRIF-IFNβ-STAT pathway and by potentiating an anti-inflammatory negative feedback process via STAT3 (Kozela et al. 2010). The possibility of modulating or inhibiting these proinflammatory signaling networks makes CBD a promising anti-inflammatory agent (Juknat et al. 2011, 2012).

7.6 Evidence for Anti-inflammatory Effects of Plant Cannabinoids That Are Not Mediated by CB_1R/CB_2R in Microglia

We have previously reported that CBD and THC, acting independently of CB_1R and CB_2R induce different effects in LPS-stimulated BV-2 cells (Kozela et al. 2010). LPS activates the toll-like receptor 4 (TLR4) and induces changes in gene expression leading to the release of proinflammatory cytokines and neurotoxic factors (Gay and Gangloff 2007). LPS activates two main intracellular pathways via specific adaptor proteins: (1) the myeloid differentiation factor 88 (MyD88)-adaptor protein-dependent pathway that leads to activation of NF-κB-dependent transcription, and (2) the MyD88-independent pathway which is dependent on the toll-interleukin-1 receptor (TIR) domain-containing adaptor-inducing interferon-beta (TRIF) protein. TRIF turns on the interferon-regulated factor 3 (IRF3)-dependent pathway and enhances IFNβ production (Kawai et al. 2001). Through an autocrine response, IFNβ then acts via the type I interferon receptor and via signal transducers and activators of transcription (STAT)-dependent pathways, activating a second wave of gene expression that includes *Ccl2* mRNA.

Kozela et al. (2010) showed that pretreatment with THC or CBD significantly reduced the expression of LPS-upregulated IL-1β mRNA. Similarly, the level of IFNβ mRNA was decreased by THC and CBD. The effects of THC and CBD on IL-1β release were not blocked by CB_1R and CB_2R antagonists, (SR141716 and SR144528), or by abn-CBD, suggesting that these effects were not mediated through these targets. Additionally, CBD markedly decreased the LPS-upregulated mRNA expression of *Ccl2* by 58% whereas *Ccl2* mRNA expression was unaffected by THC (Kozela et al. 2010; Juknat et al. 2012).

LPS activation of TLR4 leads to IκB inactivation via IRAK-1-dependent phosphorylation of IκB, which is followed by ubiquitin-dependent degradation of both IRAK-1 and IκB (Gay and Gangloff 2007). Kozela et al. (2010) reported that CBD, but less so THC, partially reverses the LPS-induced degradation of IRAK-1 and of IκB in LPS-stimulated BV-2 cells. Several earlier studies suggested the involvement of the NF-κB pathway in cannabinoid-induced immunosuppression in macrophages (Jeon et al. 1996), thymocytes (Herring and Kaminski 1999), monocytes (Rajesh et al. 2007) and granuloma tissue (De Filippis et al. 2007), all of which were CB_2R-mediated. However, in our studies using BV-2 cells, neither CB_1R (which as described above is present in BV-2 in a low concentration; Pietr et al. 2009; Rimmerman et al. 2012) nor the CB_2R seem to be involved. Interestingly, the non-CB_1R/non-CB_2R-mediated anti-inflammatory effects of cannabinoids (mediated via the NF-κB pathway) were also detected in other cells, including astrocytes and neuronal PC12 cells (Curran et al. 2005; Esposito et al. 2006). Regarding the IFNβ pathway, we observed that although both THC and CBD reduce the activation of the proinflammatory STAT1, CBD (but not THC) strengthens the LPS-induced activation of STAT3. Thus, CBD seems to decrease the ongoing proinflammatory processes as well as intensify events counteracting inflammation (Kozela et al. 2010). In summary, these studies show that both THC and CBD exert inhibitory effects on the production and release of inflammatory cytokines in activated microglial cells in culture. However, their activities seem to involve both different and overlapping intracellular pathways; pathways that are not mediated through CB_1R/CB_2R or abn-CBD-sensitive receptors (Kozela et al. 2010). These results are in line with many other studies that point out that cannabinoids mediate CB_1R/CB_2R-independent mechanisms (Felder et al. 1992; Berdyshev 2000; Puffenbarger et al. 2000; Breivogel et al. 2001; Kaplan et al. 2003; Price et al. 2004; Chiba et al. 2011; Karmaus et al. 2011).

7.7 Relevance to Experimental Autoimmune Encephalomyelitis, an Animal Model of Multiple Sclerosis

It is well established that microglia become rapidly activated when the integrity of the CNS is disrupted as a consequence of lesions, neurotoxicity, infections, and autoimmune diseases (Hanisch and Kettenmann 2007). Microglial cells are considered to be key players in multiple sclerosis (MS), a neurodegenerative disease induced and

driven by dysfunctional immune system activity (Ponomarev et al. 2005). Arrest of microglial activation and function is thought to be beneficial in MS treatment (Heppner et al. 2005; Koning et al. 2009). Using the experimental autoimmune encephalomyelitis (EAE) animal model of MS, Kozela et al. (2011) demonstrated that CBD injections to myelin oligodendrocyte glycoprotein (MOG)-immunized C57BL/6 mice ameliorate EAE disease symptoms and diminish the activation of microglia (as measured by Iba-1 and Mac-2 expression in the spinal cords of these animals). Both Mac-2 and particularly Iba-1 are expression markers for cells belonging to the monocytic cell lineage, which includes microglia. These proteins are also expressed on some perivascular macrophages and on macrophages which infiltrate the CNS during pathological conditions. This suggests that the inhibitory activity of CBD may apply not only to microglia but possibly more generally to macrophage-like cells. Moreover, in in vitro experiments, we observed that CBD decreased the MOG-induced proliferation of encephalitogenic T cells (originally isolated from EAE mice previously immunized with MOG). This effect was not mediated via either the CB_1R or CB_2R. This potent anti-proliferative activity of CBD seems to have an important role in the CBD ameliorating effects in EAE, and agrees with the lower amounts of T cells present in the spinal cords of CBD-treated EAE mice (Kozela et al. 2011). Together these observations suggest that CBD and other cannabinoids, acting through non-CB_1R/CB_2R, could hold great potential for alleviating MS. Moreover, in similarity with the effect on EAE, these cannabinoids could relieve the symptoms of other autoimmune diseases. Indeed, reports from other groups showed beneficial effects of CBD on inflammatory bowel disease (Capasso et al. 2008) and on uveitis, a degenerative retinal disease (El-Remessy et al. 2008).

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Part III
Ion Channels

Chapter 8
Temperature-Sensitive Transient Receptor Potential Channels as Ionotropic Cannabinoid Receptors

Vincenzo Di Marzo and Luciano De Petrocellis

8.1 Thermo-TRPs: Pain and Beyond

Transient receptor potential (TRP) channels represent a superfamily of nonselective cation channels including at least six subfamilies: TRPC ("Canonical"), TRPV ("Vanilloid"), TRPM ("Melastatin"), TRPP ("Polycystin"), TRPML ("Mucolipin"), and TRPA ("Ankyrin") channels. They are six transmembrane (TM) domain, integral plasma membrane proteins, characterized by cytosolic C- and N-termini, and a non-selective cation-permeable pore region between the TM5 and TM6 α-helices (Fig. 8.1). The various subfamilies differ in particular for the number of ankyrin repeats present in their N-terminus, which range from 0 repeats in TRPM, TRPP, and TRPML channels to a much higher number in TRPA channels. The structure of the C-terminal domain also varies among subfamilies. So far, more than 50 members of the TRP family have been characterized in invertebrates and vertebrates, and 28 in mammals. They are involved in the transduction of physical stimuli, including temperature, mechanical and osmotic stimuli, electrical charge, light, hypotonic cell swelling; and chemical stimuli, such as xenobiotic substances (including olfactive and taste stimuli) and endogenous lipids (including plasma membrane components). They are regulated by posttranslational modifications (phosphorylation, alkylation of cystein, etc.) and the formation of homo- and hetero-dimers. Importantly, mutations in different TRPs have been linked to human diseases, and TRP expression is often increased in tissues affected by pathological conditions (Nelson et al. 2011; Moran et al. 2011).

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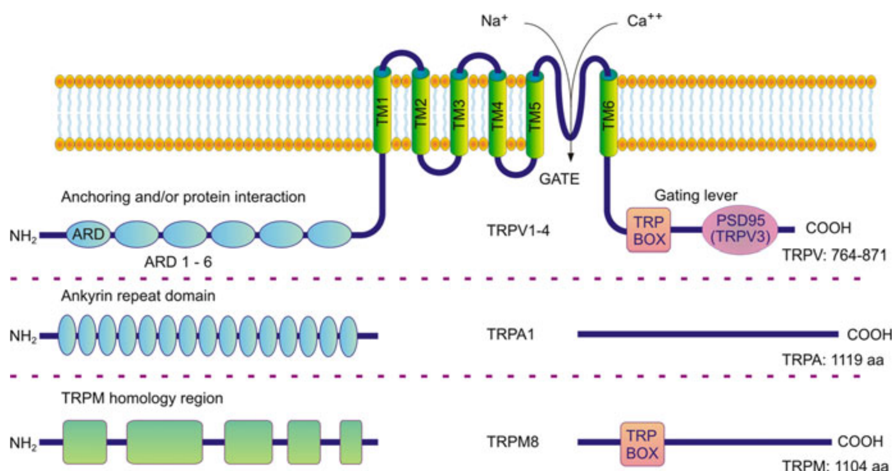

Fig. 8.1 Structure of some TRP channel families from Homo sapiens. The transient receptor potential (TRP) cation families contain different motifs in their amino and carboxyl termini. The TRP cation channel subfamily V (TRPV1, TRPV2, TRPV3, and TRPV4) and TRP cation channel subfamily A, type 1 (TRPA1) have amino terminal ankyrin repeat domains (ARD) that are not present in the TRP cation channel subfamily M, type 8 (TRPM8). The TRP box, which is found in the TRPV subfamily and TRPM8 seems to be involved in gating. *PSD95* postsynaptic density protein 95; *TM1-6* transmembrane domains

TRP channels of the vanilloid-type 1–4 (TRPV1–4), ankyrin type-1 (TRPA1), and melastatin type-8 (TRPM8) are involved in thermosensation, pain transduction, and inflammation. In fact, they are abundantly expressed in sensory fibers of Aδ and C-type, in dorsal root (DRG) and trigeminal ganglia as well as in perivascular neurons, with TRPV1 (the “capsaicin receptor”) and TRPA1 (the “mustard receptor”) being often co-expressed in the same nociceptor. Whilst TRPV1–4 are activated by temperatures higher than 37°C, TRPM8 (the “menthol receptor”) and TRPA1 (the “mustard receptor”) are activated by temperatures lower than 25°C. TRPV1 is activated by low pH and pro-inflammatory mediators, the combination of which leads to release of algogenic peptides (i.e., substance P, calcitonin gene-related peptide [CGRP]) from sensory neurons and thus contributes to neurogenic inflammation (Geppetti et al. 2008). TRPA1, instead, is activated by numerous irritant chemicals. TRPV1–4 are also expressed in central neurons. At the supra-spinal level, TRPV1 is abundant in neurons of the periaqueductal grey (PAG) and rostral ventrolateral medulla (RVM), where it modulates the descending pathway of antinociception. Contrary to its role in the spinal cord and sensory afferents, TRPV1 in the PAG-RVM contributes to descending antinociception, and it does so by enhancing both glutamatergic signaling/OFF neuron activity in the RVM and μ-opioid receptor-mediated analgesia (Starowicz et al. 2007; Maione et al. 2009). TRPV1 agonists, which usually desensitize the channel immediately after activation, together with TRPV1 antagonists, are currently under investigation for the development of new drugs against chronic and inflammatory pain. The role of TRPV1 in temperature

sensing also allows for a function in the regulation of basal temperature via both central, and, particularly, peripheral mechanisms (Moran et al. 2011).

In view of their presence in non-neuronal cells, evidence is accumulating for a role of thermo-TRPs in physiological and pathological conditions outside pain perception and in the regulation of body temperature, and their function in the etiology of cancer and bladder, skin, cardiovascular, pulmonary, and metabolic disorders has been recently outlined (see Moran et al. 2011 for a recent review). Furthermore, recent data point to the possible participation of TRPV1 in female (Cella et al. 2008) and, particularly, male (Francavilla et al. 2009) reproductive biology, osteoclast proliferation and activation (Rossi et al. 2011), and kidney disorders (Woudenberg-Vrenken et al. 2009). TRPV channels are also involved in skeletal muscle function (Iwata et al. 2009), and thermo-TRPs in gastrointestinal disorders (see Boesmans et al. 2011 for review). Finally, despite some recent controversy regarding its expression in central neurons, brain TRPV1 is emerging as a modulator of synaptic strength in various brain areas, including hippocampus, nucleus accumbens, and superior colliculus (see Di Marzo 2010 for review). Its role in regulating peripheral neuron microtubule disassembly, neuronal cone growth, synaptic sites, and cytoskeleton reorganization in general has also been established (Goswami et al. 2010; Han et al. 2007).

The observation of the ligand recognition, anatomical and functional similarities between TRPV1 channels and proteins of the endocannabinoid system (Di Marzo et al. 1998; Melck et al. 1999), namely cannabinoid CB_1 receptors and the putative membrane endocannabinoid transporter (Di Marzo 2008), raised the possibility that the thermo-TRP and endocannabinoid "worlds" could cross-talk at several levels (Table 8.1). Indeed, the first "endocannabinoid" (i.e., an endogenous ligand of cannabinoid receptors) to be discovered, anandamide (Devane et al. 1992) (Fig. 8.1), was also the first endogenous ligand of TRPV1 channels ever reported (Zygmunt et al. 1999), and therefore also designated as an "endovanilloid" (Di Marzo et al. 2001). After this important discovery, hundreds of papers have been published suggesting that thermo-TRPs, and TRPV1 in particular, might behave as "cannabinoid ionotropic receptors."

8.2 Effects at Thermo-TRPs of Endocannabinoids and Related Mediators

8.2.1 Anandamide and TRPV1

Although initially controversial because they were often observed at high concentrations and dosages, the TRPV1-mediated effects of anandamide observed since 1999 in both peripheral tissues and brain, and under both physiological and pathological conditions, have been the subject of over 200 reports and several reviews (reviewed in Ross 2003; Starowicz et al. 2007; Tóth et al. 2009), and will not be discussed in the present article. In the last 4 years, however, several studies have appeared in the literature (reviewed below), that have strengthened the hypothesis that anandamide

Table 8.1 Summary of overlapping TRPV1 and endocannabinoid system features

	CB_1		CB_2		Fatty acid amide hydrolase		Putative anandamide transporter
TRPV1	Common agonists (including endogenous agonists)	Co-expression in the spinal cord, DRG neurons, brain and osteoclasts	Common agonists (including endogenous agonists)	Co-expression in osteoclasts	Common inhibitors	Co-expression in the spinal cord and brain	Some inhibitors activate TRPV1
	Some CB_1 inverse agonists activate or antagonize TRPV1		Some CB_2 inverse agonists activate TRPV1				
References	Zygmunt et al. (1999), Huang et al. (2002), and Appendino et al. (2007)	Ahluwalia et al. (2000), Lever et al. (2009), Cristino et al. (2006), and Rossi et al. (2009, 2011)	Zygmunt et al. (1999), Huang et al. (2002), and Appendino et al. (2006)	Rossi et al. (2009, 2011)	Maione et al. (2007)	Lever et al. (2009) and Cristino et al. (2008)	Di Marzo et al. (1998) and Melck et al. (1999)

is an endovanilloid. A recent study, for example, showed that the hypolocomotor and hypothermic effects of this compound, which are present in CB_1 receptor null mice (Di Marzo et al. 2000), are instead absent in TRPV1 null mice (Garami et al. 2011).

With regard to sensory (and perivascular) neurons, in which the action of anandamide was demonstrated for the first time (Zygmunt et al. 1999), an elegant study reported how anandamide increases the responses to heat of carotid artery sinus nerve (petrosal) afferents by acting at TRPV1, thus possibly contributing to the physiological responses to mild hyperthermia of carotid bodies, and to the possible abnormal respiratory chemosensitivity in recurrent apnea syndromes (Roy et al. 2012). It was also shown that anandamide, via mild activation of TRPV1, can sensitize rat lung vagal afferents to capsaicin, adenosine, and mechanical (i.e., lung inflation) stimulation, and enhance adenosine-induced apneic responses (Lin et al. 2009). Anandamide, like other TRPV1 agonists, also causes nasal pain (Alenmyr et al. 2012). Finally, intra-plantar injection of anandamide in rats excite cutaneous C-nociceptor activity and produce nocifensive behaviors via TRPV1 activation, without altering withdrawal latency to radiant heat (Potenzieri et al. 2009). As shown through the use of selective antagonists and genetically modified mice, anandamide also inhibits and stimulates CGRP release from primary nociceptive mouse and rat neurons, at low and high concentrations, via CB_1 and TRPV1, respectively (Engel et al. 2011). Remarkably, the stimulatory effect was followed by desensitization to heat responses, suggesting that anandamide may inhibit pain and inflammation by activating and subsequently desensitizing TRPV1 channels (Engel et al. 2011). Also when administered intrathecally, or when its levels are increased through inhibition of fatty acid amide hydrolase (FAAH, the anandamide hydrolytic enzyme) with the compound URB597, anandamide can reduce hyperalgesia and allodynia in rats with neuropathic pain caused by chronic constriction injury of the sciatic nerve. This effect is mediated by either CB_1 or TRPV1 depending on whether the spinal levels of anandamide are low or high (Starowicz et al. 2012). In keratinocytes, anandamide produces anti-proliferative effects via a sequential activation of CB_1 and TRPV1, and subsequent Ca^{2+} influx (Tóth et al. 2011). This response may have possible relevance to the treatment of cutaneous disorders such as psoriasis and keratinocyte-derived tumors.

In reproduction biology, although it is now accepted that anandamide and methanandamide (a metabolically stable analogue) promote sperm capacitation by activating TRPV1, the role of CB_1 in this key function remains controversial (Maccarrone et al. 2005; Gervasi et al. 2011). On the other hand, anandamide seems to play opposing actions at the two types of receptors in rat placenta when it comes to nitric oxide (NO) synthase activity, which is stimulated via TRPV1 channels and inhibited via cannabinoid CB_1 (and CB_2) receptors (Cella et al. 2008).

In the brain, where biosynthetic and degrading enzymes for anandamide often co-localize with TRPV1 (Cristino et al. 2008), the opposing roles of this compound at CB_1 and TRPV1 receptors in the regulation of anxiety and fear have emerged from behavioral studies. These studies have investigated the effects of the anandamide analogue, arachidonoyl-2′-chloro-ethanolamide (ACEA), or the inhibitor of anandamide enzymatic hydrolysis, URB597, injected in the dorsal PAG or the prefrontal cortex in the presence of selective CB_1 and TRPV1 antagonists. The former antagonists

usually unmasked the contribution of TRPV1 to anxiety-like and panic-like behaviors in rats, whereas TRPV1 antagonists strengthened the anxiolytic and panicolytic effects of the compounds administered per se (Rubino et al. 2008; Casarotto et al. 2012). Systemic anandamide also produces behavioral disruption in rats, consisting of increased omission errors and decreased responding during intertrial intervals in the five-choice serial reaction-time task (Panlilio et al. 2009). This response was antagonized by the TRPV1 antagonist, capsazepine. Anandamide was recently reported to inhibit and exacerbate marble burying behavior in mice via CB_1 and TRPV1 receptors, respectively (Umathe et al. 2012). Likewise, activation of CB_1 or TRPV1 receptors by anandamide attenuates or stimulates the flight responses (a defensive behavioral reaction) induced in rats by the injection of an NO donor into the dorsolateral PAG (Lisboa and Guimarães 2012).

Studies employing TRPV1 null mice or the inhibitor URB597 (to increase its endogenous levels of anandamide) suggest that anandamide influences synaptic plasticity by acting at both post- and presynaptic TRPV1 channels. Postsynaptically, TRPV1 activation by anandamide hyperpolarizes neurons by: (1) reducing the biosynthesis of the other endocannabinoid, 2-arachidonylglycerol (2-AG), thereby counteracting the metabotropic (glutamatergic and cholinergic)-induced, CB_1-mediated retrograde inhibition of GABA release onto striatal medium spiny neurons (MSN) (Maccarrone et al. 2008; Musella et al. 2009); or (2) stimulating AMPA receptor endocytosis, thus impacting glutamate signaling and inducing long-term depression (Grueter et al. 2010; Chávez et al. 2010). Both effects are Ca^{2+}-dependent. A recent study showed that long-term depression in the extended amygdala is mediated by postsynaptic mGluR5-dependent release of anandamide acting on postsynaptic TRPV1 receptors (Puente et al. 2011). Presynaptic TRPV1 activation, instead, stimulates glutamate release in, among others, the PAG (Kawahara et al. 2011). This results in the facilitation of metabotropic glutamate receptor-induced impairment of GABA release (retrograde- and CB_1-mediated) and stimulates the descending antinociceptive pathway (Liao et al. 2011). This latter effect was previously suggested by Maione et al. (2006) and Starowicz et al. (2007) to occur also through other TRPV1-mediated mechanisms. As shown through the use of mice with genetically impaired expression of FAAH, tonic presynaptic TRPV1 activation by anandamide can also directly facilitate glutamatergic signaling in striatal MSN neurons (Musella et al. 2009). Finally, a recent study carried out using rat brain cortex astroglial gliosomes suggested that TRPV1 activation by anandamide can reduce aspartate release in this system (Bari et al. 2011). A previous study had shown the presence of TRPV1 in cortical astrocytes and its coupling to Na^+, rather than Ca^{2+}, influx (Huang et al. 2010).

Possibly given their stimulatory effects on Ca^{2+} influx and glutamate signaling, TRPV1 channels have been implicated in glutamate excitotoxicity and have been proposed as a target for the development of new neuroprotective drugs (Kim et al. 2007). As opposed to 2-AG, anandamide, at low (0.1–1 μM) concentrations, exacerbated oxygen-glucose deprivation-induced injury in rat organotypic hippocampal slices in a way mediated by TRPV1 (Landucci et al. 2011). Like capsaicin, it also evoked the apoptotic death of a human neuron-like cell line, in a TRPV1-dependent and caspase-independent manner (Davies et al. 2010). As assessed by measuring spontaneous and miniature excitatory postsynaptic currents, anandamide enhanced

glutamate release onto dentate gyrus granule cells prepared from a mouse model of temporal lobe epilepsy. The effect was observed only in the presence of a CB_1 antagonist, and was antagonized by TRPV1 blockade (Bhaskaran and Smith 2010). Accordingly, activation of TRPV1 was recently suggested to underlie the pro-convulsant effect of anandamide in pentylenetetrazole-induced seizures (Manna and Umathe 2012).

8.2.2 NADA and TRPV1

Unlike anandamide, the other proposed endovanilloid with activity at CB_1 receptors, *N*-arachidonoyl-dopamine (NADA), has lesser affinity for these latter proteins and higher affinity for, and efficacy at, TRPV1 (Bisogno et al. 2000; Huang et al. 2002). This possibly explains why lower concentrations are usually sufficient, and why it is not necessary to block CB_1 receptors, in order to observe NADA-induced and TRPV1-mediated stimulatory effects at nociceptors (Huang and Walker 2006; Sagar et al. 2004) or central neurons (Marinelli et al. 2007). In agreement with the vasodilatory actions of TRPV1 in perivascular neurons, NADA has been shown to reduce blood pressure response to high salt in rats in a TRPV1- and CGRP-dependent manner, an effect ascribed at least in part to the up-regulatory effect of high salt on TRPV1 expression in mesenteric arteries (Wang and Wang 2007). Furthermore, concordant with the stimulatory effects of TRPV1 in central neurons (Marinelli et al. 2007), NADA induces a prolonged elevation of presynaptic $[Ca^{2+}]_i$ and a concomitant enhancement of glutamate release at sensory synapses. This response required Ca^{2+} entry, primarily via TRPV1, although its sustained phase was independent of extracellular Ca^{2+} and instead due to mitochondrial Ca^{2+} uptake/release mechanisms. The authors suggested that mitochondria control TRPV1-mediated neurotransmission by "translating the strength of presynaptic TRPV1 stimulation into duration of the postsynaptic response" (Medvedeva et al. 2008). NADA instead shares with anandamide the capability of sensitizing capsaicin-sensitive lung vagal afferent to the action of capsaicin, adenosine and mechanical stimulus in a TRPV1-mediated manner (Hsu et al. 2009). Both NADA and anandamide produce antiemetic actions in ferrets by acting at CB_1 and TRPV1 receptors co-localized in the nucleus of the solitary tract, dorsal motor nucleus of the vagus and area postrema (Sharkey et al. 2007).

As discussed above for anandamide, NADA also induces the apoptotic death of neurons (Davies et al. 2010). Furthermore, NADA causes the death of peripheral blood mononuclear cells from both control and, particularly, end-stage kidney disease patients, the latter of which express higher levels of TRPV1 channels (Saunders et al. 2009). In the latter case, the cause of cell death (apoptosis vs. cytotoxicity) was not investigated in detail, and appeared to follow activation of both TRPV1 and CB_2 receptors (for which NADA has very low affinity (Bisogno et al. 2000)). On the other hand, NADA was recently shown to induce oxidative stress-mediated cell death in hepatic stellate cells but not in hepatocytes in a manner insensitive to cannabinoid receptor and TRPV1 antagonists (Wojtalla et al. 2012).

8.2.3 2-AG and TRPV1

Although the other most studied endocannabinoid, 2-AG, has very little functional activity at human and rat TRPV1 overexpressed in HEK-293 cells (Zygmunt et al. 1999; De Petrocellis et al. 2000), some of its effects, i.e. the anti-proliferative action in C6 glioma cells (Jacobsson et al. 2001) and the stimulation of Ca^{2+} influx in cerebromicrovascular endothelial cells (Golech et al. 2004), in vitro, and the pro-inflammatory effect in a rat model of colitis (McVey et al. 2003), in vivo, are attenuated by the TRPV1 antagonist, capsazepine. This discrepancy between molecular and pharmacological data can be explained either with the lack of selectivity for TRPV1 by capsazepine, or by the possible rapid conversion of 2-AG into the corresponding diacylglycerols (Di Marzo et al. 1999), which have been suggested to activate TRPV1, although at high μM concentrations (Woo et al. 2008; Kim et al. 2009). In much the same way that not all proposed endovanilloids share the capability of anandamide and NADA to activate the "metabotropic" CB_1 and CB_2 receptors, some of the proposed endocannabinoids, such as 2-AG and noladin ether, do not activate TRPV1. Anandamide and NADA interact with additional non-CB_1/non-CB_2/non-TRPV1 receptors at sub-μM concentrations, at least in vitro. The best established such interaction is possibly represented by their direct inhibition of T-type Ca^{2+} channels (Ross et al. 2009). Indeed, a certain "redundancy" of molecular targets is typical of lipid mediators.

8.2.4 Anandamide- and NADA-Like Molecules and TRPV1

Both anandamide and NADA are accompanied in tissues by compounds belonging to the same fatty acid amide families, i.e., *N*-acyl-ethanolamines and *N*-acyl-dopamines, the fatty acid chains of which differ from arachidonic acid (Fig. 8.2). Some of these "endovanilloid congeners" exert their effects also via non-CB_1/non-CB_2/non-TRPV1 targets, as in the case of *N*-oleoyl-ethanolamine (OEA) and *N*-palmitoyl-ethanolamine (PEA), whereas other congeners still bind selectively to either CB_1 or TRPV1 receptors, as in the case of *N*-di-homo-γ-linoleoyl-ethanolamine or *N*-7,10,13,16-docosatetraenylethanolamine, and *N*-oleoyl-dopamine, respectively (Pertwee et al. 1994; Chu et al. 2003). However, some congeners with negligible activity per se at CB_1 and TRPV1 potentiate the respective effects of anandamide and NADA at TRPV1, as shown in HEK-293 cells overexpressing the human recombinant channel (De Petrocellis et al. 2001b, 2004; Smart et al. 2002). PEA was recently found to exert a similar facilitatory effect (known as "entourage" effect) on anandamide vasodilation of isolated rat mesenteric arteries in vitro (Ho et al. 2008), as well as on the hypotensive effects of intrathecal anandamide in vivo (García Mdel et al. 2009). Likewise, *N*-stearoyl-dopamine (STEARDA) potentiated the TRPV1-mediated nociceptive effects of NADA when co-injected into the rat hindpaw, and, if administered per se, enhanced the inflammatory hyperalgesia

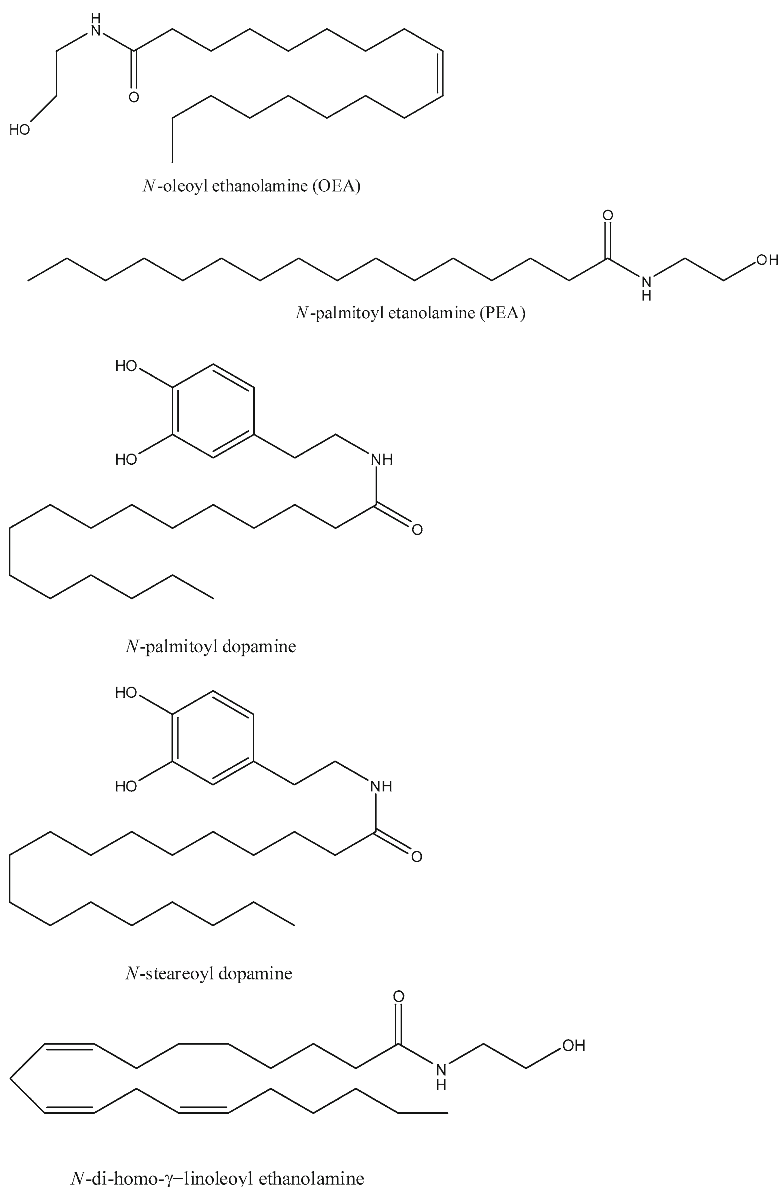

Fig. 8.2 Chemical structure of some "entourage" compounds for anandamide and *N*-arachidonoyl-dopamine

induced by carrageenan (De Petrocellis et al. 2004). This finding suggested that these "entourage" compounds may exert effects in vivo by potentiating the action of endogenous activators of TRPV1, such as those that participate in inflammatory hyperalgesia (De Petrocellis et al. 2004). In fact, by enhancing endogenous TRPV1

modulator activity, compounds like PEA and STEARDA might also help desensitize the channel and hence produce anti-hyperalgesic and anti-inflammatory effects. Accordingly, PEA was recently shown to inhibit neuropathic pain (Costa et al. 2008) and contact allergic dermatitis (Petrosino et al. 2010) in a way partly mediated by TRPV1 activation/desensitization.

8.2.5 *Anandamide, NADA, and Other Thermo-TRPs*

Of all thermo-TRPs, TRPV1 is the only one activated by capsaicin, a compound that shares chemical similarity and binding site on the channel similar to those of anandamide and NADA (Jordt and Julius 2002; Gavva et al. 2004). Accordingly, no other thermo-TRP is potently activated by these compounds and only weak stimulatory activity has been observed so far with anandamide at rat recombinant TRPA1 (EC_{50} ~ 5 μM) (De Petrocellis and Di Marzo 2009; De Petrocellis, unpublished observations). However, TRPM8-mediated elevation of $[Ca^{2+}]_i$, which is inhibited by capsaicin as well as by some TRPV1 antagonists, is also potently reduced by anandamide and NADA, as observed in HEK-293 cells overexpressing the rat recombinant channel (De Petrocellis et al. 2007).

8.3 Effect of Phytocannabinoids at Thermo-TRPs

8.3.1 *TRPV1*

Several of the over 70, olivetol-derived terpenes isolated from *Cannabis sativa* and known as "cannabinoids," including the psychotropic component, Δ^9-tetrahydrocannabinol (THC), have been tested on thermo-TRPs using either patch-clamp or Ca^{2+} imaging techniques. Historically, the first such compound to be proposed as a TRP channel ligand was cannabidiol (CBD), which exhibits similar affinity and efficacy, but significantly lower potency, as capsaicin when using as readout $[Ca^{2+}]_i$ elevated via the human recombinant TRPV1 in HEK-293 cells (Bisogno et al. 2001). More recently, the activity of CBD at TRPV1 and other cannabinoids was re-evaluated, and it was found that cannabigerol (CBG), cannabigivarin (CBGV) and tetrahydrocannabivarin (THCV) (Fig. 8.3) exhibit potencies and/or efficacies similar to those of CBD (De Petrocellis et al. 2011). The sesquiterpene derivative of CBG, which exhibits higher affinity at cannabinoid CB_2 receptors than the parent compound, exhibited instead less potency at TRPV1, and so did the acid derivative of CBD with respect to CBD (Pollastro et al. 2011; De Petrocellis et al. 2011). The TRPV1-activating phytocannabinoids were also capable of potently desensitizing TRPV1 to the subsequent stimulation by capsaicin. Indeed, TRPV1 desensitization by CBD might explain why the cannabinoid exerts anti-hyperalgesic

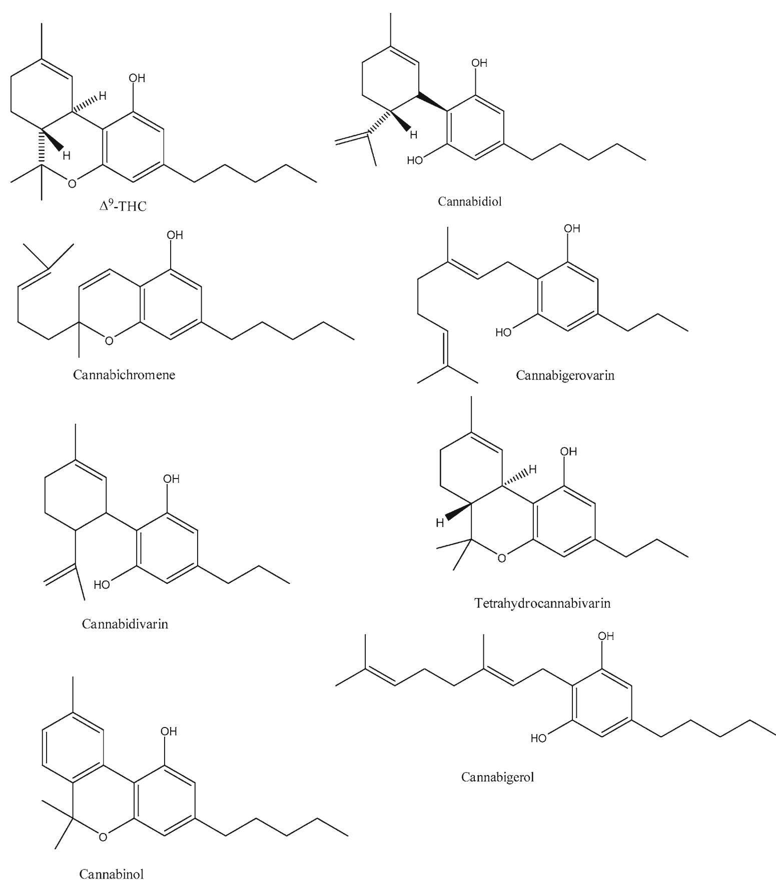

Fig. 8.3 Chemical structure of some of the most abundant phytocannabinoids

effects in a manner attenuated by the TRPV1 antagonist capsazepine in animal models of neuropathic (Costa et al. 2007; Comelli et al. 2008) and inflammatory (Costa et al. 2004) pain. Given the proposed role of TRPV1 in promoting anxiety (Rubino et al. 2008; Micale et al. 2009), these data provide a possible explanation of studies showing that TRPV1 antagonism by capsazepine unmasks the anxiolytic effect of a high dose of CBD injected into the dorso-lateral PAG of rats undergoing the elevated plus maze test (Campos and Guimarães 2009). Finally, other pharmacological effects of CBD that seem to be mediated by TRPV1 activation are the antipsychotic action in the MK-801-induced disruption of prepulse inhibition test in mice (Long et al. 2006), the inhibition of lung and cervical cancer cell invasive activity in vitro (Ramer et al. 2010a, b), and the induction of myeloid-derived suppressor cells and subsequent amelioration of experimental autoimmune hepatitis in vivo (Hegde et al. 2011). In the latter case, the involvement of TRPV1 in the actions of CBD was confirmed through the use of TRPV1 null mice.

8.3.2 TRPV2

The first phytocannabinoid to be proposed as a TRPV2 agonist was again CBD, which was found to produce TRPV2-mediated elevation of $[Ca^{2+}]_i$ and currents via the rat recombinant TRPV2 overexpressed in HEK-293 cells, and TRPV2-mediated release of CGRP in sensory neurons (Qin et al. 2008). The desensitization of this response occurred at low μM concentrations (Qin et al. 2008). The authors also reported significantly lower efficacy and potency at human recombinant TRPV2. More recently, the activity of CBD together with several other cannabinoids was reevaluated using again the rat recombinant TRPV2 in HEK-293 cells (De Petrocellis et al. 2011). Interestingly, CBG, CBGV, THC and THCV (Fig. 8.3) were more efficacious and/or potent than CBD at both activating and desensitizing the TRPV2-mediated Ca^{2+} responses in this system. The sesquiterpene derivative of CBG exhibited less efficacy and potency than CBG (Pollastro et al. 2011). Thus far, the only other known example of TRPV2-mediated effect of CBD is the induction of apoptosis in human T24 bladder cancer cells (Yamada et al. 2010).

8.3.3 TRPV3 and TRPV4

Some cannabinoids were recently shown to induce elevation of $[Ca^{2+}]_i$ in cells overexpressing either the rat recombinant TRPV3 or TRPV4 channel (De Petrocellis et al. 2012). In particular, CBD and THCV produced an efficacious TRPV3-mediated response at low μM concentrations, whereas only the latter compound was very efficacious at TRPV4. Cannabidivarin (CBDV), CBD and cannabichromene (CBC) (Fig. 8.3) were potent but much less efficacious at TRPV4. Interestingly, some cannabinoids, such as CBGV act on TRPV3, and CBG act on TRPV4, desensitized the channels to the activation by respective agonists at concentrations significantly lower than those required to elicit significant agonist-like effects per se.

8.3.4 TRPA1

Two studies from our group (De Petrocellis et al. 2008, 2011) suggest an agonist-like effect of several cannabinoids at the rat recombinant TRPA1 channel overexpressed in HEK293 cells, thus extending the results of the original report showing that THC and cannabinol (CBN) (Fig. 8.3) activate this channel at μM concentrations (Jordt et al. 2004). Most of the compounds tested exhibited, under our experimental conditions, both high potency and efficacy at inducing TRPA1-mediated elevation of $[Ca^{2+}]_i$, with CBC and CBD being the most potent (EC_{50}~0.1 μM). Most cannabinoids also potently desensitized the channel to the action of one class of agonists, the mustard oil isothiocyanates (De Petrocellis et al. 2008, 2011).

Interestingly, sesqui-CBG was slightly less potent than CBG at activating TRPA1, but significantly (~10-fold) more potent at desensitizing it (Pollastro et al. 2011). However, it should be noted that other groups have either reported no activity for CBD, or observed a TRPA1-like activity for this compound only at very high μM concentrations (Jordt et al. 2004; Qin et al. 2008). Lastly, the $[Ca^{2+}]_i$-elevating activity of CBC via TRPA1 was supported in DRG neurons expressing this channel, albeit at concentrations (EC_{50} ~ 20 μM) higher than those required to activate the channel in TRPA1-expressing HEK-293 cells (De Petrocellis et al. 2008).

8.3.5 TRPM8

Unlike other thermo-TRPs, but similar to what is observed with anandamide and NADA, TRPM8 channels are inhibited, rather than activated, by some phytocannabinoids (De Petrocellis et al. 2008, 2011). CBD, CBG, CBN, THC, and THC-acid are the most potent functional antagonists (IC_{50} = 0.06–0.21 μM) of rat recombinant TRPM8-mediated, and menthol- or icilin-induced, elevation of $[Ca^{2+}]_i$, whereas CBC was the only compound of those tested that was completely inactive. CBG also counteracts icilin-induced elevation of $[Ca^{2+}]_i$ in DRG neurons expressing this channel, although at concentrations higher than in HEK-293 cells (EC_{50} ~ 10 μM).

In conclusion, “thermo-TRPs” are potential targets for plant cannabinoids, and their modulation might underlie some of the pharmacological effects of these compounds, which are often promising from a therapeutic point of view. However, evidence for a direct interaction of phytocannabinoids with these channels is still partial or lacking.

8.4 Effect of Synthocannabinoids at TRP Channels

“Synthocannabinoids” can be defined as synthetic compounds that bind to either CB_1 and/or CB_2 receptors. These compounds have chemical structures both similar to and completely different from those of cannabinoids and endocannabinoids. Synthocannabinoids include compounds originally designed as agonists, inverse agonists and antagonists of CB_1 and/or CB_2 receptors; however, several were found to interact with thermo-TRPs. A clear example are some synthetic anandamide analogues with higher selectivity for CB_1 receptors, such as R(+)-methanandamide and, particularly, ACEA (Fig. 8.4), which, like anandamide, activate TRPV1 (Nieri et al. 2003; Price et al. 2004). By partly acting at TRPV1, ACEA was suggested to reduce osteoarthritic pain in rats (Schuelert and McDougall 2008), increase quantal release at the frog neuromuscular junction (Silveira et al. 2010), and cross-desensitize TRPA1 channels, thereby reducing mustard oil-induced CGRP release in the rat hind paw (Ruparel et al. 2011). Furthermore, as mentioned above, ACEA, injected in the dorsal-PAG, produces opposing effects on anxiety-like behavior in rats via CB_1 and TRPV1 receptors (Casarotto et al. 2012).

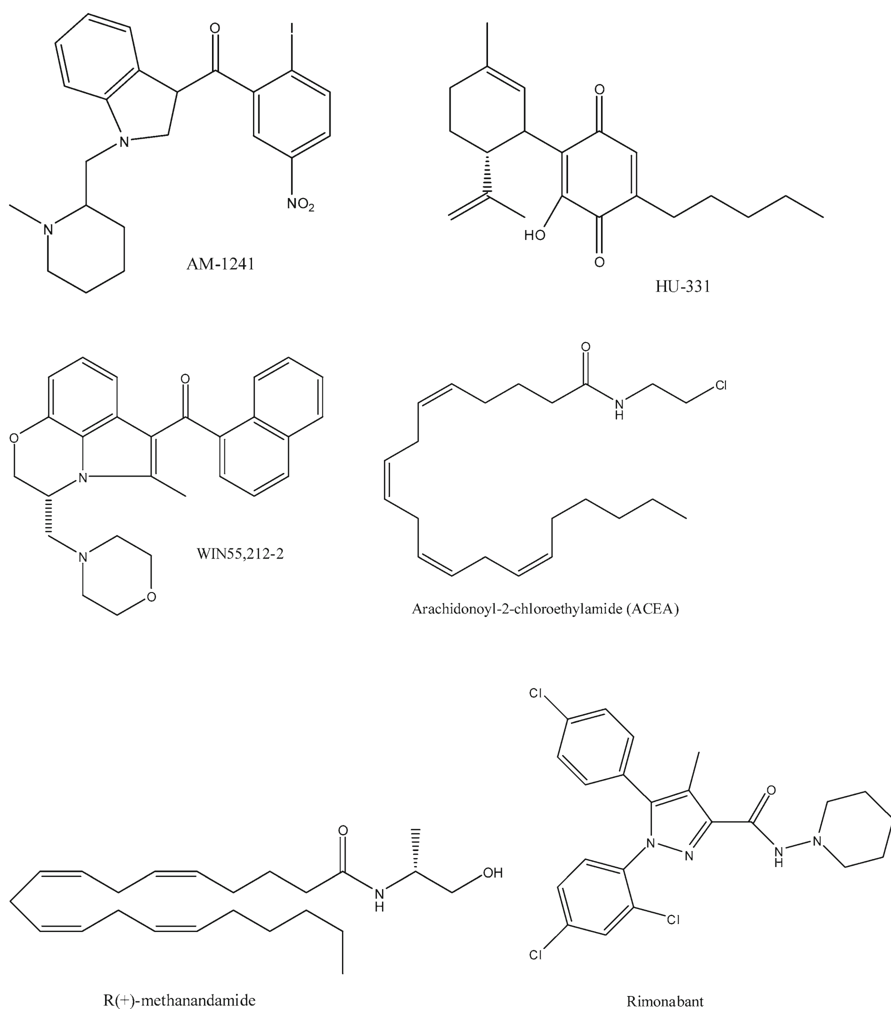

Fig. 8.4 Chemical structure of the "synthocannabinoids" found so far to interact with TRP channels

The widely used CB_1/CB_2 agonist, and aminoalkylindole compound, WIN55.212-2 (Fig. 8.4), was initially suggested to act as a functional TRPV1 antagonist via an indirect mechanism involving Ca^{2+} mobilization, calcineurin activation and TRPV1 dephosphorylation (Patwardhan et al. 2006). More recently, it has been proposed that the effects of WIN55.212-2 are due to its direct interaction with TRPA1, although at concentrations (~20–30 μM) much higher than those necessary to activate cannabinoid receptors (Jeske et al. 2006; Akopian et al. 2008). In support, Qin et al. (2008) reported an EC_{50} of 9.8 μM for WIN55.212-2 at elevating $[Ca^{2+}]_i$ in HEK-293 cells overexpressing the rat recombinant TRPA1. Its isomer, WIN55.212-3, which is inactive at cannabinoid receptors, is also active at rat TRPA1

but with reduced potency (EC_{50} = 21.7 μM). Other synthocannabinoids that activate TRPA1 channels include abnormal-CBD, a compound that has little or no activity at CB_1/CB_2 receptors, but activates rat TRPA1 (EC_{50} = 9 μM) (Qin et al. 2008) and the quinine-cannabinoid, HU-331 (EC_{50} = 3.2 μM) (Fig. 8.4), which is also inactive at CB_1/CB_2 receptors (Kogan et al. 2006). Finally, the CB_2-selective agonist AM1241 (Fig. 8.4) activates rat TRPA1 at high μM concentrations (Akopian et al. 2008). Despite their low potency at TRPA1 in vitro, both WIN55.212-2 and AM1241 did exert anti-hyperalgesic effects in vivo against capsaicin-induced nociception in mice in a manner greatly reduced in TRPA1 null mice (Akopian et al. 2008).

Some potent CB_1 and CB_2 inverse agonists, such as the di-aryl-pyrazoles SR141716A (rimonabant) and SR144528 (Fig. 8.4), seem to interact with some thermo-TRPs. We first reported that rimonabant activates and desensitizes human recombinant TRPV1-mediated $[Ca^{2+}]_i$ elevation in HEK-293 cells at concentrations higher than 1 μM (De Petrocellis et al. 2001a). Later, we found that both rimonabant and SR144528 antagonize the rat recombinant TRPM8 $[Ca^{2+}]_i$ response to icilin, but not to menthol, with IC_{50} in the low nM range (De Petrocellis et al. 2007). Some effects of rimonabant in vivo (i.e., inhibition of adult neurogenesis in mice and neuroprotection against global cerebral ischemia in gerbils) and in vitro (i.e., inhibition of long-term depression at hippocampal GABAergic interneurons) were ascribed to its interaction with TRPV1 channels (Jin et al. 2004; Gibson et al. 2008; Pegorini et al. 2006). Finally, two CB_1- and CB_2-selective antagonists chemically related to rimonabant and SR144528, i.e., AM251 and AM630, were recently found to activate and desensitize TRPA1, and, although their intra-paw injection in mice did not produce nocifensive behaviors, both compounds inhibited capsaicin-induced thermal hyperalgesia in wild-type mice and rats, but not in TRPA1 null-mutant mice (Patil et al. 2011).

8.5 Conclusions

A wealth of evidence suggests that several thermo-TRPs, in particular TRPV1 channels, can act as ionotropic receptors for endo-, phyto- and syntho-cannabinoids, and hence for “cannabimimetic” substances of endogenous, xenobiotic or synthetic origin. However, with the exception of anandamide, the physiological, pathological and even pharmacological significance of several of the interactions described in this chapter still needs to be fully investigated. For example, with the exception of the effects of CBD at TRPV1 and TRPV2, and of THC, ACEA, WIN55.212-2, and AM214 at TRPA1, we still do not know if phyto- and syntho-cannabinoids physically and directly interact with TRP channels, and even for the above compounds such interactions were shown to occur only at high μM concentrations. On the other hand, even though the evidence for the physiopathological roles exerted by anandamide via TRPV1 is very strong, it must be emphasized that this compound, as well as NADA, is not selective for cannabinoid and vanilloid receptors. Conversely, other endogenous compounds have also been suggested to act as endovanilloids, with

perhaps higher selectivity (see Starowicz et al. 2007 for review). Nevertheless, we believe that TRPV1 satisfies all the requirements necessary to be considered a "cannabinoid receptor" as recently outlined by the International Union of Basic and Clinical Pharmacology (IUPHAR) in an authoritative article on cannabinoid receptor nomenclature (Pertwee et al. 2010). Specifically: (1) TRPV1 is activated at an orthosteric site and with significant potency by established CB_1/CB_2 receptor ligands (i.e., anandamide, NADA, ACEA); (2) TRPV1 is activated by at least one established endogenous CB_1/CB_2 receptor agonist at "physiologically relevant" concentrations. Note that anandamide potency at TRPV1 is in the sub-μM range, which is not too dissimilar from its potency at CB_1 receptors and from the endogenous concentrations often reached by this lipid during certain physiopathological conditions. Also note that NADA is more potent at TRPV1 than CB_1 receptors, but is also less abundant in tissues than anandamide; (3) TRPV1 was not a orphan non-CB_1/CB_2 receptor or channel when its interactions with a cannabimimetic compound was discovered. Note that it was not shown previously to be activated endogenously by a non-CB_1/CB_2 receptor ligand with appropriate potency and relative intrinsic activity. Further note that anandamide was the first endovanilloid to be discovered, while other endovanilloids with no activity at cannabinoid receptors were discovered only later; and (4) TRPV1 is expressed by mammalian cells that are known to be exposed to concentrations of endogenously released endocannabinoid molecules capable of eliciting a response. This has been shown for anandamide in several studies, by using FAAH inhibitors or FAAH "knock-out" mice.

Since the pharmacology of non-THC cannabinoids is now better understood, one might envisage the opportunity to encompass in the name "cannabinoid receptors," apart from CB_1 and CB_2, also thermo-TRP channels that interact specifically with non-THC cannabinoids. For example, the finding, if confirmed, that TRPV2–4 have no other potent xenobiotic activator than, e.g., THCV, which also interacts with CB_1 and CB_2 receptors (Thomas et al. 2005), would strengthen further the possibility that these channels are identified in the future as "ionotropic cannabinoid receptors."

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Chapter 9
Nonpsychoactive Cannabinoid Action on 5-HT_3 and Glycine Receptors

Li Zhang and Wei Xiong

Abbreviations

5-HT	5-Hydroxytryptamine
AEA	Anandamide
GABA	γ-Aminobutyric acid
I_{Gly}	Glycine-activated current
THC	Δ^9-Tetrahydrocannabinol
TM	Transmembrane domain
VTA	Ventral tegmental area
WT	Wild type

9.1 5-HT_3 Receptor

9.1.1 Molecular Composition and Distribution

The 5-HT_3 receptor was identified as the first serotonin-activated receptor in a study of 5-hydroxytryptamine (5-HT)-induced smooth muscle contraction in guinea pig ileum (Gaddum 1953). Unlike other subtypes of 5-HT receptors, the 5-HT_3 receptor

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belongs to the Cys-loop ligand-gated ion channel (LGIC) superfamily (LIGC), which includes neuronal nicotinic acetylcholine receptors (nAChRs), γ-aminobutyric acid (GABA) type A receptors ($GABA_A$Rs), and glycine receptors (Maricq et al. 1991). The topology of a typical LGIC subunit contains a large extracellular N-terminus, four transmembrane domains (TMs), a short extracellular C-terminus, and a large cytoplasmic domain between TM3 and TM4 (Maricq et al. 1991). Five subtypes of 5-HT_3 receptors (A–E) have been identified to date (Niesler et al. 2007). The functional form of the 5-HT_3 receptors can be either homomeric or heteromeric pentameric oligomers (Barnes et al. 2009). The homomeric 5-HT_{3A} receptor has received the most attention as compared to its counterparts. First, this is the dominant form expressed in the brain (Maricq et al. 1991). Second, homomeric and heteromeric 5-HT_3 receptors do not significantly differ in their pharmacological profile (Davies et al. 1999; Hu and Peoples 2008; Barnes et al. 2009). There is evidence to suggest that agonists bind to the interface across the 3A+ and 3A-subunits at both homomeric and heteromeric 5-HT_3 receptors (Thompson et al. 2011). Third, our knowledge about the functional role of 5-$HT_{3C–D}$ subunits is relatively sparse (Karnovsky et al. 2003; Niesler et al. 2003; Barnes et al. 2009). The 5-HT_{3A} subunits are expressed on post- and presynaptic sites in the peripheral and central nervous systems (CNSs) (Morales et al. 1998). The 5-HT_{3A} receptors are abundant in cortex, hippocampus, nucleus accumbens, substantia nigra, ventral tegmental area (VTA), and brain stem (Lundeberg et al. 2002). While human 5-HT_{3B} subunits are detectable at either mRNA level or protein level in brain tissue (Davies et al. 1999; Brady et al. 2007), the question remains open about the presence of these subunits in the CNS in rodents (Lundeberg et al. 2002; van Hooft and Yakel 2003). Consistent evidence shows that the native 5-HT_3 receptors comprise both 5-HT_{3A} and 5-HT_{3B} subunits in dorsal root, superior cervical, and nodose ganglion neurons (NGN) (Morales et al. 2001).

9.1.2 Biological and Therapeutic Role of 5-HT_3 Receptor

Using selective 5-HT_3 receptor antagonists is one of the most popular treatments for chemotherapy-induced emesis in the last decade (Aapro et al. 2006). Selective-5-HT_3 receptor antagonists are also effective in alleviating symptoms such as pain and diarrhea in irritable bowel syndrome (Delvaux et al. 1998; Jones et al. 1999). Growing evidence has suggested that 5-HT_3 receptors play roles in drug addiction and in several neurological disorders such as schizophrenia, anxiety, psychosis, and cognitive function (Thompson and Lummis 2007). Mice with depletion of the 5-HT_{3A} receptor gene exhibited decreased sensitivity to tissue injury-induced persistent nociception, suggesting that the 5-HT_3 receptors are pronociceptive (Zeitz et al. 2002). Consistent with this idea, clinical evidence shows that 5-HT_3 receptor antagonists can produce analgesic effects in patients with fibromyalgia, a chronic pain illness, and chronic pain (Koeppe et al. 2004; Spath et al. 2004a, b; Stratz and Muller 2004).

9.1.3 Cannabinoid Inhibition of 5-HT_3 Receptors

The endocannabinoid anandamide (AEA) modulation of 5-HT_3 receptors was first described by Fan (1995) in NGN. AEA reduced the amplitude of 5-HT-activated current ($I_{5\text{-HT}}$) with an IC_{50} of 94 nM. Both the psychoactive CB_1 receptor agonist, CP55,940, and its nonpsychoactive enantiomer, CP56,667, produced a similar inhibitory effect on $I_{5\text{-HT}}$. The inhibition of $I_{5\text{-HT}}$ by cannabinoids developed slowly and required a sustained cannabinoid incubation to reach a maximum. This response was reported in separate studies of nonpsychoactive cannabinoid inhibition of 5-HT_3 receptors (Barann et al. 2002; Butt et al. 2008; Xiong et al. 2008; Yang et al. 2010a; Xiong et al. 2011a). Cytoplasmic application of GDP-βγ, an inhibitor of G-proteins, failed to alter AEA inhibition of 5-HT_3 receptors (Fan 1995). The author concludes that the inhibition of 5-HT_3 receptors by cannabinoids is not mediated by CB receptors expressed in NGN. This hypothesis has received favorable support from different studies in *Xenopus* oocytes or HEK-293 cells expressing recombinant 5-HT_{3A} receptors (Barann et al. 2002; Oz et al. 2002; Butt et al. 2008; Xiong et al. 2008; Yang et al. 2010a; Xiong et al. 2011a). In these cells, D^9-tetrahydrocannabinol (THC) and cannabidiol (CBD), major psychoactive and nonpsychoactive components of marijuana, and AEA inhibited $I_{5\text{-HT}}$ in a CB_1-independent mechanism. Some of these studies have shown that the IC_{50} values of cannabinoid inhibition of 5-HT_3 receptors are in a nanomolar range. In this regard, allosteric modulation of 5-HT_3 receptors by cannabinoids is physiologically and clinically relevant.

9.1.3.1 Factors Influencing Inhibition of 5-HT_3 Receptor: Receptor Density

The IC_{50} values for AEA inhibition of 5-HT_3 receptors can vary significantly over a range from 94 nM in NGN to 3.7 μM in native neurons and in cell lines expressing recombinant 5-HT_{3A} receptors (Fan 1995; Barann et al. 2002; Oz et al. 2002; Xiong et al. 2008). To address this particular issue, a previous study examined the interrelationship between receptor expression at the cell surface and AEA inhibition (Xiong et al. 2008). The authors of this study suggest that the magnitude of the AEA inhibition of 5-HT_3 receptors depends on expression levels of receptor proteins at cell membrane surfaces (Xiong et al. 2008). The magnitude of AEA inhibition of $I_{5\text{-HT}}$ is inversely correlated with surface expression and density of 5-HT_3 receptors expressed in both *Xenopus* oocytes and HEK-293 cells. For instance, the maximal AEA inhibition was 95% in *Xenopus* oocytes injected with 2.5 ng of 5-HT_{3A} receptor cRNA, whereas the maximal inhibition was only 25% in oocytes injected with 50 ng of 5-HT_{3A} receptor cRNA. Moreover, a change in receptor expression levels can alter 5-HT_3 receptor desensitization kinetics. As receptor expression level decreases, the desensitization and AEA-induced inhibiting effect on $I_{5\text{-HT}}$ increase. A similar mechanism has been shown in recent studies of THC and CBD inhibition of 5-HT_3 receptors expressed in *Xenopus* oocytes and HEK-293 cells (Yang et al. 2010a, b; Xiong et al. 2011a).

Together, these studies have suggested that cannabinoid inhibition of 5-HT_3 receptors vary with expression levels of receptor protein at cell surfaces.

9.1.3.2 Factors Influencing Inhibition of 5-HT_3 Receptor: Receptor Desensitization

The magnitude of cannabinoid inhibition of $I_{5\text{-HT}}$ is also found to depend on the state of 5-HT_3 receptor desensitization (Xiong et al. 2008). There are a number of factors that can influence 5-HT_3 receptor desensitization. Besides changes in receptor density, pretreatment with nocodazole, a microtubule disruptor, 5-hydroxyindole and a point-mutation in the large cytoplasmic domain of 5-HT_{3A} receptor slowed receptor desensitization without significantly affecting receptor density (Xiong et al. 2008). Reducing receptor desensitization by nocodazole, 5-hydroxyindole and a point-mutation in the large cytoplasmic domain of the receptor significantly decreased AEA and CBD-induced inhibition (Xiong et al. 2008, 2011a). Thus, cannabinoids inhibit 5-HT_3 receptors through a mechanism that is dependent on receptor desensitization.

9.1.4 Mechanism of Action

The precise mechanism of cannabinoid inhibition of 5-HT_3 receptors remains unknown. One hypothesis suggests that 5-HT_3 receptors may contain a motif structurally similar to the binding pockets for the CB_1 receptor agonists since all three CB receptor agonists, THC, WIN55,212-2, and AEA, inhibit 5-HT_3 receptors with a reasonable potency (38–129 nM). These values are close to the binding affinity of the ligands for CB_1 and CB_2 receptors (Howlett et al. 2002). However this hypothesis is unlikely since several cannabinoids that blocked 5-HT_3 receptors did not significantly alter specific binding of [^{3}H]-GR65630, a selective 5-HT_3 receptor antagonist in HEK-293 cells (Barann et al. 2002; Yang et al. 2010a, b).

The 5-HT_{3A} and glycine receptors share a high level of amino acid sequence homology, especially within the transmembrane domains. A single amino acid residue of S296 in the TM3 region of the glycine receptor α1 and α3 subunits has been identified as a critical site for cannabinoid potentiation of Glycine-activated current (I_{Gly}) (Xiong et al. 2011b, 2012). However, it is unlikely that this residue is involved in AEA inhibition of 5-HT_{3A} receptors expressed in HEK-293 cells as the S296A mutation did not alter AEA inhibition of 5-HT_{3A} receptors (Xiong et al. 2012). Although the S296 residue is conserved between glycine and 5-HT_{3A} receptors, molecular modeling at the two dimensional level suggests that the S296 residue between 5-HT_{3A} and glycine receptors differs with respect to its orientation. The S296 residue of the glycine receptor is facing outside of the ion channel protein and lipid–protein interface, whereas the S296 residue of 5-HT_{3A} receptors is buried inside of the channel protein and away from lipid–protein interfaces (Xiong et al. 2012).

9.1.5 In Vivo Consequence

9.1.5.1 Antinociception

A recent study has provided evidence for the role of 5-HT_3 receptors in cannabinoid-induced analgesia (Racz et al. 2008). An analgesic action of AEA remained in knockout mice depleted of both CB_1 and CB_2 receptors, whereas THC-induced analgesia was completely abolished in these mice. The analgesic effect induced by AEA was reduced after administration of the 5-HT_3 antagonist ondansetron. This study suggests that 5-HT_3 receptors may be involved in AEA but not THC-induced analgesia.

9.1.5.2 Cocaine Hyperlocomotion

Microinjection of the 5-HT_3 receptor antagonist ondansetron into the nucleus accumbens attenuated stimulatory effects on locomotor activity induced by peripheral administration of cocaine (Herges and Taylor 2000). Like ondansetron, intraperitoneal injection of the CB_1 receptor agonist WIN55,212-2-inhibited chronic cocaine-induced hyperlocomotor activity in rats (Przegalinski et al. 2005). However, WIN55,212-2-induced inhibition was not reduced by the selective CB_1 receptor antagonist SR141716. Instead, ondansetron reversed WIN55,212-2-induced inhibition of cocaine-induced hyperlocomotion (Przegalinski et al. 2005).

9.1.5.3 Bradycardia

There is evidence that CB_1 receptor agonists can directly inhibit the function of peripheral 5-HT_3 receptors in vivo (Godlewski et al. 2003). In this study, WIN55,212-2 and CP55,940 inhibited the peripheral 5-HT_3 receptor-mediated Bezold–Jarisch reflex, i.e., a decrease in heart rate, in rats, whereas the vanilloid VR1 receptor-mediated Bezold–Jarisch reflex was unaffected. The actions of the CB_1 receptor agonists were not mediated through actual CB_1 receptors since the animals were pretreated with SR141716A. These findings together suggest that nonpsychoactive cannabinoid action on 5-HT_3 receptors may contribute to some of the cannabinoid-induced behaviors in the central and peripheral nervous system.

9.2 Glycine Receptor

9.2.1 Molecular Composition and Distribution

The glycine receptors consist of α and β subunits, which combine to form a pentameric receptor complex (Betz et al. 1999). To date, five glycine receptor subunits

have been identified including four α subunits and one β subunit (Lynch 2009). The α subunits exhibit a high degree of similarity in amino acid sequence (>90%). It is difficult to distinguish these subunits using functional assays in native neurons since there is no subunit-specific agonist or antagonist, and these subunits do not differ significantly in either agonist binding affinity or channel properties (Lynch 2004).

The α2 subunit represents the dominant homomeric glycine receptor at embryonic and early development stages (Becker et al. 1988, 1993). While the α2 subunit is less abundant later in development, the α1β subunits become the dominant subunits expressed in brainstem and spinal cord at the adult stage in rats (Malosio et al. 1991). This switch between the α1 and α2 subunits occurs at about postnatal day 20. However, the α2 subunits appear to be a dominant subunit in forebrain even at the adult stage (Jonsson et al. 2009). The α3 subunit is found to distinctly express in superficial layers of the spinal cord dorsal horn, a formation center for pain sensation (Harvey et al. 2004). The native glycine receptors can be formed by either homomeric α subunits or heteromeric α and β subunits. It is well accepted that the postsynaptic glycine receptors are heteromeric α1β or α3β subunits since these receptors are mainly located in the postsynaptic sites through an interaction of the β subunit with the cytoskeleton protein gephyrin (Meyer et al. 1995). There is strong evidence to suggest that presynaptic and extrasynaptic glycine receptors are likely homomers (Xu and Gong 2010). While the postsynaptic glycine receptors have been the interest of many studies, relatively less is known about the roles of presynaptic glycine receptors under physiological and pathological processes. As inhibitory neurotransmitter receptors, the glycine receptor α1 and α3 subunits are predominantly expressed in spinal cord and brain stem. This expression pattern is correlated with the distinct functional roles of these subunits in neuromotor activity and antinociception. The physical identity and functional role of presynaptic glycine receptors could be the interest of future study.

9.2.2 Behavioral Roles and Therapeutic Target

The inhibitory action of glycine receptors regulates several important physiological and pathological processes such as pain transmission and neuromotor activity. The role of the α3 subunit in modulating inflammatory pain has been the focus of many reviews (Harvey et al. 2004; Zeilhofer and Zeilhofer 2008; Zeilhofer et al. 2012). The α3-containing glycine receptors are abundantly located in the lamina II of the spinal dorsal horn, an area known for integrating nociceptive information (Harvey et al. 2004). Experimental evidence suggests that prostaglandin E_2 (PGE_2), a critical mediator of central and peripheral pain sensitization, selectively inhibits α3 glycine receptor function (Harvey et al. 2004). PGE_2 inhibits the glycinergic inhibitory postsynaptic currents (IPSCs) in spinal cord slices of wild-type, but not in α3-glycine receptor knockout mice. Such inhibition of the α3 glycine receptors is found to contribute to the mechanism of chronic inflammatory pain induced by the intra-plantar

injection of complete Freund's adjuvant (CFA). A recent study has provided evidence for a role of the α3 subunits in regulating rhythmic breathing movements in mice (Manzke et al. 2010).

The glycine receptor α1 subunit mediates the major inhibitory neurotransmission in spinal cord and brain-stem motor neurons. Missense point-mutations in the human glycine receptor α1 subunit gene disrupt glycine receptor function which causes familial startle disease, an autosomal dominant disorder (Shiang et al. 1993; Harvey et al. 2008). Although rare, this disease is often characterized by an exaggerated startle reaction to sudden, unexpected auditory, tactile stimuli, and hypertonia. The most frequently occurring mutation causing human hyperekplexia is the R271Q/L mutation in the α1 subunit (Harvey et al. 2008). Mice carrying the R271Q mutation exhibit severe neuromotor defects that resemble human startle disease (Becker et al. 2002).

Accumulating evidence has shown that glycine receptors are also involved in the regulation of dopamine release upon exposure to ethanol in nucleus accumbens and the VTA (Molander and Soderpalm 2005a, b; Chau et al. 2009; Adermark et al. 2010; Li et al. 2012). These observations have contributed to the idea that glycine receptors play a role in drug addiction and reward mechanisms.

9.2.3 Cannabinoid Inhibition of Glycine Receptors

AEA and another major endocannabinoid, 2-arachidonylglycerol (2-AG), at 1 μM have been shown to inhibit and accelerate the desensitization of the amplitude of current activated by 100 μM glycine in isolated rat hippocampal pyramidal and Purkinje cerebellar neurons (Lozovaya et al. 2005). The effects induced by endocannabinoids on I_{Gly} were unaffected by either cytoplasmic application of the G-protein inhibitor GDP-β – S, or of CB_1 and TRPV1 receptor antagonists, suggesting a direct action of cannabinoids on glycine receptors. Consistent with these observations, 2-AG was also found to inhibit I_{Gly} in CHO cells expressing human glycine receptor α1 subunits and in brainstem slices from CB_1 receptor knockout mice (Lozovaya et al. 2011).

9.2.4 Cannabinoid Potentiation of Glycine Receptors

Hejazi and colleagues first reported that both THC and AEA enhanced the amplitude of I_{Gly} in a CB_1 receptor-independent mechanism in *Xenopus* oocytes expressing homomeric α1 and heteromeric α1β glycine receptors, and in acutely isolated VTA neurons (Hejazi et al. 2006). This conclusion has been tested and supported by numerous subsequent studies showing that various psychoactive and nonpsychoactive cannabinoids potentiate I_{Gly} in amygdala neurons, cultured spinal neurons and in HEK-293 cells expressing various recombinant glycine receptors (Yang et al. 2008;

Ahrens et al. 2009a; Delaney et al. 2009; Xiong et al. 2011b, 2012; Yevenes and Zeilhofer 2011). The EC_{50} values for the THC-induced potentiation of glycine receptors are 73 nM for human α1 glycine receptors, 109 nM for human α1β glycine receptors, and 320 nM for native glycine receptors in rat VTA neurons (Hejazi et al. 2006). THC at concentrations of 100 and 300 nM can significantly enhance I_{Gly} in HEK-293 cells expressing the α1 and α3 subunits (Table 9.1). This concentration range of THC has been found to induce psychotropic and antinociceptive effects in humans (Huestis and Cone 2004). Specifically, the concentrations of THC in human blood can peak as high as 800 nM 15 min after a casual marijuana inhalation and remain significantly elevated at 100 nM 60 min after smoking.

However, there is a notable inconsistency regarding the nature of cannabinoid modulation of glycine receptors among different laboratories. For instance, AEA potentiated I_{Gly} in HEK-293 cells expressing the α3 subunits (Yevenes and Zeilhofer 2011; Xiong et al. 2012), whereas AEA was ineffective in potentiating the α3 subunit in a separate study (Yang et al. 2008). Similarly, AEA has been shown to produce different effects on I_{Gly} in different cell lines expressing the α1 subunits (Hejazi et al. 2006; Lozovaya et al. 2011; Yevenes and Zeilhofer 2011; Xiong et al. 2012). The potency of cannabinoid potentiation also varied substantially in different studies (Hejazi et al. 2006; Yang et al. 2008; Xiong et al. 2011b, 2012). Several factors that may contribute to this discrepancy are discussed below.

9.2.4.1 Factors Influencing Potentiation: Agonist Concentrations

The degree of potentiation of I_{Gly} by either exogenous or endogenous cannabinoids is dependent on glycine concentration in *Xenopus* oocytes and HEK-293 cells expressing recombinant glycine receptors (Hejazi et al. 2006; Yang et al. 2008; Xiong et al. 2011b, 2012; Yevenes and Zeilhofer 2011). Maximal potentiation induced by cannabinoids occurs at the lowest concentration of glycine (at EC_2–EC_{10}, 3–15 μM) for the glycine receptor α1 subunit. With increasing glycine concentrations, the cannabinoid potentiation decreases. There is considerable evidence that basal levels of synaptic and extrasynaptic glycine are at concentrations that produce low occupancy of glycine receptors (Gomeza et al. 2003; Bradaia et al. 2004; Eulenburg et al. 2005). For example, extracellular glycine concentrations in rat spinal cord tissues and cerebrospinal fluid are in the range of 2–6 μM (Whitehead et al. 2001). In this regard, cannabinoid-induced potentiation of glycine receptors in the presence of low glycine receptor occupancy should be physiologically relevant.

9.2.4.2 Factors Influencing Potentiation: Subunit Specificity

Both endogenous and exogenous cannabinoids modulate glycine receptors in a subunit-specific manner (Yang et al. 2008; Xiong et al. 2011b, 2012; Yevenes and Zeilhofer 2011). AEA was initially found to produce various effects on I_{Gly} in different neurons (Lozovaya et al. 2005; Hejazi et al. 2006; Xiong et al. 2012). Among

Table 9.1 Effects of cannabinoids on GlyRs

Cannabinoids	Expressing system	GlyR subunits	Effects	Maximum Potentiation (folds)	EC_{50} (μM)	Drug application	References
Δ^9-THC	HEK-293 cells	Human α1	Potentiation	32±6.0	2.5±0.7	Sustained	Xiong et al. (2011b)
	HEK-293 cells	Rat α2	Potentiation	7.5±2.2	9.1±1.8	Sustained	Xiong et al. (2011b)
	HEK-293 cells	Rat α3	Potentiation	30±5.8	2.6±1.1	Sustained	Xiong et al. (2011b)
	Oocytes	Human α1	Potentiation	0.9±0.1	0.086±0.009	–	Hejazi et al. (2006)
	Oocytes	Human α1β1	Potentiation	0.98±0.08	0.073±0.008	–	Hejazi et al. (2006)
	VTA neurons	Rat native	Potentiation	0.54±0.06	0.12±0.013	–	Hejazi et al. (2006)
AEA	Oocytes	Human α1	Potentiation	1.1±0.1	0.32±0.031	–	Hejazi et al. (2006)
	Oocytes	Human α1β1	Potentiation	1.1±0.07	0.32±0.024	–	Hejazi et al. (2006)
	VTA neurons	Rat native	Potentiation	0.18±0.04	0.23±0.029	–	Hejazi et al. (2006)
	Hippocampal pyramidal and Purkinje cerebellar neurons	Native	Inhibition	–	0.3	–	Lozovaya et al. (2005)
	Spinal neurons	Native	Potentiation	8.0±0.7	5.5±2.0	Sustained	Xiong et al. (2012)
	HEK-293 cells	Human α1	Potentiation	0.8	0.038±0.011	–	Yang et al. (2008)
	HEK-293 cells	Human α1	Potentiation	8.2±2.8	4.2±2.0	Sustained	Xiong et al. (2012)
	HEK-293 cells	Human α2	No effect	–	–	–	Yang et al. (2008)
	HEK-293 cells	Rat α3	No effect	–	–	–	Yang et al. (2008)
2-AG	Hippocampal pyramidal and Purkinje cerebellar neurons	Native	Inhibition	–	–	–	Lozovaya et al. (2005)
	Hippocampal pyramidal neurons	Native	Inhibition	–	–	–	Yatsenko and Lozovaya (2007)
	CB_1 knockout mice brainstem slices	Glycine IPSC	Inhibition	–	–	–	Lozovaya et al. (2011)
Win 55,212-2	Hippocampal pyramidal and Purkinje cerebellar neurons	Native	Weak potentiation	–	–	–	Lozovaya et al. (2005)
	HEK-293 cells	Human α1	No effect	–	–	–	Yang et al. (2008)
	HEK-293 cells	Human α2	Inhibition	–	0.22±0.05	–	Yang et al. (2008)
	HEK-293 cells	Rat α3	Inhibition	–	0.086±0.026	–	Yang et al. (2008)

(continued)

Table 9.1 (continued)

Cannabinoids	Expressing system	GlyR subunits	Effects	Maximum Potentiation (folds)	EC_{50} (μM)	Drug application	References
Ajulemic acid	HEK-293 cells	Human α1	Potentiation	–	–	–	Foadi et al. (2010)
	HEK-293 cells	α1	Potentiation	I_{max}	9.7 ± 2.6	–	Ahrens et al. (2009b)
CBD	HEK-293 cells	Human α1	Potentiation	–	–	–	Foadi et al. (2010)
		α1	Potentiation	I_{max}	12.3 ± 3.8	–	Ahrens et al. (2009a)
		α1β1	Potentiation	I_{max}	18.1 ± 6.2	–	Ahrens et al. (2009a)
HU210	HEK-293 cells	Human α1	Potentiation	–	–	–	Foadi et al. (2010)
	HEK-293 cells	Human α1	Potentiation	1.0	0.27 ± 0.05	–	Yang et al. (2008)
	HEK-293 cells	Human α2	Inhibition	–	0.09 ± 0.021	–	Yang et al. (2008)
	HEK-293 cells	Rat α3	Inhibition	–	0.05 ± 0.006	–	Yang et al. (2008)
	HEK-293 cells	α1	Potentiation	I_{max}	5.1 ± 2.6	–	Demir et al. (2009)
HU-308	HEK-293 cells	Human α1	Weak inhibition	–	1.0	–	Yang et al. (2008)
	HEK-293 cells	Human α2	Inhibition	–	1.13 ± 0.3	–	Yang et al. (2008)
	HEK-293 cells	Rat α3	Inhibition	–	0.097 ± 0.017	–	Yang et al. (2008)
NA-Gly	HEK-293 cells	Human α1	Complex effects[a]	–	–	–	Yang et al. (2008)
	HEK-293 cells	Human α2	Inhibition	–	3.03 ± 0.09	–	Yang et al. (2008)
	HEK-293 cells	Rat α3	Inhibition	–	1.32 ± 0.10	–	Yang et al. (2008)

[a]Complex effects with initial potentiation and subsequent inhibition

the three glycine receptor α subunits (α1, α2 and α3) expressed in HEK-293 cells, the α1 subunit is most sensitive to AEA-induced potentiation (Yang et al. 2008; Yevenes and Zeilhofer 2011; Xiong et al. 2012). In addition to AEA, other cannabinoids and cannabinoid mimic lipids such as *N*-arachidonyl-glycine (NA-glycine) exhibited complex action (both potentiation and inhibition) of I_{Gly} in a subunit-specific manner (Yevenes and Zeilhofer 2011). NA-glycine potentiated the amplitude of I_{Gly} in HEK-293 cells expressing the α1 subunits and inhibited the amplitude of I_{Gly} in HEK-293 cells expressing the α2 and α3 subunits (Yevenes and Zeilhofer 2011). Similarly, THC has been shown to potentiate glycine receptors in a subunit-specific manner expressed in HEK-293 cells (Xiong et al. 2011b). The most significant difference among the three subunits appears to be the efficacy of the THC potentiation (Xiong et al. 2011b). For instance, the magnitudes of THC (1 μM)-induced potentiation of I_{Gly} were 1,156, 1,127, and 232% in HEK-293 cells expressing the α1, α3, and α2 subunits, respectively. It should be mentioned that heteromeric α1β1 subunits are less sensitive than their counterpart homomeric α1 receptors to THC-induced potentiation (Hejazi et al. 2006; Xiong et al. 2011b).

9.2.4.3 Factors Influencing Potentiation: Simultaneous Cannabinoid Application vs. Sustained Cannabinoid Incubation

The variation in the potency of cannabinoid potentiation can be caused by different methods of cannabinoid application. While most previous studies co-applied cannabinoids with glycine simultaneously, two recent studies have suggested that sustained cannabinoid incubation is critical for obtaining the maximal potentiation induced by THC or AEA (Xiong et al. 2011b, 2012). The magnitude of potentiation gradually increases over the first few min of sustained THC or AEA exposure with intermittent glycine applications every min. The maximal potentiation of I_{Gly} was reached 5 min after sustained THC or AEA incubation in both spinal neurons and in HEK-293 cells expressing homomeric and heteromeric glycine receptors.

Different protocols for THC or AEA application (sustained application vs. simultaneous application with agonists) could cause several notable differences between previous and more recent studies (Hejazi et al. 2006; Yang et al. 2008; Xiong et al. 2011b, 2012). First, the maximal magnitude of potentiation of the α1 glycine receptor by 1 μM THC was around 80–100% when this compound was applied simultaneously with glycine (Hejazi et al. 2006). In contrast, the maximal potentiation of the α1 glycine receptor was 700–800% when THC was applied continuously for 5 min with intermittent supplementation of glycine (Xiong et al. 2011b). Second, because of a pronounced changes in E_{max} for THC potentiation and the maximally efficacious concentration of THC, the EC_{50} value of THC potentiation increased from 86 nM as described in an earlier report to 1.2 μM as reported in a recent study (Hejazi et al. 2006; Xiong et al. 2011b). A similar effect has been reported for AEA-induced potentiation of glycine receptors (Xiong et al. 2012). Third, there is evidence to suggest that distinct molecular processes are involved in the effects induced by simultaneous and sustained cannabinoid incubation. Simultaneous AEA application did not significantly

alter I_{Gly} in HEK-293 cells expressing the α3 glycine receptor in a previous study (Yevenes and Zeilhofer 2011), whereas sustained AEA incubation significantly potentiated α3 glycine receptors (Xiong et al. 2012). It is likely that the larger effect of sustained AEA application explains this difference. In fact, sustained cannabinoid incubation has been used in studies of cannabinoid modulation of 5-HT_3 receptors, nAChRs and $GABA_A$Rs (Fan et al. 1995; Barann et al. 2002; Spivak et al. 2007; Xiong et al. 2008; Sigel et al. 2011).

9.2.5 Molecular Mechanisms

The first analysis of potential molecular sites of cannabinoid potentiation focused on the S267Q mutation in the TM2 domain of the α1 subunit. The S267Q mutation is found to abolish ethanol and volatile anesthetic-induced potentiation of I_{Gly} and thus is thought to be critical for the actions of alcohol and volatile anesthetics on glycine receptors (Mihic et al. 1997). There are inconsistent findings coming out from studies using *Xenopus* oocytes and HEK-293 cells as expression systems (Hejazi et al. 2006; Foadi et al. 2010). The S267Q mutation did not significantly alter THC or AEA-induced potentiation of I_{Gly} when the S267Q mutant receptors were expressed in *Xenopus* oocytes (Hejazi et al. 2006). In contrast, the S267Q mutation abolished the potentiation induced by three compounds (CBD, HU-210, ajulemic acid) structurally similar to THC when the mutant receptors were expressed in HEK-293 cells (Foadi et al. 2010). Thus, the effect of the S267A mutation on cannabinoid potentiation of glycine receptor α1 subunits appears cell type specific. Nevertheless, there is considerable evidence showing that the S267Q mutation significantly impairs properties of the glycine receptor channel and causes severe neurological defects in mice carrying the S267Q mutation (Findlay et al. 2002, 2003, 2005). In this regard, the idea that the S267Q mutation is an interacting site of glycine receptors with cannabinoids should be made with caution.

Two recent studies by Xiong and colleagues have revealed that a serine residue (S) in the TM3 domain of the α1 and α3 subunits is critically involved in the interaction between THC or AEA and glycine receptors (Xiong et al. 2011b, 2012). Substituting the serines (S) at positions 296 and 307 within the TM3 domain of the α1 and α3 subunits, respectively, with an alanine (A) as found at the equivalent position within the α2 subunit reduced the sensitivity of α1/α3 receptors to cannabinoids to that of α2 containing receptors (i.e., less sensitive to cannabinoid potentiation) (Fig. 9.1).

The idea that S296 is a molecular determinant of cannabinoid potentiation of glycine receptors has gained further support. In an experiment involving NMR chemical shift measurement, THC shifted the S296 residue in a concentration-dependent manner in the purified protein containing the full-length 4 TM regions of the human α1 subunit (Fig. 9.1). Mutagenesis analysis suggests that THC interacts with S296 through a hydrogen bond (Xiong et al. 2011b). Consistent with this idea, didesoxy-THC, a modified THC with removal of both hydroxyl and oxygen groups failed to affect I_{Gly} when applied alone but competitively inhibited the potentiation

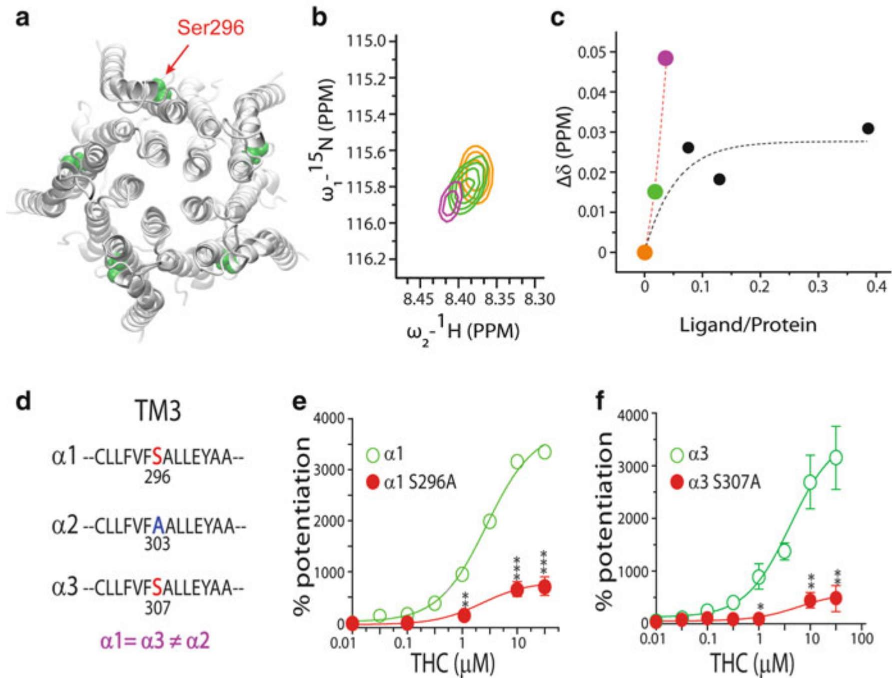

Fig. 9.1 S296 is critical for interaction with THC. (**a**) Molecular modeling of the four transmembrane domains of the α3 glycineR protein. (**b**) NMR analysis: the S296 of ^{15}N-^{1}H HSQC resonance. Chemical shift in three representative HSQC spectra of 420 μM glycine receptor-TM titrated by 0 μM (*orange*), 8.5 μM (*green*), and 17 μM (*pink*) of THC. (**c**) Observed chemical shift changes (Δ*δ*) as a function of the ligand (THC) to protein (glycine receptor-TM) concentration ratio (*colored solid circles*, low ligand-to-protein ratio; *black filled circles*, high ligand-to-protein ratio). (**d**) Amino acid alignment of the TM3 region flanking S296 (α1) or equivalent residues in the α2 and α3 subunits. (**e**) The concentration response curves of THC potentiation in cells expressing the wild-type (α1) and S296A mutant receptors. $^{**}P<0.01$, $^{***}P<0.001$, ANOVA against α1 ($n=7$–9). (**f**) The concentration response curves of THC potentiation in cells expressing the wild-type (α3) and S307A mutant receptors. $^{**}P<0.01$, ANOVA against α3 ($n=5$–6)

of I_{Gly} induced by AEA and THC (Xiong et al. 2011b, 2012). This finding also suggests that exogenous and endogenous cannabinoids potentiate glycine receptors via a common molecular basis involving the S296 residue in the TM3 region of the α1 and α3 subunits.

Besides S296, other residues that differ between the α1/α3 and α2 subunits may also contribute to differential AEA or THC potentiation of glycine receptors. The S296A mutation appears to selectively contribute to the mechanism underlying sustained cannabinoid-induced potentiation since the S296A mutation did not significantly alter the potentiation of the α1 and α3 subunits induced by simultaneous AEA application (Yevenes and Zeilhofer 2011; Xiong et al. 2012).

There is evidence to suggest that some point-mutations in the TM domains in the α2 subunit can turn cannabinoid potentiation to inhibition of I_{Gly} (Yevenes and Zeilhofer 2011). In addition, the K385A mutation in the large cytoplasmic domain

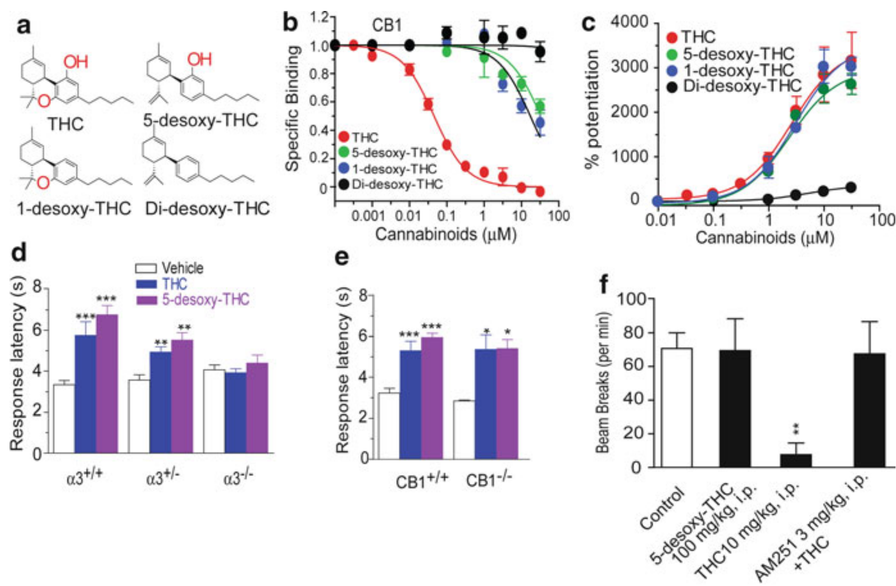

Fig. 9.2 Functional characterization of 5-desoxy-THC and didesoxy-THC. (**a**) Chemical structure of 5-desoxy-THC, 1-desoxy-THC, and didesoxy-THC. (**b**) The concentration response curves of THC, 5-desoxy-THC, 1-desoxy-THC, and didesoxy-THC in suppressing specific binding of [^{3}H]-CP55940 in purified brain membranes. (**c**) The concentration response curves of cannabinoid potentiation of I_{Gly} in HEK-293 cells expressing the α1 glycine receptors. (**d**) The analgesic effect of THC and 5-desoxy-THC in the TFR in the wild-type litter mates ($\alpha3^{+/+}$), heterozygotes ($\alpha3^{+/-}$) and homozygotes ($\alpha3^{-/-}$) of α3 glycine receptor-KO mice. $^{**}P<0.01$, $^{***}P<0.001$ ($n=5$–7). (**e**) The analgesic effect of THC and 5-desoxy-THC in the TFR in $CB1^{+/+}$ and $CB1^{-/-}$ mice. $^{*}P<0.05$, $^{***}P<0.001$ ($n=6$). (**f**) The effects of THC and 5-desoxy-THC on locomotor activity. $^{**}P<0.01$ ($n=6$–7). Note that THC but not 5-desoxy-THC induces hypolocomotion. This effect induced by THC is reversed by the CB1 antagonist AM251, respectively

of the α1 subunit was found to reduce AEA and NA-glycine-induced potentiation of I_{Gly} (Yevenes and Zeilhofer 2011). The K385A mutation in the α1 subunit is also reported to critically contribute to the sensitivity of glycine receptors to ethanol-induced potentiation (Yevenes et al. 2008, 2010).

9.2.5.1 In Vivo Consequence

The first evidence for an in vivo effect of cannabinoid potentiation of glycine receptors was recently provided by Xiong et al. (2011b). THC and 5-desoxy-THC, a chemically modified THC that shows significantly reduced CB_1 receptor binding affinity, produced analgesia in a tail flick reflex test in mice (Fig. 9.2). The analgesic effect of the cannabinoids remained intact in knockout mice lacking CB_1 and CB_2 receptors. However, the analgesic effect was absent in mice lacking glycine receptor α3 subunits. Moreover, the cannabinoid-induced analgesia was prevented by administration

of strychnine, a selective glycine receptor antagonist, as well as by didesoxy-THC, but not by SR141716. Collectively, these observations suggest that cannabinoid potentiation of glycine receptors may contribute to cannabis-induced analgesia. Because 5-desoxy-THC lacks CB_1 receptor binding affinity and retains the potency in potentiating glycine receptors, 5-desoxy-THC can provide pain relief without causing psychoactive behavioral effects (Fig. 9.2).

9.3 Summary

It is evident from the data summarized in this chapter that nonpsychoactive cannabinoids critically regulate the functions of the 5-HT_3 and glycine receptors. These CB_1 receptor-independent effects should be given additional attention since these effects, in some cases, were obtained in the nanomolar concentration range in vitro. Currently, the widespread medical use of cannabis is controversial because the plant can produce both therapeutic and unwanted effects. The cannabinoid–glycine receptor interaction appears to represent a novel mechanism through which cannabis-induced analgesic effects can be separated from cannabis-induced psychoactive effects (Fig. 9.3) (Christie and Vaughan 2011). This could open up a new avenue for developing novel analgesic agents based on cannabinoids that are selective allosteric modulators of glycine receptors. Besides antinociceptive actions, cannabinoids

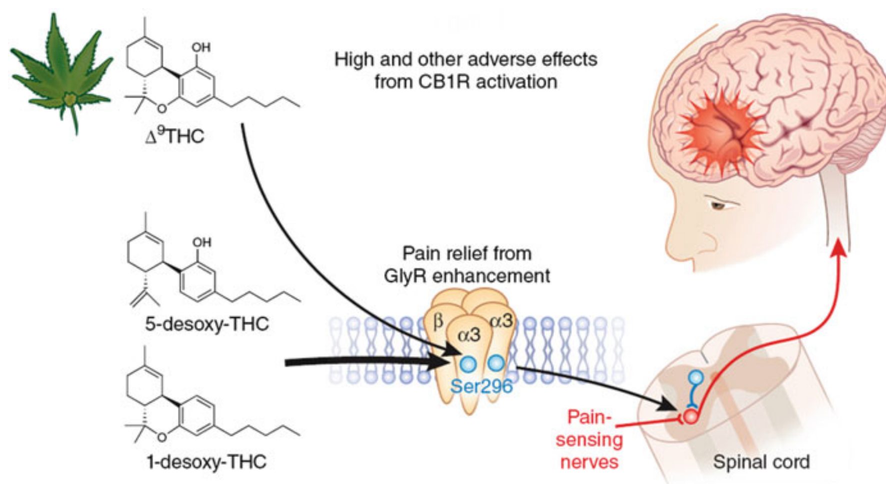

Fig. 9.3 A glycine receptor-dependent mechanism of THC and 5-desoxy-THC-induced analgesia in the spinal cord. Both cannabinoids interact with S296 in the α3 subunits, thereby enhancing inhibition of pain transmission neurons (*red*) via glycinergic neurons (*blue*). 5-desoxy-THC lacks CB_1 receptor activity and does not produce psychoactive side effect in the brain. Cited from Christie and Vaughan (2011)

and glycine receptors play similar roles in the processes of neuromotor activity, seizure, anxiety, drug abuse, and muscle relaxation (Pacher et al. 2006; Lynch 2009). We currently lack subunit-specific agonists and antagonists for glycine receptors, and the development of novel glycinergic cannabinoids with specificity for single glycine receptor subtypes may prove useful in treating these disorders. In addition, identification of the molecular sites for cannabinoid modulation of glycine receptors will inform the development of novel genetically modified mice. These transgenic mice could be a valuable research tool for exploring the role of glycine receptors in some of the nonpsychotropic cannabinoid-induced behaviors.

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Part IV
Transcription Factors

Chapter 10
Peroxisome Proliferator-Activated Receptors and Inflammation

James Burston and David Kendall

Abbreviations

IL-1β	Interleukin 1 beta
PEA	Palmitoylethanolamide
PPARα	Peroxisome proliferator-activated receptors alpha
PPARβ/δ	Peroxisome proliferator-activated receptors beta
PPARγ	Peroxisome proliferator-activated receptors gamma

10.1 Introduction

After a short introduction to the topic, this chapter discusses evidence that the nuclear peroxisome proliferator-activated receptors (PPARs) are involved in and modulate inflammation, the inflammatory processes, and immune cell migration.

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10.2 PPAR Structure and Control of Gene Transcription

The PPARs belong to the superfamily of nuclear hormone receptors which produce their effects via regulation of the transcription of many genes. There are three PPAR isoforms; α, δ (also known as β) and γ, all of which, when activated by appropriate ligands, heterodimerize with a partner protein, the 9-*cis*-retinoic acid (retinoid X, RXR) receptor, and bind to DNA sequences in target genes, thereby modulating transcription. Although each of the PPAR isoforms is a separate gene product, they show substantial amino acid sequence similarity.

PPARs have three main domains: LBD (ligand binding domain), AF-1 (activation function-1), and DBD (DNA binding domain). The LBD is located at the C-terminal forming a ligand-binding pocket with 13 α-helices and a small 4-stranded β-sheet (Xu et al. 1999). The ligand-binding domains of the PPARs are unusually large and, thus, accommodating to a wide variety of ligands of quite different structures, resulting in the receptors being rather promiscuous in relation to agonist activation. The AF-1 region is N-terminally located and has ligand-*independent* transcriptional regulation ability. The DBD, which is adjacent to AF-1, is the part of the protein by which PPARs, via two zinc fingers, bind to the PPREs (peroxisome proliferator responsive elements) of the regulated genes. The DBD and the LDB regions are the most highly conserved of the three PPAR isoforms. There is an AF-2 domain at the C-terminal of the LBD which, in contrast to AF-1, is a ligand-*dependent* activation domain. After agonist binding, PPARs undergo a conformational change and fuse with RXR forming an asymmetrical heterodimer that binds to the PPREs of the numerous PPAR-inducible genes. The majority of PPREs are direct repeat (DR)-1 elements consisting of two hexanucleotides with the consensus sequence AGGTCA separated by a single nucleotide spacer (e.g., AGGTCAxAGGTCA). Other protein partners are required for transcriptional control and PPARs interact with a number of co-activators and co-repressors which either aid or suppress transcription (Fig. 10.1).

In the non-activated state, in the absence of agonist binding, the heterodimer complex is associated with multicomponent co-repressors which have histone deacetylase activity. These include nuclear receptor co-repressor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Chen and Evans 1995; Horlein et al. 1995). In the deacetylated state histone inhibits transcription, then, upon ligand binding, co-activators such as CBP/p300 and steroid receptor co-activator (SRC)-1 which have histone acetylase activity replace the co-repressors and initiate a sequence of events which induces gene transcription (Xu et al. 1999). These co-activators interact with the nuclear receptors in an agonist-dependent and gene-specific manner through a conserved LXXLL motif (where X is any amino acid) by binding to a hydrophobic cleft in the surface of the receptor formed by helices 3, 4, and 5 and the AF-2 helix (Berger and Moller 2002). It is likely that the pattern of interaction of the PPARs with different cofactors allows a variety of downstream signaling pathways to be engaged, resulting in agonist-selective physiological responses (or agonist biased signaling).

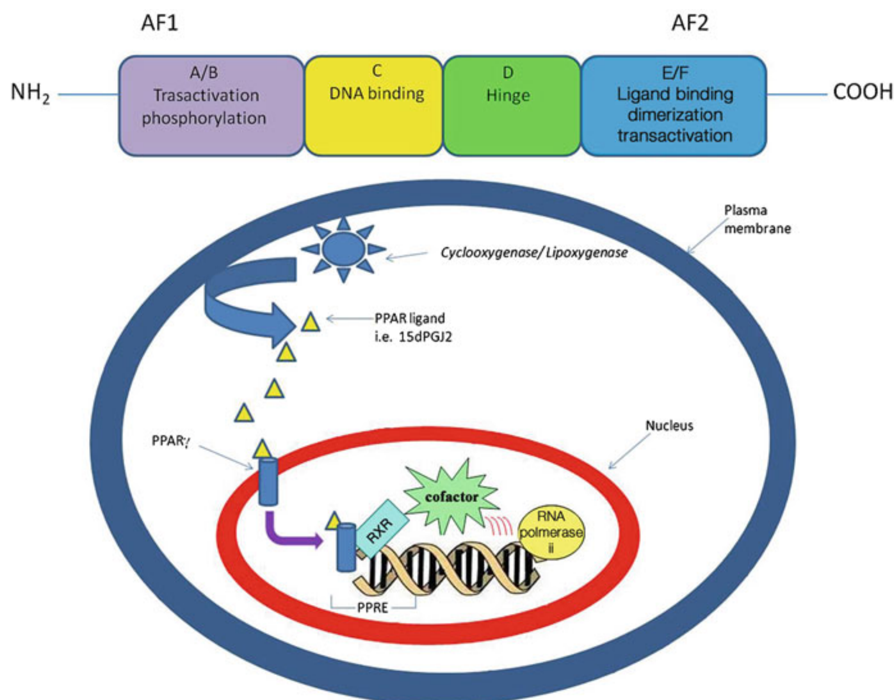

Fig. 10.1 The structure and transcription-modulating activities of PPARs. PPARs have three main domains: AF-1, DBD, and LBD. PPARs act on the PPREs by forming heterodimers with another nuclear hormone receptor, RXR. After binding to certain agonists, PPARs change the structure and bind with RXR to form an asymmetrical dimer. This binding changes the conformation of the PPARs and induces it to bind the PPRE, which have been found in numerous PPAR-inducible genes. With the help of certain co-activators and co-repressors, related genes transcription is induced (or suppressed)

10.3 PPAR Ligands

To date a number of compounds have been suggested to act as endogenous and exogenous activators of PPAR receptors; Tables 10.1 and 10.2 list some of these compounds as well as their receptor subtype selectivity.

10.3.1 PPARα

There are numerous studies indicating that peroxisome proliferator-activated receptors alpha (PPARα) appears to be involved in the control of inflammation. Activation of PPARs inhibits the expression of pro-inflammatory genes and reduces the

Table 10.1 Endogenous PPAR ligands

PPARα	PPARγ	PPARβ/δ
Palmitic acid	Linoleic acid	Dihomo-γ-linolenic acid
Stearic acid	Arachidonic acid	EPA
Palmitoleic acid	EPA	Arachidonic acid
Oleic acid	DHA	Eicosanoids
Linoleic acid	9-HODE	(PGA1 and PGD2)
Arachidonic acid	13-HODE	
Eicosapentaenoic acid	15dPGJ2	
8(S)-HETE	azPC	
LTB4		

EPA eicosapentaenoic acid; *DHA* docosahexaenoic acid; *9-HODE* 9-hydroxy-10,12-octadecadienoic acid; *13-HODE* 13-hydroxy-9,11-octadecadienoic acid; *15dPGJ2* 15-deoxy-Δ-12,14-prostaglandin J2; *azPC* hexadecyl azelaoyl phosphatidylcholine; *8(S)-HETE* 8-hydroxyeicosa-5,9,11,14-tetraenoic acid; *PGA1* prostaglandin A1; *PGD2* prostaglandin D2; *LTB4* leukotriene B4

Table 10.2 Synthetic PPAR ligands

PPARα	PPARγ	PPARβ/δ
Fibrates	TZDs	L-165041
WY-14.643	JTT-501 (isoxazolidinedione)	GW 501516
Gemfibrozil	GW-7845	
Nafenopin	BRL49653	
LY518674	PA-082 (partial agonist)	
	BADGE (antagonist)	
	LG-100641 (antagonist)	
	GW9662 (antagonist)	

Interestingly many of the synthetic ligands listed here have much higher potencies/affinities than the naturally occurring compounds, such as the fatty acid derivatives. Ligands shown are agonists unless otherwise indicated *TZDs* thiazolidinediones; *BADGE* bisphenol A diglycidyl ether

production of cytokines, metalloproteases, and acute-phase proteins. Regulation of the NF-κB pathway is a key feature of the anti-inflammatory effects of PPARα and activation both increases I-κB and reduces the expression of the NF-κB p50 subunit (Zandbergen and Plutzky 2007). Importantly, PPAR activation stimulates the catabolism of pro-inflammatory eicosanoids (Delerive et al. 2001) and PPARα-null mice have been shown to be more responsive than their wild-type littermates to leukotriene B4 (LTB4) and its precursor arachidonic acid (Devchand et al. 1996). PPAR-α knockout mice also display significantly elevated levels of neutrophils, macrophages, and tumor necrosis factor alpha (TNF-α), following intranasal administration of lipopolysaccharide, compared with wild-type (Delayre-Orthez et al. 2005). Furthermore, Woerly et al. (2003), reported that ovalbumin challenge in previously sensitized mice resulted in increased eosinophilia (approximately 5.3 fold) in PPAR-α knockout mice as compared to wild-type controls.

PPARα also controls lipid metabolism via a number of mechanisms. They regulate fatty acid transport by inducing expression of transport proteins, such as FATP (Martin et al. 1997) and FAT (Motojima et al. 1998) and modulate lipid oxidation by controlling the transcription of a variety of mitochondrial genes such as carnitine palmitoyltransferase I (CPT I) (Brady et al. 1989), acyl-CoA dehydrogenases, and the hydroxymethylglutaryl-CoA synthase (Aoyama et al. 1998). These effects on lipid oxidation may further contribute to the control of the inflammatory response, especially as lipid peroxidation is associated with modulation of inflammatory cell activity as well as induction of COX-2 (Kumagai et al. 2004). Interestingly, given the modulator effects of PPARα on lipid oxidation and metabolism, one can speculate that targeting PPARα may prove a useful strategy to counteract inflammatory processes, specifically, chronic, low-grade tissue inflammation in diabetes (Wellen and Hotamisligil 2005).

10.3.2 PPARγ

There is growing evidence of a role for peroxisome proliferator-activated receptors gamma (PPARγ) in the control of inflammation. The receptors modulate the production of inflammatory cytokines such as TNF-α and IL-6/IL-12 and free radicals such as nitric oxide (NO) and superoxide by monocytes and macrophages, and also appear to control immune cell differentiation and function (Cunard et al. 2002a, b; Jiang and Dhib-Jalbut 1998). PPARγ expression is increased in activated murine peritoneal macrophages and T cells and upon the differentiation of monocytes into macrophages; ligand activation inhibits the expression of inducible nitric oxide synthase (iNOS), gelatinase B, and scavenger receptor A genes (Chinetti et al. 1998; Ricote et al. 1998). PPARγ activation appears to be important in controlling neuroinflammation via modulation of microglial function, for instance in Alzheimer's Disease (Shie et al. 2009). Numerous studies (Mendez and LaPointe 2003; Shiojiri et al. 2002) have revealed that PPARγ agonists inhibit the expression (both mRNA and protein) of enzymes involved in producing inflammatory mediators, for example, cyclooxygenase and nitric oxide synthase. In addition to modulating expression of these two enzymes, PPARγ agonists have also been shown to alter the production of prostanoids (Sawano et al. 2002) and of nitric oxide (Shiojiri et al. 2002). To date, there are limited data on inflammatory processes in PPARγ knockout mice, due to the lethal nature of complete PPARγ deletion (Woerly et al. 2003). In one study (Natarajan et al. 2003), PPARγ-deficient heterozygous mice display an exacerbated experimental allergic encephalomyelitis in comparison with wild-type controls, which lead to CNS inflammation and demyelination. In the same study, PPARγ heterozygous mice showed increased T cell proliferation and Th1 responses to MOGp35-55 (myelin oligodendrocyte glycoprotein p35-55), suggesting that PPARγ may be a critical regulator of CNS inflammation, and possibly involved in the genesis of multiple sclerosis.

More recent work has suggested that targeting PPARγ may provide a therapy for reducing pro-inflammatory mediators after neuronal injury, including trauma to the spinal cord (McTigue 2008). Indeed, targeting PPARγ receptors may also be effective in reducing CNS trauma-induced inflammation as PPARγ agonists modulate CNS glial cell (microglia and astrocyte) activity (Carta et al. 2011). PPARγ has also been shown to have a modulatory function in Alzheimer's disease (Xu et al. 2008), as the PPARγ receptor agonist 15-deoxy-Δ12,14-prostaglandin J2 attenuated microglial production of pro-inflammatory cytokines. *In vivo* treatment with the PPARγ agonists 15-deoxy-Δ12,14-prostaglandin J2 and cigitazone ameliorates experimental allergic encephalomyelitis by inhibition of IL-2 production and signaling (Diab et al. 2002; Natarajan and Bright 2002). Another report (Raikwar et al. 2005) that used the same model found that BADGE (Table 10.2; a PPARγ antagonist) reversed the protective effect of PPARγ. This result suggests that the effect was mediated by PPARγ, and that PPARγ is involved in the pathological development of this inflammatory state. Additional information on the roles of PPARs in CNS inflammation is available in the excellent review by Bright et al. (2008).

10.3.3 PPARβ/δ

There is far less evidence for anti-inflammatory effects of peroxisome proliferator-activated receptors beta (PPARβ/δ) activation. To the best of our knowledge, the only available relevant report is that of Defaux et al. (2009) in which GW501516 (a selective PPARβ agonist), when applied to brain cell cultures, reduced GFAP expression and reversed interferon-gamma (IFNγ)-induced upregulation of TNF-α and iNOS expression. However, it is difficult to conclude from this work that PPARβ activation reduces inflammatory mediators, as, in the same report, GW501516 induced a significant increase in interleukin-6 (IL-6) expression in both control cultures and in those treated with IFNγ.

10.4 Immune Cell Modulation and Infiltration

Up to this point the chapter has provided specific examples of the links between PPAR activation and modulation of pro-inflammatory mediators or the effectiveness of PPAR ligands in inflammatory models. However, it is equally important to discuss PPAR-induced modulation of immune cells and the ability of PPAR ligands to inhibit immune cell infiltration and activity. Within the last 10 years there has been considerable research investigating the involvement of PPARs (particularly PPARα and PPARγ) in the immune cascade. Previous work (Chinetti et al. 1998) has shown that PPARα and PPARγ are expressed in differentiated human monocyte-derived macrophages, and that ligand binding and activation of PPARγ results in apoptosis

of unactivated differentiated macrophages. This study also suggested that PPAR activators induce macrophage apoptosis by negatively regulating the anti-apoptotic NF-κB signaling pathway.

A more recent study (Sethi et al. 2002) has shown that oxidized eicosapentaenoic acid reduces leukocyte rolling and adhesion to vascular endothelium in mice treated with intraperitoneal lipopolysaccharide. This could be ascribed to PPARα activation as non-oxidized eicosapentaenoic acid was unable to reproduce the effect; however, a specific PPARα antagonist was not used in the experiments. Furthermore, this study found that (in human umbilical vein endothelial cell culture) oxidized eicosapentaenoic acid not only inhibited LPS-induced neutrophil and monocyte adhesion, but also reversed LPS-stimulated increases in the chemoattractant molecules intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1). Frode et al. (2009) showed that pioglitazone, a PPARγ agonist, was able to reduce myeloperoxidase (a surrogate for neutrophil activity/infiltration), adenosine-deaminase, TNF-α, and interleukin 1 beta (IL-1β) levels in the first phase of a mouse pleurisy model involving intra-pleural carrageenan administration. Furthermore, pioglitazone reduces these same mediators (apart from myeloperoxidase) in the second phase of the model.

In 2006, Genovese et al. proposed that PPARα might be involved in modulating a number of factors in an animal model of pancreatitis (i.p. administration of cerulean) (Genovese et al. 2006). In this report, the authors showed that cerulean-induced pancreatic edema is significantly elevated in PPARα knockout vs. wild-type controls. Interestingly, in the PPARα knockout mice, cerulean induced a greater elevation (than in wild-type controls) of pancreatic TNF-α, ICAM-1, P-selectin, transforming growth factor beta (TGF-β), vascular endothelial growth factor (VEGF), and myeloperoxidase activity, which are associated with immune cell infiltration, inflammation, and vascularization. Perhaps the most dramatic effect reported in the latter study was seen with regard to survival rate. Two days after cerulean treatment, a number of mice in the PPARα knockout group had died, yet no mortality was seen in the wild-type group at the same time point. By day 5 post-cerulean, 30% of the wild-type mice had survived compared with none in the PPARα knockout group. Taken together, these data suggest that PPARα and PPARγ may be important in modulating immune/inflammatory actions and dampening the "immune barrage" (Fig. 10.2). This proposed immune barrage is of interest in relation to the PPARs as the work summarized thus far in this chapter suggests that, in an inflammatory condition that includes immune cell infiltration into tissue space (e.g., bacterial-induced tissue inflammation), targeting PPARs would be appealing as this could offer multiple anti-inflammatory targets especially when the inflammation becomes pathological. For example, targeting PPARs could (1) decrease pro-inflammatory molecules such as prostaglandins, cytokines, and reactive oxygen species, (2) decrease immune cell invasion by altering chemotactic signals generated from tissue-resident cells (macrophages), (3) directly inhibit immune cell invasion/migration by interacting with PPARs expressed on immune cells, and (4) modulate tissue repair. Thus, targeting PPARs to alter inflammation, especially inflammation that has a specific immune component, could be therapeutically useful.

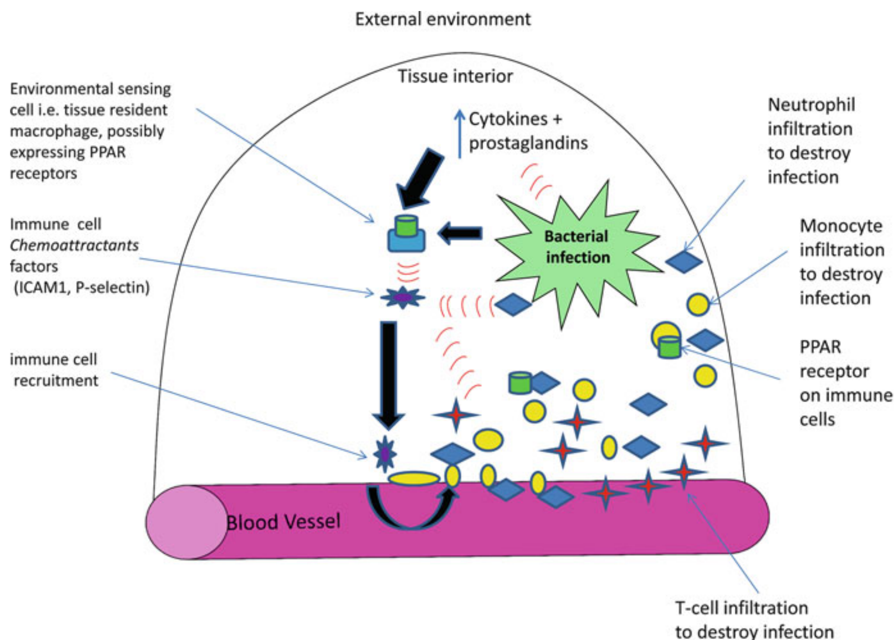

Fig. 10.2 The inflammatory/immune barrage. A visual representation of bacterial-induced elevation of chemotactic factors, possibly released from tissue-resident macrophages as well as other cells, which would then be expected to induce invasion of immune cells (macrophages, neutrophils, and t-cells amongst others) into the tissue to destroy the bacterial threat. Given that PPARs are expressed on immune cells (*green cylinders*) and that activation of PPARs has been shown to reduce pro-inflammatory cytokines and inhibit immune cell invasion, there are multiple points within this pathway that PPAR activation could potentially modulate

For further information on the interactions between PPARs and other inflammatory responses and immune cells (including T-cell interaction) readers are directed to the excellent reviews by Yang et al. (2008) and Clark (2002).

10.5 Cannabinoids and PPARs

Although the anti-inflammatory/analgesic properties of endogenous and exogenous synthetic cannabinoids are mainly mediated through the cannabinoid CB_1 and CB_2 receptors, there is a growing evidence to suggest that both endogenous and exogenous cannabinoids can interact with PPARs. Kozak et al. (2002) showed that micromolar concentration of 15-hydroxyeicosatetraenoic acid glyceryl ether (a 15-lipoxygenase metabolite of the endocannabinoid 2-AG) was able to increase the transcriptional activity of PPARα. Interestingly, a number of cannabinoid agonists including THC and anandamide exhibit appreciable binding affinity (Sun et al. 2006) to mouse PPARα. Subsequent to this report, anandamide was shown to

increase PPARα transcriptional activity when examined in a HeLa cell luciferase activity assay (Sun et al. 2007). The potential functional importance of the interaction between endocannabinoids and PPARs was highlighted in a behavioral study by Jhaveri et al. (2008) which showed that the fatty acid amide hydrolase (FAAH) inhibitor URB-597 was able to reduce intraplantar carrageenan-induced inflammatory hyperalgesia, and that this effect was reversed by the PPARα agonist GW6417, which suggests that URB-597 induced elevation of anandamide as well as palmitoylethanolamide (PEA) probably activates PPARα as well as the CB_1 receptor. However, the effect of a CB_1 receptor selective antagonist was not examined in this report. Nevertheless, a study reported in the same year (Costa et al. 2008) concluded that the anti-hyperalgesic effects of PEA (in a chronic constriction injury model) was mediated both through the CB_1 receptor as well as PPARα.

Other lines of research demonstrate interactions between cannabinoids and PPARγ. O'Sullivan et al. (2005) showed that Δ^9-tetrahydrocannabinol at concentrations as low as 100 nM activates the transcriptional activity of PPARγ. Furthermore, this same group (O'Sullivan et al. 2006) showed that Δ^9-tetrahydrocannabinol-induced vasodilation in isolated arteries was inhibited by a PPARγ antagonist GW9662. It has also been shown (Bouaboula et al. 2005; Gasperi et al. 2007; Rockwell and Kaminski 2004; Rockwell et al. 2006) that both anandamide and 2-AG bind directly to and activate PPARγ. Interestingly, both cannabinoids induced a decrease in the secretion of the pro-inflammatory cytokine interleukin-2, which was reversed by PPARγ antagonism. In contrast to the cannabinoid interactions with PPARα and PPARγ, there is, to the best of our knowledge, no evidence of specific interactions of endocannabinoids with the PPARδ/β.

Given the existing evidence of the links between cannabinoids and PPARs, it is fair to conclude that activation of PPARs may contribute (along with CB_1/CB_2 receptor activation) to the numerous anti-inflammatory effects seen with cannabinoid ligands, and may suggest that anti-inflammatory therapies that target both systems may be more beneficial than those therapies that target only CB_1/CB_2. Additional information on cannabinoid interactions with PPARs can be found in the review by O'Sullivan and Kendall (2010).

10.6 Concluding Remarks

The evidence presented in this chapter makes it clear that the PPAR signaling system clearly has a modulating effect on immune cell function and inflammation and is most likely involved in the consequent physiological processes of tissue repair and apoptosis. Despite the vast amount of data available on PPAR ligands and inflammation, there appear to be some knowledge gaps in this field. There is a general lack of information concerning PPAR expression on immune cells, although some information does exist on particular immune cell types (for example, PPARα protein expression on neutrophils). There is also a lack of validation in some published accounts in which authors have failed to pair specific agonists and antagonists making interpretations relating to the involvement of PPAR subtypes difficult.

Nevertheless, the majority of the published data on PPARs and inflammation/immune modulation are remarkably consistent and, in our opinion, suggest that further work on the PPAR system is likely to produce clinical leads for therapeutic interventions. Ongoing work in this field, such as investigations of PPAR modulation via sumoylation (Ohshima et al. 2004) as well as current work using specific agonists and antagonists to study the effect of PPAR activation in animal models of osteoarthritis, will surely suggest new avenues in which targeting PPARs may offer new therapeutic leads (Costa et al. 2011). Despite the information gathered on PPARs and inflammation, there are few clinically approved PPAR ligands to treat pathological inflammation, or to modulate the immune response. Some possible reasons for this include safety issues (including cardiovascular risk, edema, weight gain, carcinogenesis, and myopathy) and have been discussed in prior publications (LoVerme et al. 2005; Shearer and Billin 2007).

However, one note of caution should be raised in regard to PPAR modulation of immune function and inflammation. Given that (as already discussed) PPARs have a number of mechanisms through which modulation of inflammation is possible, drugs that target PPARs could also have detrimental effects on the resolution of inflammation and tissue healing. Thus drugs that directly target PPARs may enhance the time course of inflammation and as a result cause cell damage or incomplete resolution. This is an important consideration as "normal" physiological inflammation/immune cell infiltration is an important pathway to achieve pathogen removal and resolution of damaged tissue, and in acute inflammation is responsible for maintaining normal physiology. Therefore with this in mind, there is a need to be cautious when considering PPAR activation as a therapeutic avenue for the development of new anti-inflammatory agents.

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Chapter 11
Peroxisome Proliferator-Activated Nuclear Receptors and Drug Addiction

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11.1 Introduction

There is a growing body of evidence showing that the rewarding effects of abused drugs, which underlie their addictive potential, are modulated by the endocannabinoid system. Pharmacological blockade or genetic deletion of cannabinoid CB_1 receptors reduces or eliminates many abuse-related behavioral and neurochemical effects of nicotine, heroin, morphine, methamphetamine, delta-9-tetrahydrocannabinol (THC; the psychoactive ingredient in marijuana), and alcohol, and can modulate dependence development, withdrawal, and even relapse (see reviews by Maldonado et al. 2006; Solinas et al. 2008). For example, the selective cannabinoid CB_1-receptor inverse agonists/antagonists rimonabant (SR141716) and AM251 decrease self-administration of nicotine (Cohen et al. 2002, 2005a, b; Shoaib 2008), opioids (Navarro et al. 2001; De Vries et al. 2003; Solinas et al. 2003; Caillé et al.

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2007), cocaine (Xi et al. 2008), methamphetamine (Vinklerová et al. 2002), and alcohol (Freedland et al. 2001; Gessa et al. 2005; Economidou et al. 2006), and prevent relapse to drug-seeking behavior in abstinent rats. Also, cannabinoid CB_1-receptor inverse agonists/antagonists block conditioned place preference (CPP) induced by opioids (Chaperon et al. 1998; Singh et al. 2004) and nicotine (Le Foll and Goldberg 2004; Forget et al. 2006), and attenuate the reinstatement of extinguished nicotine place conditioning in rats (Cohen et al. 2005a, b; Budzyńska et al. 2009; Forget et al. 2009). Moreover, cannabinoid CB_1-receptor inverse agonists/antagonists have been shown to block dopamine elevations in the nucleus accumbens shell produced by nicotine (Cohen et al. 2002; Cheer et al. 2007) and THC (Tanda et al. 1997), but not the dopamine elevations produced by heroin (Tanda et al. 1997), morphine, or cocaine (Caillé and Parsons 2003, 2006), in the nucleus accumbens shell (but see Li et al. 2009).

Although initial clinical trials indicated that cannabinoid CB_1-receptor inverse agonists/antagonists might have significant efficacy as a treatment for tobacco addiction in cigarette smokers (Cahill and Ussher 2007; Rigotti et al. 2009), and preclinical studies indicated potential efficacy against addiction to other drugs, development of cannabinoid CB_1-receptor inverse agonists/antagonists for these indications essentially ceased in 2008 when rimonabant, which had been in clinical use for treatment of obesity in Europe, was removed from the market due to the risks involved with its use including increased incidence of depression, psychiatric side effects, nausea, and negative impacts if taken with other drugs (see review by Le Foll et al. 2009).

It would be of great interest to test newly developed "neutral" antagonists of CB_1 receptors (Bergman et al. 2008; Järbe et al. 2008, 2012; Sink et al. 2009, 2010) which might produce a blockade of abuse-related behavioral and neurochemical effects of abused drugs similar to that seen with CB_1-receptor inverse agonists/antagonists like rimonabant and AM251, but without the unwanted side effects of these compounds. Another approach to studying endocannabinoid system modulation of abuse-related behavioral and neurochemical effects of abused drugs, in particular nicotine, would be to study compounds that increase extracellular levels of endocannabinoids in the brain (Solinas et al. 2007; Scherma et al. 2008). Endocannabinoids are rapidly cleared from the extracellular space by specific enzymes involved in their degradation, and inhibitors of these enzymes are particularly useful tools for studying endocannabinoid system modulation of abuse-related behavioral and neurochemical effects of abused drugs.

Currently, there are two well-characterized endogenous ligands for cannabinoid receptors (Fig. 11.1), *N*-arachidonoylethanolamide (anandamide; Devane et al. 1992) and 2-arachidonoylglycerol (2-AG; Sugiura et al. 1995), but there are several other candidates that require better characterization, including virodhamine (Porter et al. 2002), noladin ether (Hanus et al. 2001), and *N*-arachidonoyldopamine (Bisogno et al. 2006). Anandamide has been the endocannabinoid most frequently studied. It is synthesized upon demand from N-arachidonoyl phosphatidylethanolamine in cell membranes in almost all cells and tissues of the body, including neurons (Di Marzo et al. 1994). The biological activity of anandamide is terminated

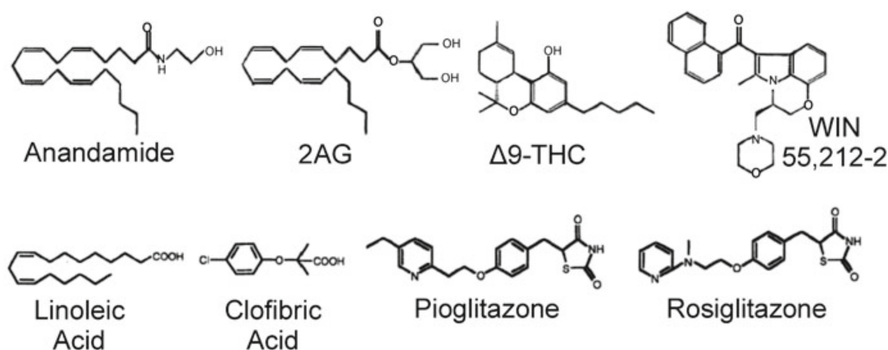

Fig. 11.1 Chemical structure of natural and synthetic cannabinoids and PPAR ligands

by two important processes: internalization of anandamide into cells, likely by a carrier-mediated uptake system (Beltramo et al. 1997; Piomelli et al. 1999), followed by intracellular degradation to ethanolamine and arachidonic acid (Cravatt and Lichtman 2002; Cravatt et al. 1996), which is primarily catalyzed by fatty acid amide hydrolase (FAAH), an enzyme that is distributed throughout the body, including the CNS (Thomas et al. 1997; Morozov et al. 2004).

During the last 10 years, researchers have focused on this two step process of anandamide degradation and have developed a number of compounds that inhibit anandamide uptake or degradation by FAAH in order to avoid the side effects resulting from the use of drugs that directly activate or block cannabinoid CB_1 receptors. For example, Scherma et al. (2012) recently reported that AM404, a compound thought to inhibit cell-membrane anandamide transport, prevents development of nicotine-induced CPP, impedes nicotine-induced reinstatement of extinguished CPP, and reverses nicotine-induced increases in extracellular dopamine levels in the nucleus accumbens shell in rats. Moreover, Gamaleddin et al. (2011) recently reported that another anandamide uptake inhibitor, VDM11, attenuates reinstatement of extinguished nicotine-seeking behavior induced either by nicotine-associated cues or by a priming dose of nicotine in rats.

Researchers have also developed highly selective inhibitors of intracellular FAAH activity during the last 10 years (Tarzia et al. 2003; Mor et al. 2004) and newer FAAH inhibitors continue to be developed by many pharmaceutical companies and research groups (e.g., Clapper et al. 2009; Godlewski et al. 2010). Selective inhibition of FAAH leads to accumulation of anandamide, and in turn to local elevated anandamide levels, and based on physiological actions of endocannabinoids, these drugs are currently being pursued for the treatment of pain, anxiety, and depression. FAAH inhibitors have also been suggested as medications for the treatment of nicotine (Scherma et al. 2008; Mascia et al. 2011) and cannabis (Schlosburg et al. 2009) dependence, based on findings in rats that FAAH inhibition or genetic deletion of FAAH reduces the intensity of withdrawal signs precipitated by the CB_1 receptor antagonist/inverse agonist rimonabant in THC-dependent mice (Schlosburg et al. 2009). FAAH inhibition also prevents the acquisition of nicotine self-administration

behavior and the development of nicotine-induced CPP, reduces nicotine-induced dopamine elevations in the nucleus accumbens shell, prevents the reinstatement of nicotine-seeking behavior in both CPP and self-administration models of relapse (Scherma et al. 2008; Forget et al. 2009), and suppresses nicotine-induced activation of dopamine neurons in the ventral tegmental area (VTA) (Melis et al. 2008).

11.2 FAAH Inhibition and PPAR Activation

In addition to hydrolyzing the endocannabinoid anandamide, FAAH is also responsible for the hydrolysis of structurally related, non-cannabinoid acylethanolamides, OEA and PEA, which are endogenous ligands for alpha-type peroxisome proliferator-activated nuclear receptors (PPARα) (Fu et al. 2003; Lo Verme et al. 2005). PPARα are nuclear receptors that are ubiquitously expressed in many tissues including the brain (Moreno et al. 2004) have anti-inflammatory and neuroprotective effects (Pistis and Melis 2010), and regulate lipid metabolism (O'Sullivan 2007). Thus, FAAH inhibition increases OEA and PEA levels in the brains of experimental animals leading to activation of PPARα (Kathuria et al. 2003; Fegley et al. 2005; Ahn et al. 2008; Justinova et al. 2008). It has been known for over 10 years that many physiological responses to cannabinoid-like compounds in the CNS and periphery were not mediated by cannabinoid receptors (Howlett et al. 2002) and that PPARα receptors were involved in a significant portion of these non-cannabinoid effects. In 2005, for example, it was shown that PEA has anti-inflammatory and analgesic effects, similar to cannabinoid receptor agonists, which are mediated through activation of PPARα (Lo Verme et al. 2005). Subsequently, researchers have investigated beneficial effects of cannabinoid actions at PPARs in many diseases such as diabetes, cancer, hyperlipidemia, atherosclerosis, metabolic syndrome, and neurodegenerative disorders (for reviews, see Francis et al. 2003; Fenner and Elstner 2005; Barish et al. 2006; Glass 2006; Lleo et al. 2007; Stienstra et al. 2007) and recently have explored the role of endocannabinoids acting as PPAR ligands in drug addiction (Melis et al. 2008; Luchicchi et al. 2010; Mascia et al. 2011; Stopponi et al. 2011; Scherma et al. 2012).

11.3 PPAR Subtypes

PPARs are a family of nuclear receptors, which consists of three isoforms: α, δ, and γ. Upon activation by endogenous or exogenous ligands, PPARs heterodimerize with the retinoid X receptor, and bind to DNA sequences called PPAR response elements, which leads to the transcription of target genes (Moraes et al. 2006).

The α subtype of PPAR, which is activated by the endogenous FAAH substrates OEA and PEA, is expressed by metabolically active tissues such as liver, heart, muscle, and brain, and is involved in the regulation of fatty acid catabolism and inflammatory processes (Stienstra et al. 2007). A wide variety of natural and synthetic

compounds (Fig. 11.1) activate PPARα in addition to OEA and PEA (Göttlicher et al. 1992; Forman et al. 1996), including natural fatty acids (e.g., linoleic acid and arachidonic acid), and a diverse group of synthetic PPARα agonists, including WY14643 and GW9578. These ligands (such as clofibrate, fenofibrate, and gemfibrozil) are clinically used to treat dyslipidemia, as they restore lipid balance in lipid metabolism disorders (see reviews by Berger and Moller 2002; Filippatos and Elisaf 2011; Keating 2011).

The γ subtype of PPAR is expressed predominantly in adipose tissue and has direct actions on adipose cells with secondary actions on insulin-responsive tissues such as liver and skeletal muscle (Fiévet et al. 2006; Stienstra et al. 2007). PPARγ agonists, such as the thiazolidinediones (TZDs) (e.g., pioglitazone and rosiglitazone, see Fig. 11.1), are primarily used clinically in the treatment of type 2 diabetes to improve insulin sensitivity, but also have anti-inflammatory, anti-cancer, and anti-hypertensive effects (see reviews by Breidert et al. 2002; Chang et al. 2007; Kapadia et al. 2008; Tontonoz 2008).

The third subtype of PPAR, PPARδ (also known as PPARβ), is also ubiquitously expressed. The exact functions of PPARβ/δ are not yet established, but it has been shown to be involved in keratinocyte, adipocyte, and oligodendrocyte differentiation (Matsuura et al. 1999; Jehl-Pietri et al. 2000; Saluja et al. 2001). Recent evidence suggests that PPARβ/δ is a powerful metabolic regulator (Barish et al. 2006).

These three PPAR isoforms are expressed in the brain and peripheral nervous system in rats (Moreno et al. 2004; Cimini et al. 2005). However, a direct role for central PPARs in brain function has only recently been identified.

11.4 Localization of PPARs in the Rat Brain

In situ hybridization and immunohistochemistry studies have determined the distribution of PPARs in the adult rat CNS. With respect to cell type, PPARs are expressed by neurons (Braissant et al. 1996; Krémarik-Bouillaud et al. 2000; Woods et al. 2003; Moreno et al. 2004; Cimini et al. 2005), and glial cells, including oligodendrocytes (Kainu et al. 1994; Granneman et al. 1998; Woods et al. 2003) and astrocytes (Cullingford et al. 1998; Granneman et al. 1998).

The PPAR subtype α is expressed in layer V of the cerebral cortex (Moreno et al. 2004), in granular cells, in the molecular layer, and in the olfactory tubercle (Kainu et al. 1994). In the hippocampus, PPARα is prevalent in the dentate gyrus and in CA1 pyramidal cells (Kainu et al. 1994; Braissant et al. 1996; Moreno et al. 2004). Furthermore, PPARα is abundant in the oculomotor nucleus of the mesencephalon, in the facial nucleus of the rhombencephalon, in the deep cerebellar nuclei, and in the neurons of laminae VII–IX in the spinal cord (Moreno et al. 2004). Braissant et al. (1996) have shown that PPARα is also expressed in the retina and in the cells of the choroid plexus.

The PPAR subtype γ is mainly expressed in microglia (Bernardo et al. 2003) and astrocytes (Cristiano et al. 2001; Cullingford et al. 1998), and its localization in the

brain has been related to inflammation and neurodegeneration (Heneka et al. 2000; Kitamura et al. 1999). PPARγ is also expressed in key neuronal subsets regulating glucose metabolism and energy homeostasis (Sarruf et al. 2009). Interestingly, in the VTA, PPARγ receptors colocalize with tyrosine hydroxylase, suggesting the expression of this receptor in dopaminergic cells (Sarruf et al. 2009; Stopponi et al. 2011). According to Braissant et al. (1996), PPARγ is present in the retina (ganglion cells) but is barely detectable in the hippocampus. They also reported that PPARγ was expressed in the cerebellum, but only at a low level in the granular layer. The piriform cortex and olfactory tubercle, as well as the medial thalamic subdivision, are also rich in the γ isotype (Moreno et al. 2004). Finally, PPARγ has been detected in many regions of the rhombencephalon and in layers II and IX of spinal cord (Moreno et al. 2004).

The PPAR subtype β/δ (PPARβ/δ) is abundantly expressed in the whole brain. PPARβ/δ is strongly expressed in immature oligodendrocytes (OL) where it is involved in cellular differentiation (Granneman et al. 1998; Saluja et al. 2001) and in myelin maturation and turnover (Cimini et al. 2003). PPARβ/δ was found to be prevalent in the mesencephalic division, hippocampus (highly expressed in the dentate gyrus, CA1 to CA3 pyramids, and the hilus), and in the retina and the spinal cord (Braissant et al. 1996; Woods et al. 2003; Moreno et al. 2004). In the cerebellum, PPARβ/δ was detected in the three layers (Purkinje, granular, and molecular cells) of the cortex (Braissant et al. 1996; Krémarik-Bouillaud et al. 2000), where it could be involved in the pathophysiology of the degeneration of Purkinje cells (Krémarik-Bouillaud et al. 2000).

11.5 Actions of Cannabinoids at PPARs

After the discovery of cannabinoid CB_1 and CB_2 receptors in the 1990s and the development of genetically modified mice with deletions of one or both of the receptors, it became increasingly clear that cannabinoid drugs exert some activities that were not related to activation of CB_1 and/or CB_2 receptors (Howlett et al. 2002). Among the most likely candidate receptors for mediating these non-cannabinoid actions of cannabinoid drugs were the transient receptor potential vanilloid (TRPV1) ion channel, the G-protein-coupled receptor GPR55, and PPARs.

It is interesting to note that, independently from their source (endogenous, plant-derived, or synthetic), most cannabinoid drugs bind and activate PPARγ and/or PPARα. For example, anandamide, noladin ether, virodhamine, and WIN 55,212-2 show affinities for PPARα of the same order of magnitude shown by fenofibrate (Sun et al. 2006). THC, instead, shows no affinity for PPARα, but a substantial affinity for PPARγ, which is also a binding target for anandamide, 2-AG, cannabidiol, and *N*-arachidonoyl-dopamine (NADA) (O'Sullivan and Kendall 2010). Thus, cannabinoids may bind to PPARs and produce PPAR-related activation and functions that are not under the control of cannabinoid receptors (i.e., cannot be blocked by CB_1 antagonists). Transcriptional activation of PPARα by cannabinoids may result in different actions, for example anti-inflammatory responses, lipolysis,

anorexia, and analgesia (O'Sullivan and Kendall 2010), while the transcriptional activation of PPARγ by cannabinoids has been related, for example, to stimulation of adipogenesis, vasorelaxation, or anti-inflammatory effects (O'Sullivan and Kendall 2010).

11.6 PPAR in Learning and Memory Processes

Previous studies have shown the potential neuroprotective role of the nuclear receptor subtype α against oxidative damage which causes neurodegeneration (Maccioni et al. 2001; Gilgun-Sherki et al. 2001), suggesting that PPARα may play an important role in neurotransmission by regulating H_2O_2 production (Chen et al. 2001; Avshalumov and Rice 2002). Moreover, as has been already considered by Moreno et al. (2004), there is evidence that PPARα and acetylcholine closely interact. For example, localization of PPARα and acyl-CoA oxidase in cholinergic neurons often overlaps, suggesting an involvement of this nuclear receptor in acetylcholine biosynthesis (Farioli-Vecchioli et al. 2001). Apo-E-deficient mice exhibit reduced levels of both PPARα mRNA and acetylcholine (Hung et al. 2001). Finally, the PPARα activator dehydroepiandrosterone enhances cognitive impairing effects related to decreases in acetylcholine levels (Racchi et al. 2001a, b; Zambrzycka et al. 2002).

In 2009, Mazzola et al. demonstrated a potential role for PPARα in the learning and memory processes that contribute to the development of drug addiction (Mazzola et al. 2009). They tested both the selective FAAH inhibitor URB597 and the selective synthetic PPARα agonist WY14643 using a passive avoidance procedure in rats and found that both compounds enhanced memory acquisition and retention in contrast to THC and the cholinergic antagonist scopolamine which impaired memory acquisition and retention. The effects of URB597 and WY14643 were reversed by both the cannabinoid CB_1 inverse agonist/antagonist rimonabant and the PPARα antagonist MK886, indicating that both CB_1 receptors and PPARα were involved in the effects. PPARα, like cannabinoid CB_1 receptors, are abundantly expressed in the hippocampus and amygdala (Moreno et al. 2004), key brain areas for learning and memory processes. Notably, the behavioral effects produced by FAAH inhibition in these experiments on learning and memory in rats were similar to the effects produced by PPARα activation and, more importantly, the effects of FAAH inhibition were reversed by a PPARα antagonist. These findings suggest that the effects of URB597 on learning and memory in the study by Mazzola et al. (2009) might be mediated primarily by enhanced levels of OEA and PEA acting at PPARα, rather than by enhanced levels of the endocannabinoid anandamide acting at cannabinoid CB_1 receptors.

Endogenous ligands of PPARα in the brain may also represent new targets for the treatment of cognitive deficits induced by MDMA (3,4-methylenedioxymethamphetamine, Ecstasy) abuse. MDMA is an amphetamine derivative that is widely abused. Plaza-Zabala et al. (2010) recently demonstrated that repeated treatment with high doses of MDMA for four consecutive days impaired subsequent acquisition and recall of an active avoidance task in mice, and that dopamine transporter

(DAT)-binding sites significantly decreased 4 days after the last MDMA administration. Pretreatment with OEA at 5 mg/kg ameliorated and at 25 mg/kg worsened these cognitive deficits. However, pretreatment with both doses of OEA prevented the decreases in DAT-binding sites. These results suggest that OEA administration can modulate the cognitive deficits induced by MDMA in a DAT-independent manner.

11.7 Dopamine Neuron Signaling and Drug Addiction

The dopaminergic system, especially the mesolimbic dopaminergic system, plays a fundamental role in different aspects of drug abuse and addiction (Wise 1998; Di Chiara et al. 1993; Koob and Volkow 2010), including in abuse and addiction related to nicotine (Dani and De Biasi 2001; De Biasi and Dani 2011). The mesolimbic dopamine system includes the VTA, where dopamine cell bodies are located, and from which dopamine axon projections reach limbic dopaminergic terminal brain regions, like the nucleus accumbens shell and core, the tuberculum olfactorium, and the medial prefrontal cortex. This dopaminergic system is involved in the neurobiology underlying many aspects of drug abuse. Indeed, virtually all drugs abused by humans increase dopamine neurotransmission in the nucleus accumbens shell in rodent in vivo microdialysis experiments (Pontieri et al. 1995, 1996; Tanda et al. 1997). Moreover, it has been shown in humans that dopamine neurotransmission in the ventral striatum (which corresponds to the nucleus accumbens in animals) is activated after administration of abused psychostimulants. The exact function of dopamine transmission in drug abuse and addiction has not been completely elucidated, and there are many different theories on how different important dopaminergic functions are involved. For example learning of the incentive properties of drugs or drug-associated stimuli, memory, emotional or motivational effects, all mediate the rewarding effects of both natural and drug reinforcers (Wise and Bozarth 1987; Di Chiara et al. 1993, 1998; Tanda and Di Chiara 1998; Koob and Volkow 2010). Consequently, the VTA and nucleus accumbens shell are critical brain areas for the rewarding/reinforcing effects of nicotine and other abused drugs (Di Chiara and Imperato 1988; Pontieri et al. 1996). Surprisingly, recent studies demonstrate that PPARs appear to modulate dopamine signaling in the mesolimbic dopamine system, suggesting that the reinforcing effects of abused drugs could be modulated by PPAR ligands.

11.8 PPARα and Nicotine Abuse/Addiction

Nicotine is the primary drug in tobacco products that causes addiction. Addiction is characterized by: repeated use of a drug despite known harmful effects, difficulty in quitting the use of the drug, and often developing physical dependence on the drug and showing withdrawal signs when drug use ceases abruptly. Numerous studies

have documented that nicotine has reinforcing effects in animals and humans (Corrigall 1999; Harvey et al. 2004). A related concept to reinforcement is reward, which is defined as the subjective, hedonic value given to a drug (Everitt and Robbins 2005). Nicotine reliably increases subjective ratings of drug liking, good effects, and feeling relaxed (Garrett and Griffiths 2001; Kalman and Smith 2005; Perkins et al. 2003).

The rewarding/reinforcing effects elicited by nicotine are centrally mediated by its pharmacological activity as an agonist for nicotinic-acetylcholine receptors (nAChR) (Dani 2001; Picciotto et al. 1998). Nicotinic receptors are composed of different transmembrane subunits which are capable of organizing into pentamers, heteromers, or homomers and forming ligand-gated ion channels (Millar 2003). Among the possible subunit combinations, α4-β2-nicotinic receptors have been shown to play a major role in mediating nicotine's rewarding/reinforcing effects (Picciotto et al. 1998; Maskos et al. 2005; Tapper et al. 2004).

Several brain transmitter systems might be involved in mediating the rewarding/reinforcing effects of nicotine and its addictive effects. Among them, the dopaminergic system, especially the mesolimbic dopamine system, has been shown in preclinical studies to react to nicotine administration in a similar manner to other drugs abused by humans (Pontieri et al. 1995, 1996; Tanda et al. 1997; Pich et al. 1997). Specifically, nicotine facilitates dopaminergic neurotransmission and dopamine release in the nucleus accumbens shell by directly stimulating nAChR located on cell bodies of VTA dopaminergic neurons. Nicotine also indirectly stimulates glutamate release (Yin and French 2000), which in turn stimulates VTA dopaminergic neuron firing and dopamine release from their terminals located in the nucleus accumbens shell (Mereu et al. 1987; Pidoplichko et al. 1997; Di Chiara and Imperato 1988; Pontieri et al. 1996), thus increasing dopamine neurotransmission.

Also, clinical studies showed that smoking cigarettes containing nicotine stimulates the release of dopamine in the ventral striatum to a greater extent than smoking denicotinized cigarettes (Brody et al. 2009). Further, stimulation of dopamine significantly correlates with verbal reports of feelings of "high" in human smokers (Barrett et al. 2004). Thus, it appears that the mesolimbic dopamine system plays a pivotal role in nicotine dependence.

11.9 Electrophysiological Studies with Nicotine and PPARα Agonists

As noted above, dopaminergic neurotransmission, especially in terminal areas of the mesolimbic dopaminergic system, plays fundamental roles in different aspects of drug abuse and addiction. The main components of the mesolimbic system are the dopamine neurons located in the VTA and their projections to terminal areas, like the nucleus accumbens shell. The activities of dopaminergic neurons have been the subject of many different scientific studies using both in vitro and in vivo electrophysiology techniques to explore distinct dopaminergic functions related to drug

abuse and addiction. These include basal and stimulated dopaminergic firing, different firing modalities associated with different neuronal functions and modulation of neuronal activities by drugs and natural reinforcers (Mereu et al. 1987) including nicotine (Pidoplichko et al. 1997). Furthermore, Melis et al. (2008) studied the electrophysiological responses to nicotine administration of isolated dopamine neurons in the VTA of anesthetized rats, following either blockade of CB_1 receptors with rimonabant or enhancement of brain endocannabinoid levels by inhibition of FAAH. They obtained four surprising findings. One, URB597, but not rimonabant, prevented the nicotine-induced stimulation of both firing rate and burst firing of VTA dopamine neurons. Two, methanandamide, the metabolically stable analog of anandamide, did not alter the response of VTA dopamine neurons to nicotine, suggesting that CB_1 receptor activation was not involved in these actions of URB597. Three, when animals were pretreated with the CB_1 inverse agonists/antagonists rimonabant or AM251 to eliminate the contribution of CB_1 receptor activation by elevated anandamide levels, URB597 blocked only the nicotine-induced increases in dopamine neuron firing rate but not increases in burst firing. Four, when animals were treated with the PPARα antagonist MK886 to test for the involvement of PPARα in nicotine-induced increases in dopamine neuron firing rate and burst firing, MK886 pretreatment reversed the blockade by URB597 of nicotine-induced increases in dopamine neuron burst firing but not the increases in firing rate. These unexpected results suggested that anandamide was not responsible for the anti-nicotine effects of URB597 in rodent models of drug addiction (Scherma et al. 2008; Forget et al. 2009). URB597 inhibits FAAH, and this inhibition, in turn, increases levels of other endogenous lipids metabolized by FAAH. Melis et al. (2008) tested two such acylethanolamides, OEA and PEA, which are FAAH substrates and PPARα ligands with no actions at cannabinoid receptors. Results from in vivo and in vitro experiments converged; OEA and PEA prevented nicotine-induced excitation of mesolimbic dopamine neurons and MK886 antagonized the OEA- and PEA-induced blockade of nicotine's response. Moreover, the OEA and PEA responses were mimicked in vitro by the synthetic PPARα agonist WY14643, and effects of WY14643 were also reversed by MK886 indicating the involvement of PPARα in actions of nicotine relevant for nicotine abuse and addiction.

11.10 In Vivo Microdialysis Studies with Nicotine and PPARα Agonists

Mascia et al. (2011) conducted in vivo microdialysis studies showing that nicotine-induced dopamine elevations in the nucleus accumbens shell in unanesthetized rats were blocked by methOEA (a metabolically stable analog of OEA). Furthermore, the action of methOEA was mimicked by WY14643, and the effects of WY14643 and methOEA were reversed by MK886. In the same study, additional in vivo electrophysiological experiments were conducted to verify that the PPARα agonists,

methOEA and WY14643, which blocked nicotine-stimulated accumbal dopamine release in the microdialysis experiments, blocked nicotine-induced excitation of VTA dopaminergic neurons in electrophysiology experiments. In line with the microdialysis results, the PPARα agonists methOEA and WY14643 blocked nicotine-induced increases in firing rate and burst firing in VTA dopamine neurons, and these responses were reversed by MK886 suggesting that the effect of the PPARα agonists was taking place mainly in the VTA.

11.11 Behavioral Studies with Nicotine and PPARα Agonists

Nicotine plays a major role in tobacco dependence by acting directly as a reinforcer of drug-seeking and drug-taking behavior. In rats, nicotine reinforces drug self-administration behavior (Corrigall and Coen 1989; Shoaib et al. 1997; Le Foll and Goldberg 2006), induces CPP (Le Foll and Goldberg 2005), and triggers relapse to previously acquired drug-seeking behavior (Shaham et al. 1997). As mentioned above, the FAAH inhibitor URB597 blocks many of those effects caused by nicotine (see Scherma et al. 2008; Forget et al. 2009) and these rewarding/reinforcing and relapse-inducing effects of nicotine may be modulated by PPARα. In a series of recent experiments in rats and squirrel monkeys, Mascia et al. (2011) found that the PPARα agonists WY14643 and methOEA significantly decreased ongoing nicotine self-administration in both rats and monkeys (Fig. 11.2). Moreover, PPAR-α activation with WY14643 suppressed reinstatement of extinguished drug-seeking behavior when abstinent rats and monkeys were reexposed to nicotine. Pretreatment of monkeys with the PPAR-α antagonist MK886 prevented the effects of WY14643 in this model of relapse, demonstrating the receptor specificity of these effects. However, when WY14643 was tested in a nicotine-discrimination procedure, it failed to alter the percentages of responses on the nicotine-appropriate lever or the rate of lever-press responding for food (Mascia et al. 2011). The fact that the PPAR-α agonist WY14643 did not alter the interoceptive effects of nicotine in the drug-discrimination procedure is consistent with previous findings showing that nicotine's reward-related dopaminergic effects are not well captured by this procedure (Corrigall and Coen 1994). For example, even though the cannabinoid CB_1-receptor inverse agonist/antagonist rimonabant blocks both nicotine reward (self-administration and CPP behavioral assays) and nicotine-induced increases in extracellular dopamine levels in the nucleus accumbens shell, rimonabant does not alter nicotine discrimination (Le Foll and Goldberg 2004; Cohen et al. 2002). Similarly, antagonism of the dopamine D3 receptor blocks nicotine-induced CPP but does not alter nicotine discrimination (Le Foll et al. 2005). The finding that WY14643 blocked nicotine's effects on dopamine levels in the nucleus accumbens shell but did not alter its discriminative effects is consistent with previous data suggesting that neurobiological substrates between reward-related and interoceptive effects of nicotine do not entirely overlap (Smith and Stolerman 2009).

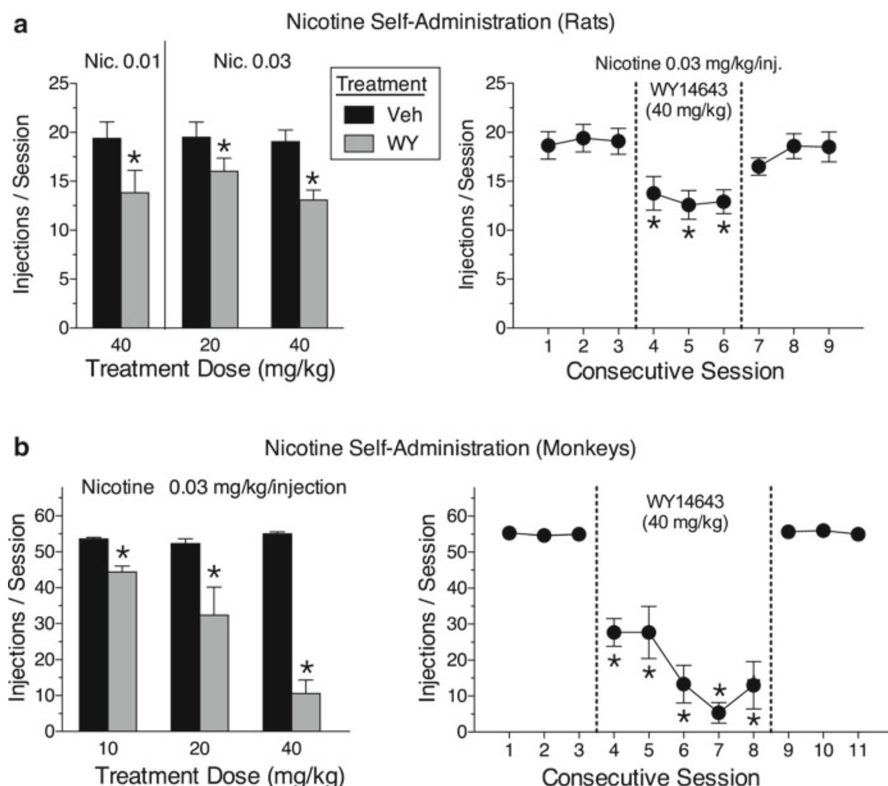

Fig. 11.2 The PPAR-alpha agonist WY14643 (20 and 40 mg/kg) reduced nicotine self-administration in (**a**) rats and (**b**) squirrel monkeys. WY14643 was given intraperitoneally 20 min before the session for three (rats) or five (monkeys) consecutive sessions. During the sessions, rats and monkeys intravenously self-administered nicotine (0.01 or 0.03 mg/kg/injection) under a five-response (rats) or ten-response (monkeys) fixed-ratio schedule. (**a**) Average rate of injection in rats over three test sessions compared with average of three sessions of vehicle treatment, *left panel*, and rates of nicotine self-administration during individual sessions under baseline conditions (sessions 1–3) and after treatment with 40 mg/kg WY14643 (sessions 4–6), *right panel*. (**b**) Average rate of injection in monkeys over five test sessions, compared with average of five sessions of vehicle treatment, *left panel*, and rates of nicotine self-administration during individual sessions under baseline conditions (sessions 1–3) and after treatment with 40 mg/kg WY14643 (sessions 4–8), right panel. Data are presented as group means plus or minus SEM. *Significant difference from vehicle treatment (modified from Mascia et al. 2011)

Because of the promising effects of PPARα agonists WY14643 and methOEA on abuse-related neurochemical and behavioral effects of nicotine, we recently conducted studies with the clinically approved PPARα agonist clofibrate, a first generation PPARα agonist, to determine whether fibrates also inhibit abuse-related behavioral and neurochemical effects of nicotine in rats and squirrel monkeys (Panlilio et al., unpublished observations). Clofibrate prevented the acquisition of nicotine-taking behavior in naive rats, substantially decreased nicotine self-administration

in experienced rats and monkeys, and counteracted the relapse-inducing effects of nicotine reexposure after a period of abstinence. Clofibrate blocked nicotine's effects on neuronal firing in the VTA and on dopamine release in the nucleus accumbens shell. These effects of clofibrate were reversed by MK886 indicating that its actions were mediated by PPARα.

11.12 Mechanisms for PPARα Modulation of Abuse-Related Effects of Nicotine

Activation of PPARα promotes transcriptional regulation of genes encoding proteins (Berger and Moller 2002). However, it is unlikely that the PPARα ligands studied in animal drug addiction models produced their effects by triggering a genomic mechanism, since the onset of their effects was very rapid. Previous studies suggested that PPARα ligands rapidly increase tyrosine kinase activity through a nongenomic mechanism (Rokos and Ledwith 1997; Mounho and Thrall 1999; Lennon et al. 2002; Teruel et al. 2003; Gardner et al. 2005). Melis et al. (2010) conducted a number of experiments to elucidate the mechanisms by which PPARα ligands suppress nicotine-induced activation of VTA dopamine neurons. They tested the general tyrosine kinase inhibitor genistein in vitro. Interestingly, genistein prevented the OEA-induced inhibition of nicotine-induced excitation of VTA dopamine neurons, supporting the hypothesis that direct activation of PPARα by agonists, or indirect activation by elevated levels of OEA and PEA resulting from FAAH inhibition, increases tyrosine kinase activities, which then phosphorylate nAChR and reduces their affinity for nicotine as well as promotes their internalization (Melis et al. 2008).

In a subsequent study, Melis et al. (2010) combined electrophysiological and behavioral experiments with the aim of understanding how PPARα modulate dopamine cell activity by interfering with intracellular events that regulate the functioning of nAChR. They found that MK886 enhanced the spontaneous activity of dopamine neurons. By testing different antagonists of α4-containing or α7-containing nAChR, and testing MK886 in β2-nAChR knockout mice, they found that the responses of dopamine neurons to MK886 were mediated by postsynaptic α4β2-nAChR. This finding was supported by behavioral experiments which showed that the synthetic PPARα agonist WY14643 reversed the nicotine-induced increases in locomotor activity in mice (Melis et al. 2010). Since the β2-containing nAChR is the key receptor through which nicotine increases locomotion, these results confirm the involvement of β2 subunits in PPARα effects. Finally, they discovered that synthetic and endogenous PPARα ligands negatively modulate dopamine neural activity by increasing hydrogen peroxide production, which in turn activates tyrosine kinase. This agrees with both the hypothesis made in 2008 by Melis et al. and the involvement of tyrosine kinases in the regulations of nAChR activity (Charpantier et al. 2005). Taken together, these findings suggest a mechanism by which PPARα may modulate the reward-related dopaminergic effects of nicotine underlying nicotine reward and nicotine-induced relapse to drug-seeking behavior.

11.13 PPAR and Other Abused Drugs

11.13.1 Electrophysiological Studies with PPARα Agonists and Other Abused Drugs

The effects of FAAH inhibition and PPARα activation on nicotine-induced increases in VTA dopamine neuron firing appear to be selective. Luchicchi et al. (2010) found that URB597 did not reverse the decreases in firing rate and burst firing caused by cocaine in in vitro experiments using single-unit electrophysiological recordings of VTA dopamine neurons. Results were consistent with microdialysis data obtained by Mascia et al. (2011) showing that WY14643 did not prevent cocaine-induced dopamine elevations in the shell of the nucleus accumbens. Furthermore, Luchicchi et al. (2010) found that URB597 did not affect morphine-induced increases in both firing rate and burst firing of VTA dopamine neurons, indicating again that FAAH inhibition selectively blocks nicotine-induced excitation of VTA dopamine neurons.

Luchicchi et al. (2010) also compared the effects of URB597 on nicotine-, cocaine- and morphine-induced depression of medial spiny neurons in the shell of the nucleus accumbens in in vitro experiments using single-unit electrophysiological recordings. They found that URB597 blocked the effects of both nicotine and cocaine on medial spiny neurons in the accumbens shell. URB597's blockade of nicotine's depressant effect on medial spiny neurons was reversed by both rimonabant and MK886, indicating the involvement of membrane CB_1-receptors and nuclear PPARα. However, MK886, but not rimonabant, reversed URB597's blockade of cocaine-induced depression of medial spiny neurons in the accumbens shell, indicating that PPARα ligands modulate the neuronal response to cocaine in the shell of the nucleus accumbens.

11.14 PPAR and Behavioral Sensitization to Morphine, Cocaine, and Methamphetamine

Behavioral sensitization associated with development of synaptic plasticity (or neuronal adaptations) in the brain mesocorticolimbic system is thought to contribute to the transition from simple drug use to compulsive drug abuse and addiction by altering brain systems that normally regulate the attribution of incentive salience to stimuli (for reviews, see Robinson and Berridge 1993, 2008; Nestler 2001; Kalivas and O'Brien 2008). PPARα activation reduces the production of pro-inflammatory factors in brain (Combs et al. 2001), which participate in sensitization to the effects of different classes of abused drugs, including psychostimulants (Zalcman et al. 1999; Nakajima et al. 2004; Maeda et al. 2007) and opioids (Narita et al. 2008). The reduction of proinflammatory factors seen after PPARα activation, and the participation of neuroinflammation processes in the sensitizing effects of drugs of abuse, led to an investigation of PPARα involvement in morphine-induced behavioral sensitization

(Fernandez-Espejo et al. 2009). In this study, conducted with PPARα$^{-/-}$ mice and their wild-type littermates, they found that lack of PPARα shifts the dose effect curve for morphine sensitization to the left compared to that in wild-type mice, suggesting that in wild-type mice PPARα would counteract the sensitizing effects of morphine. Indeed, behavioral sensitization to morphine is blocked by systemic administration of the PPARα agonist WY14643 in wild-type mice, an effect likely mediated by a decrease in inflammation-associated changes. However, in the same report, PPARα was not involved in behavioral sensitization to cocaine (Fernandez-Espejo et al. 2009). Moreover, they showed that chronic high-dose morphine treatment, but not chronic cocaine treatment, upregulated the expression of brain PPARα, suggesting that PPARα might play a homeostatic role opposing morphine-induced behavioral sensitization.

In a different series of experiments using a sensitization protocol with repeated administration of methamphetamine in mice, brain protein levels and activity of PPARγ were significantly increased both during methamphetamine administration and after withdrawal (Maeda et al. 2007). Also, when a PPARγ agonist, either pioglitazone or ciglitazone, was injected intracerebroventricularly (i.c.v.) once daily, it prevented the expression but not the development of behavioral sensitization elicited by methamphetamine. Furthermore, when mice were pretreated i.c.v. daily with the PPARγ antagonist GW9662, the expression of methamphetamine-induced behavioral sensitization was magnified, suggesting that methamphetamine sensitization is modulated by activating PPARγ receptors (Maeda et al. 2007). PPARγ expression in the brain is also altered during methamphetamine-induced dopaminergic neurotoxicity treatments (Tsuji et al. 2009). A reduction in PPARγ and dopamine transporter expression in the striatum after methamphetamine was dose-dependently attenuated by the nonsteroidal anti-inflammatory drug ibuprofen, which is also a PPARγ ligand, and by the intrinsic PPARγ ligand 15d-PG J2, but not by aspirin, which was used as an anti-inflammatory drug control. These results indicate that the protective effects of ibuprofen in animals treated with neurotoxic doses of methamphetamine are likely provided by its anti-inflammatory PPARγ agonistic effects.

11.15 Behavioral Studies with PPARγ Agonists and Alcohol

A recent study by Stopponi et al. (2011) investigated the role of PPARγ in counteracting the effect of alcohol abuse and the ability of alcohol to trigger relapse. Using genetically selected alcohol-preferring rats as subjects, they tested two clinically approved PPARγ agonists, pioglitazone and rosiglitazone, both of which belong to the class of TZDs and are used for the treatment of insulin resistance and type 2 diabetes (Chang et al. 2007; Tontonoz and Spiegelman 2008; Breidert et al. 2002; Berger and Moller 2002). They found that both drugs significantly reduced alcohol intake, although pioglitazone had a higher efficacy than rosiglitazone (see Fig. 11.3, upper panel). As noted by the authors, pioglitazone binds to PPARα, but rosiglitazone does not (Kapadia et al. 2008), suggesting that the more pronounced effects of pioglitazone were due to an involvement of PPARα. However, this hypothesis was

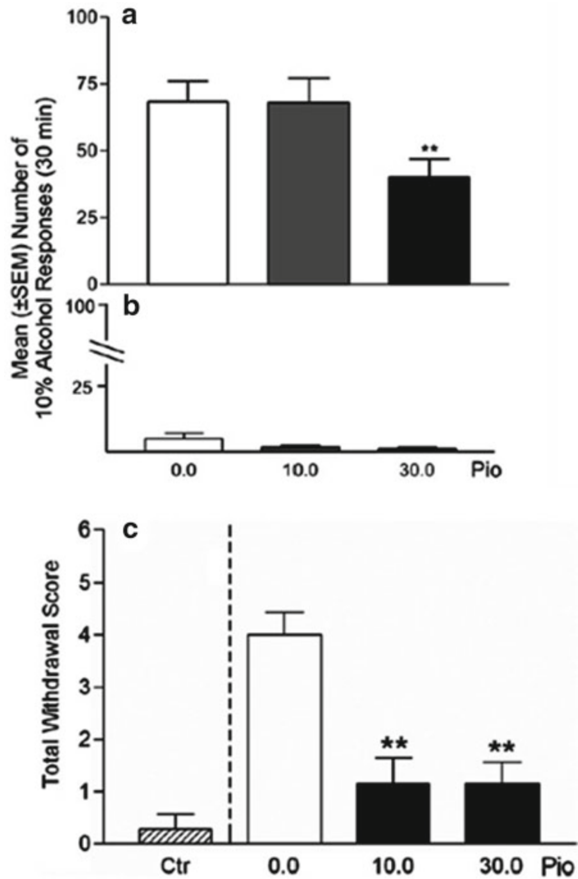

Fig. 11.3 Effect of pioglitazone (Pio; 0, 10, and 0.30 mg/kg) treatment on alcohol self-administration (**a**, **b**, *upper panels*) and *on the expression* of alcohol withdrawal (**c**, *lower panel*). Self-administration values represent the mean (SEM) number of responses at the (**a**) active or (**b**) inactive lever. Differences from vehicle-treated rats (controls), $^{**}p<0.01$. (**c**) Total withdrawal score (**c**) was obtained from each animal by cumulating the score of the three withdrawal signs: (1) presence of the ventromedial distal flexion response, (2) tail stiffness/rigidity, and (3) tremors. Total withdrawal score ranged between 0 and 6. Significant difference from vehicle (0.0), $^{**}p<0.01$ (modified from Stopponi et al. 2011)

not supported by the finding that the effects of both pioglitazone and rosiglitazone in reducing alcohol drinking were reversed by pretreatment with the selective PPARγ antagonist GW9662. Moreover, pioglitazone reduced the stress-induced reinstatement of alcohol seeking, had no effect against cue-induced relapse to drug-seeking behavior (Stopponi et al. 2011), and significantly reduced the intensity of alcohol withdrawal symptoms (see Fig. 11.3, lower panel). These findings suggest a new role of PPARs in the treatment of alcohol addiction and encourage further investigation of the relative importance of different PPAR subtypes in other forms of drug addiction.

11.16 Mechanisms for PPARγ Modulation of Alcohol Effects

Further studies need to be conducted to understand the mechanism through which PPARγ ligands counteract the rewarding effects of alcohol. PPARγ is expressed at high levels in the paraventricular nucleus of the hypothalamus, the lateral hypothalamus, the arcuate nucleus, the VTA, and by glial cells (Moreno et al. 2004).

In the lateral hypothalamus and the arcuate nucleus, PPARγ is expressed by cells producing α melanocyte-stimulating hormone (α-MSH), agouti-related protein (AgRP), and pro-opiomelanocortin. Stopponi et al. (2011) proposed a genomic mechanism whereby pioglitazone negatively modulates PPARγ localized in the paraventricular nucleus inducing a down regulation of the transcript for corticotrophin-releasing factor, which could explain the anti-relapse effects of pioglitazone in the presence of stress. PPARγ are expressed in VTA dopamine neurons (Sarruf et al. 2009), where they may regulate the neurotransmitter involved in alcohol consumption. However, this hypothesis is not supported by the finding that pioglitazone does not counteract cue-induced relapse (Stopponi et al. 2011), a process in which dopamine plays an important role. Regarding PPARγ expressed in glial cells, Stopponi et al. explained the effects of pioglitazone on alcohol addiction as due to TZDs blocking the production of some proinflammatory mediators, such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor TNF-α, and the increased expression of toll-like receptor 4, a sensor of endogenous damage signals (Dasu et al. 2009), after toxic insult. Moreover, the protective effects of pioglitazone on alcohol withdrawal and on stress-induced reinstatement (Stopponi et al. 2011) agree with a study reporting that lipopolysaccharide and cytokine treatments sensitized ethanol withdrawal-induced anxiety-like behavior (Breese et al. 2008).

11.17 Conclusions: Potential Therapeutic Actions of Clinically Used PPAR Agonists

PPARα and PPARγ agonists have become clinically relevant due to their important roles in lipid and glucose metabolism, inflammation, and neuroprotection. For example, the PPARγ agonist pioglitazone is prescribed for type 2 diabetes. Its ability to inhibit alcohol consumption in preclinical studies encourages starting clinical studies in alcohol abusers. The PPARα agonist fibrates are clinically used to reduce the risk of cardiovascular disease and other complications associated with lipid profile disorders (Bishop-Bailey 2000; Ferré 2004; Guerre-Millo et al. 2000; Jackevicius et al. 2008, 2001; Abourbih et al. 2009). Based on our preclinical findings with clofibrate (described above), we are currently starting clinical studies with the fibrate gemfibrozil in tobacco smokers on nicotine reinforcement (using a forced-choice procedure) and on reports of craving elicited by nicotine-associated cues in the laboratory. Fibrate medications could aid in the treatment of nicotine dependence, and might also reduce the risk of developing cardiovascular disease associated with tobacco smoking by virtue of their ability to normalize abnormal lipid profiles. Thus, PPAR agonists represent novel promising compounds for the treatment of nicotine and alcohol dependence, and future studies will determine whether they show promise for treatment of addiction to other abused drugs.

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Part V
Conclusions/Therapeutic Potential

Chapter 12
Conclusions: Therapeutic Potential of Novel Cannabinoid Receptors

Mary E. Abood, Roger G. Sorensen, and Nephi Stella

12.1 Introduction

For centuries the plants *Cannabis sativa* and *Cannabis indica* (commonly known as *marijuana*) have been used for recreational, religious, and medicinal purposes across diverse cultures. The first recorded attributes of *Cannabis* were on its potent therapeutic actions, which included analgesic, sedative, and anticonvulsant effects. In the 1930s *Cannabis* extracts were one of the most commonly prescribed medicines of the US pharmacopeia. Unfortunately, the early criminalization of the use of *Cannabis* resulted in a near 35-year stall in scientific research aimed at understanding and optimizing the therapeutic potential of its extracts. It was the isolation and chemical characterization of Δ^9-tetrahydrocannabinol (THC) in 1964 as the first bioactive ingredient produced by *Cannabis* that revived the scientific community's interest in further understanding and optimizing the unique therapeutic properties of phytocannabinoids (phyto-CB) (Mechoulam and Gaoni 1967).

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Based on the lipophilicity of THC, the scientific community initially hypothesized that this ingredient might mediate its biological effects by disrupting cell membrane fluidity, thereby categorizing phyto-CB_s as "partial anesthetics." However, the notion that THC induces biological effects by disrupting cell membrane fluidity was rapidly challenged, and ultimately invalidated, by two landmark studies. The first showed a stringent structure–activity relationship (SAR) exhibited by THC analogues when measuring adenylyl cyclase activity (Dill and Howlett 1988). The second used high-affinity, radioactively labeled, synthetic cannabinoid ligands (synth-CB) to demonstrate specific and reversible interactions of THC with a binding site expressed in brain (Devane et al. 1988). Both of these studies were rapidly validated by the molecular identification of the gene sequences encoding the cannabinoid CB_1 and CB_2 receptors (Matsuda et al. 1990; Munro et al. 1993). These two G protein-coupled receptors (GPCRs), together with the various enzymes controlling the production and inactivation of endocannabinoids (endo-CB) (Pertwee et al. 2010), form the basic framework of the endo-CB signaling system. Definitive pharmacological and genetic evidence has implicated this signaling system in the control of many pathophysiological processes throughout the central nervous system (CNS) and in the periphery, outlining a clear biological map that is guiding the development of drugs targeting the eCB signaling system for therapeutic benefit (Kreitzer and Stella 2009). Indeed, the most recent generation of selective drugs targeting the components that form the eCB signaling system, be the CB receptors or the enzymes controlling endo-CB levels, is showing very promising therapeutic efficacies when treating diseases as diverse as metabolic syndrome, chronic pain, chronic inflammation, neurological disease, and cancer.

Although the molecular and cellular details of how CB_1 and/or CB_2 receptors control various pathophysiological processes have been extensively studied, many basic questions still remain unanswered. There are at least four possible modes of action whereby cannabinoid-based compounds can act independently of CB_1 and CB_2 receptors to potentially regulate the pathophysiological processes. (1) Because of their hydrophobic properties, cannabinoids at relatively high concentrations may regulate cell function by changing cell membrane fluidity (Howlett and Mukhopadhyay 2000; Maingret et al. 2001; Oz 2006). (2) Cannabinoids may interact with proteins that do not directly transduce signals, but rather, control the signaling efficacy of other transmitters. Examples of this would include phyto-CBs directly inhibiting adenosine and dopamine transport, thereby boosting the local levels of these transmitters (Carrier et al. 2006; Price et al. 2007). (3) Cannabinoids might interact at a distinct binding site (or groove) present in CB_1/CB_2 receptors. Such a binding site might overlap with the binding site targeted by endo-CBs, possibly providing an allosteric modulation of CB_1/CB_2 receptor activity and biasing their signal transduction mechanism (Price et al. 2005; Pertwee et al. 2010; Atwood et al. 2012). Finally, (4) cannabinoids may produce their biological effects through other receptor targets, the non-CB_1/CB_2 receptors, some of which have been molecularly identified, while others remain orphans. The chapters in this book focused on this fourth possibility.

Considering the remarkable scientific advances made during the past decade towards the pharmacological and molecular identifications of additional, non-CB_1/CB_2 receptors activated by cannabinoids, our field of research has now the required tools to thoroughly test the biological function played by these new players in the endo-CB signaling system and in the specific pathophysiological processes it regulates. This will open alternative venues for the development of drugs that act through novel mechanisms of action. Concomitantly, the academic challenge raised by the elucidation of the molecular identities of non-CB_1/CB_2 receptors and the details of their coupling mechanisms are fuelling a very exciting period of research involving multiple disciplines and levels of investigation spanning from fundamental biology to *state-of-the-art* drug discovery and medical sciences. Below we propose conclusions on two aspects pertaining to non-CB_1/CB_2 receptors: their mechanisms of action and the opportunity for developing new therapies.

12.2 Non-CB_1/CB_2 Receptors: Mechanisms of Action

Compounds: Over 60 phyto-CB are produced by *Cannabis* (Dewey 1986), with some of these compounds behaving as agonists or antagonists with varying affinities at CB_1 and/or CB_2 receptors. Accumulating evidence shows that phyto-CB also interacts with non-CB_1/CB_2 receptors and, thus, our first challenge is to delineate the similarities and differences in the pharmacology and mechanism of action of cannabinoid compounds acting through CB_1/CB_2 receptors and non-CB_1/CB_2 receptors. Here medicinal chemistry, pharmacology, and molecular biology offer synergistic advantages to tackle this particular challenge. The medicinal chemistry and pharmacology of synth-CB are rich, consisting of numerous compounds that selectively target either CB_1 or CB_2 receptors with nanomolar (and sometimes subnanomolar) affinities. Many detail studies reporting the SAR of these compounds are available, providing a clear understanding of what chemical moieties are required for these high-affinity interactions (Pertwee et al. 2010). The chemical structures of phyto-CB, synth-CB, and endo-CB cover a broad chemical space, for they are composed of distinct (sometimes non-overlapping) chemical scaffolds, providing medicinal chemists with comprehensive building blocks and strategies to rationally develop and optimize the next generation of drugs that will exhibit reduced potency for CB_1/CB_2 receptors and enhanced potency for non-CB_1/CB_2 receptors. Here molecular biology provides the genetic and anatomical tools—for example, mice lines that lack a specific component of the endo-CB signaling system in only one cell type (Cravatt et al. 2004; Monory et al. 2007)—to thoroughly test the selectivity of any new drug developed to target non-CB_1/CB_2 receptors and probe its mechanism of action.

Ligand discovery: De-orphanization and identification of ligands for candidate CB receptors have recently been facilitated by high-throughput screening, both by pharmaceutical companies and academic laboratories. The National Institutes of

Health initiated a program in 2005 whereby investigators could submit assays for High-Throughput Screening (HTS) in the Molecular Libraries Screening Centers Network (MLSCN, http://mli.nih.gov/mli/).

One recent example of this HTS approach utilized β-arrestin reporter biosensors. β-Arrestins are intracellular proteins that bind and desensitize activated GPCRs and in the process form stable receptor/arrestin signaling complexes (Shenoy and Lefkowitz 2005; Gurevich and Gurevich 2006). β-Arrestin redistribution to the plasma membrane containing activated GPCRs represents one of the early intracellular events provoked by agonist binding, and consequently is less prone to a false positive or negative readout as compared to studying a GPCR downstream signaling event as a readout of receptor activation. β-Arrestin–green fluorescent protein chimeras make the process of screening many new drugs for GPCRs faster and, more importantly, powerful. The signal emitted by this assay is remarkably sensitive and reliable, and the recruitment of β-arrestin by GPCR activation occurs independently of downstream G protein-mediated signaling, providing an opportunity to identify a broader spectrum of drugs that interact with these targets (Barak et al. 1997; Marion et al. 2006; McGuinness et al. 2009). For example, GPR55 responsiveness to a representative panel of cannabinoid ligands and the GPR55 agonist, lysophosphatidylinositol (LPI), in the presence (and absence) of a β-arrestin2–green fluorescent protein (βarr2-GFP) biosensor was determined (Kapur et al. 2009). The evaluation (Heynen-Genel et al. 2010a) of 290,000 compounds through MLSCN using the β-arrestin recruitment assay from a GPR55-expressing cell line as the primary screen identified three new GPR55 agonist scaffolds exhibiting nanomolar (EC_{50}) potency: ML184 (CID2440433, 263 nM), ML185 (CID1374043, 658 nM), and ML186 (CID15945391, 305 nM). In addition to screening for agonism at GPR55, all compounds were also screened for both agonism and antagonism of GPR35, CB_1, and CB_2, and found to have $EC_{50s} > 32$ μM at these receptors. Further support that these compounds are GPR55 agonists was obtained from their activities in a secondary assay, activation of mitogen-activated protein kinase, ERK1/2 (Heynen-Genel et al. 2010a). The details of their interactions with GPR55 were further examined by using a recently derived molecular model of GPR55 (Kotsikorou et al. 2011). Interestingly, these three chemical scaffolds interact with GPR55 similar to LPI and quite different from the actions of cannabinoid ligands (Kotsikorou et al. 2011).

A new compound (GSK494581A, EC_{50} of 158 nM) exhibiting a similar chemical structure to ML184 was independently discovered in a high-throughput screen conducted by Glaxo Smith Kline (Brown et al. 2011). Specifically, this study evaluated 5,000 custom compounds for GPR55 activation using a yeast reporter system. In this yeast assay, receptor activation is coupled to cell growth via the pheromone-response pathway, and GPCR activation is determined by higher production of fluorescein from substrates added to the culture media. Screens were performed by incubating GPR55-expressing yeast cells with compounds and positive hits were assigned when the reporter measure surpassed an arbitrary threshold level of 25% relative to a standard agonist. Six putative GPR55 agonists were identified that conformed to a common chemotype with a benzoylpiperazine structure. To confirm their specificity for GPR55, compounds were incubated with yeast cells expressing

CB_1 receptors. Specifically, the reference CB_1 receptor agonist, HU210, activates CB_1 receptor-expressing cells, whereas the benzoylpiperazines had no significant agonist activity at CB_1 receptors.

GSK494581A and the other analogs were further confirmed as GPR55 agonists through a secondary assay, their ability to cause concentration-dependent increases in $[Ca^{2+}]_i$ in $G\alpha_{16z49}$-transfected GPR55-HEK293aeq cells. In this assay, $G\alpha_{16z49}$ confers calcium responsiveness to GPCRs. Each of the compounds increased $[Ca^{2+}]_i$ in $G\alpha_{16z49}$-transfected GPR55-HEK293aeq cells indicating their ability to activate GPR55. To confirm the selectivity of these compounds for GPR55, the compounds were also tested for their ability to increase $[Ca^{2+}]_i$ in HEK293aeq cells expressing α_{1A} adrenoceptors rather than GPR55. Phenylephrine, an α_{1A} agonist, increased $[Ca^{2+}]_i$ whereas the benzoylpiperazines were inactive. Together, this evidence shows that benzoylpiperazines are agonists of human GPR55 and that they exhibit a promising selectivity profile towards GPR55.

Several highly selective GPR55 antagonists have recently been identified using the β-arrestin technology (Heynen-Genel et al. 2010b). The three main scaffolds identified were: ML191 (CID23612552, 1,080 nM), ML192 (CID1434953, 702 nM), and ML193 (CID1261822, 221 nM). Furthermore, it was shown that ML191 is >100-fold more selective as an antagonist for GPR55 than for GPR35, CB_1, and CB_2; ML192 has >45-fold greater antagonist and agonist selectivity for GPR55 against GPR35, CB_1, and CB_2; and ML193 is >145-fold, >27-fold, and >145-fold more potent as an antagonist for GPR55 than towards GPR35, CB_1, and CB_2, respectively.

Another β-arrestin based readout, PathHunter by DiscoverRx (Fremont, CA), has been used to identify lipid and cannabinoid ligands that activate GPCRs (Yin et al. 2009). The PathHunter assay measures β-arrestin translocation to the activated GPCR using β-galactosidase enzyme fragment complementation technology (Yan et al. 2002). This technology offers a tagged receptor assay whose readout, β-arrestin binding measured by reconstituted β-galactosidase activity, is immediately downstream of receptor activation. It is a generic GPCR assay that works for receptors that couple to all classes of G proteins, as it examines the desensitization and internalization pathway, not the G protein-dependent signaling pathway. The readout is luminescence signal strength, which makes this an easy quantitative assay that does not require cell imaging, and is amenable to HTS. Here too, an advantage is the possibility to identify biased agonists as β-arrestin activates signaling pathways (i.e., ERK1/2) independently of G proteins (Violin and Lefkowitz 2007). Sixteen deorphanized and control GPCRs, including GPR55 and GPR18, were profiled against approximately 400 lipid molecules using the PathHunter assays to establish pharmacological profiles for the GPCRs and attempt to determine receptor ligand specificity (Yin et al. 2009).

Caveats to HTS: The pairing of GPCRs and ligands is highly error prone because GPCRs cannot usually be expressed and assayed as purified proteins but instead require alternative complex cell-based assay systems. Accordingly even though most studies contain thorough internal controls, studies from different groups might

not agree with each other because of the differences and complexity of their assays. The most widely used cell-based assays to measure G protein-dependent secondary messenger formation employ probes that measure Ca^{2+} flux, cAMP levels, and reporter gene activation. The host cells used for these assays express endogenous GPCRs in addition to the over-expressed receptor of interest. Thus, agonist-stimulated increases in readout might not be limited to the agonist activating the heterologously expressed GPCR but could include a sum of signals controlled by endogenously expressed GPCRs. For example, most host cell lines express some combination of endogenous GPCRs that respond to sphingosine-1-phosphate and lysophosphatidic acid, and obtaining a silent parental cell response to control for lipid molecules acting through these endogenous GPCR rather than the heterologously expressed GPCR is very difficult. Another limitation is linked to the heterologously expressed proteins themselves. The signal difference between over-expressed GPCRs and GPCRs expressed at levels found in native cells can considerably affect the coupling of GPCRs to signaling pathways. For example, increasing the expression level of CB_2 receptors increases their coupling to AKT but not to ERK1/2 (Cudaback et al. 2010). There can be many signaling steps downstream of receptor activation that cross-interact and influence the readout as well. Also, heterologous expression of promiscuous G proteins (such as $G\alpha_{16}$) and G-protein chimeras (such as G_{qi5}) are often used to artificially reroute the GPCR signaling to the Ca^{2+} pathway, yet these effecter proteins will couple to many signaling networks that can vary between host cells to differentially affect the readout. Considering these caveats and challenges, it is extremely important that multiple assay formats are used to validate the claims of receptor–ligand pairing.

Receptor expression: Molecular biology has enabled the identification and precise mapping of CB_1/CB_2 and non-CB_1/CB_2 receptor expression in tissues and cell subpopulations. It is known that CB_1 receptors are abundantly expressed by neurons and to a lower level by nearly all tissues and cell subpopulations, while CB_2 receptors are predominantly expressed by cells originating from the hematopoietic lineage (Munro et al. 1993). This evident dichotomy in the expression profile of CB_1 and CB_2 receptors fueled the development of synth-CB agonists and antagonists that selectively target CB_2 receptors and are devoid of CB_1 receptor-mediated side effects. Following the same strategy and using the tools that we have in hand, the scientific community is now comparing the expression profiles of CB_1/CB_2 and non-CB_1/CB_2 receptors in specific tissues and cell subpopulations, and astutely testing selective agonists and antagonists at CB_1/CB_2 and non-CB_1/CB_2 receptors to determine their respective roles played in the endo-CB signaling system. This work will likely redefine the textbook view of how *Cannabis* extracts produce their diverse bioactive and therapeutic effects. One exciting example is the demonstration that THC produces most of its analgesic properties through glycine receptors rather than CB_1 and CB_2 receptors (Xiong et al. 2011). These are exciting times for our field of research, as it appears that we might still have only scratched the superficial intricacy of both the physiological importance of the endo-CB signaling system and the therapeutic potential of targeting this system.

Signaling networks: Unlike classical transmitter systems in which signaling diversity is typically accomplished via multiple receptor subtypes for a single endogenous ligand, the endo-CB signaling system utilizes multiple endogenous ligands acting on multiple protein targets. In fact, it is becoming evident that the lipid signaling system acts in parallel to the signaling highways carried by water-soluble transmitters and hormones. Studies on, for example, the production and inactivation of endo-CB such as anandamide and 2-AG have been instrumental in supporting physiological roles for lipid signaling (Devane et al. 1992; Mechoulam et al. 1995; Sugiura et al. 1995). Typically enzymes that produce and inactivate these lipids are tightly, and for the most part independently, regulated (Schlosburg et al. 2010). Accordingly, membrane metabolism spawns many lipid signals on-demand that build dynamic networks carrying information to constantly fine-tune protein and cellular functions. Most likely, lipid signals act both locally on neighboring cells and over larger distances on remote cells. While most enzymes producing and degrading lipid signals are specific to one lipid subfamily, some enzymes act on a range of substrates, thereby controlling multiple arms of the lipid signaling network. These multi-substrate enzymes act as nodes (or hubs) that can fine-tune the flow of information carried by the lipid signaling network, for example, boosting the level of one subfamily of lipids while dampening another, and consequently differentially impacting pathophysiological processes. The studies summarized in this book indicate that anandamide and 2-AG engage and modulate non-CB_1/CB_2 receptors, and thus drugs that target these enzymatic “hubs” will likely judiciously control the levels of either anandamide or 2-AG, yet also fine-tune the stream of information carried by these particular lipids at multiple receptors, i.e., both the CB_1/CB_2 and non-CB_1/CB_2 receptors.

12.3 Non-CB_1/CB_2 Receptors: Therapeutic Development

The evidence obtained during the last decade on the therapeutic potential of drugs targeting non-CB_1/CB_2 receptors is pointing to specific medical areas where such drugs could provide important benefits. Below we focus on four medical needs where several studies indicate the promising therapeutic potential of drugs targeting non-CB_1/CB_2 receptors: alcohol and drug addition, chronic pain, cardiovascular disease, and cancer.

Alcohol and drug addiction: There is a great need for the development of pharmacotherapies for the treatment of substance use disorders. In 2010, the past year rates of use of alcohol, tobacco, and illicit drugs by individuals aged 12 and older were 51.8%, 27.4%, and 8.9%, respectively (SAMHSA 2011). Seven percent of the population in the United States met the DSM-IV diagnostic criteria for alcohol abuse or dependence, and 8.7% for any substance abuse or dependence, excluding nicotine and tobacco use (SAMHSA 2011). Despite these high use rates, smoking and substance use disorders are under-treated. Pharmacotherapies for treating alcohol

and drug abuse disorders have been problematic. There are currently three medications approved by the US Food and Drug Administration to treat alcohol use disorders: naltrexone (Revia), an opioid receptor antagonist, which is effective in reducing heavy drinking, increasing abstinence, and reducing craving and relapse (Ray et al. 2010); acamprosate (Campral), an analogue of amino acid neurotransmitters that appears to largely exert its actions at *N*-methyl-D-aspartate (NMDA) receptors to alter glutamatergic neurotransmission and restore the neuronal hyperexcitability observed during abstinence, which is used to maintain (continuously extend) abstinence in detoxified alcohol-dependent patients (Mason and Heyser 2010); and disulfuram (Antabuse), an inhibitor of aldehyde dehydrogenase (ALDH), which functions as an aversive or avoidant agent (Barth and Malcolm 2010). However, each is not without its problems. Non-adherence to treatment, especially in taking disulfuram, is one issue. Naltrexone use may cause an upregulation of opioid receptors with implications for reduced opioid analgesia in patients also needing pain management. Finally, despite their approval for the treatment of alcohol use disorders, the evidence to support the effectiveness of these agents towards increasing abstinence rates, decreasing relapse, and reducing cravings remains weak.

There are several pharmacological treatments for tobacco use and nicotine addiction. Nicotine replacement therapy (NRT), in which the nicotine obtained by tobacco and smoking is replaced by safer options (such as a nicotine patch or gum), is used to reduce the severity of nicotine withdrawal symptoms and craving. Varenicline (Chantix), which has dual effects as both a partial agonist and an inhibitor of nicotinic acetylcholine receptors, has also been shownl effective in reducing withdrawal symptoms and craving. Bupropion (Zyban), which blocks dopamine uptake through the dopamine transporter, decreases the reinforcing effects of tobacco after initial cessation (Polosa and Benowitz 2011; Nides 2008; D'Souza and Markou 2011). Both varenicline and bupropion have concerns with adverse effects, including suicidal ideation and increased risk of psychiatric symptoms. Few pharmacotherapies have been developed to treat addictive disorders of other drugs of abuse.

The recent several decades of research in neuroscience demonstrated that changes in synaptic transmission and neural networks underlie the initiation, development, and escalation of substance use. We now have a much better understanding of the molecular and cellular mechanisms underlying the risk of drug seeking and relapse after extended abstinence. As described within this volume and elsewhere, many cannabinoids alter and modulate neuronal excitability independently of CB_1/CB_2 receptors. It is now well established that cannabinoids activate hippocampal non-CB_1/CB_2 receptors and decrease excitatory postsynaptic potential (EPSP) amplitude and glutamatergic transmission in $CB_1^{-/-}$ mice (Hajos and Freund 2002). Endocannabinoids acting through a non-CB_1 receptor-mediated mechanism contribute to short-term depression of excitatory transmission mediated by metabotropic glutamate receptor (mGluR) activation (Rouach and Nicoll 2003). GPR55 activation inhibits M-type potassium channels (Lauckner et al. 2008). TRPV1 modulates synaptic transmission within the brain (Matta and Ahern 2011; see also Chap. 8) and cortical excitability in humans (Mori et al. 2012). Anandamide and WIN55,212-2 increase GABAergic transmission (mIPSC), with this effect being present in $CB_1^{-/-}$

mice and not involving CB_2 receptors or TRPV1 receptors (Hofmann et al. 2011). Additional evidence suggested that this effect was mediated through an unknown GPCR. Behaviorally, anandamide elicited analgesia, catalepsy, and locomotor hypomotility in $CB_1^{-/-}$ mice (Di Marzo et al. 2000). Cannabinoids have been reported to act at various ligand-gated and voltage-gated ion channels, and these actions have the potential to contribute to the regulation of neuronal excitability (reviewed in Pertwee et al. 2010). Lastly, cannabinoids act as ligands for classic neurotransmitters that have defined behavioral functions. For example, serotonin ($5\text{-}HT_3$) receptors contribute to drug addiction and to neurological disorders such as schizophrenia, anxiety, psychosis, and cognitive function (Thompson and Lummis 2007). Glycine receptors serve a role in pain transmission, neuromotor activity, and in drug addiction and reward (Li et al. 2012, discussed in Chap. 9). 2-AG activates $GABA_A$ receptors which may be important in locomotion and sedation (Sigel et al. 2011). Despite all these landmark studies, data are still lacking on whether cannabinoids acting at ion channels and neurotransmitter receptors might participate in the development or expression of more complex cognitive behaviors and neuropathologies. Better understanding of the biological consequences of cannabinoids activating non-CB_1/CB_2 receptors in the brain will be especially important for the development of future therapeutics designed to treat severe neuropathologies, and the evidence collected so far indicates that there is an untapped potential that the actions of cannabinoids at non-CB_1/CB_2 receptors may offer a novel approach in developing new pharmacological treatments for substance abuse and addiction.

This might seem a paradox, but the strongest evidence for cannabinoids acting on non-CB_1/CB_2 receptors that might offer therapeutic benefit in the context of alcohol and drugs of abuse is from the studies on antagonists targeting CB_1 receptors in treating substance use disorders (Moreira and Lutz 2008; Onaivi 2008). Specifically, given the limitations of current pharmacotherapies for treating abuse and addictive behaviors, it was with great expectations that the CB_1 cannabinoid receptor antagonist, rimonabant, might represent the next generation of therapeutics for treating these major health problems (Le Foll et al. 2009). Preclinical evidence pointed to that outcome. Initially, it was thought that CB_1 receptor antagonists might be used as appetite suppressants to treat obesity because of the observation that *Cannabis* stimulates appetite. The idea was that its active ingredient, (−)-Δ^9-tetrahydrocannabinol (Δ^9-THC), binds to CB_1 receptors and stimulates appetite, and thus one needed to block this receptor. Rimonabant (SR141617A) was the first CB_1 receptor antagonist (inverse agonist) developed and made available to the scientific community, and also the first to be tested as an antiobesity drug (Di Marzo and Després 2009; Kirilly et al. 2012). Clinical trials showed that rimonabant was effective in promoting weight loss and treating metabolic abnormalities (for example, type 2 diabetes mellitus). The results were so promising that the European Medicines Agency approved rimonabant in 2006 for the treatment of obesity and metabolic complications.

At the same time, rimonabant was being touted as a promising pharmaceutical agent for treating alcohol and other drug (nicotine, alcohol, marijuana, cocaine, methamphetamine, and heroin and other opiates) addictions (Pacher et al. 2006; Wiskerke et al. 2008). In particular, rimonabant entered clinical trials for smoking

cessation and proved to be effective in improving the chances of quitting and in moderating weight gain associated with smoking cessation (Cahill and Ussher 2011). Government funding agencies (National Institute on Alcohol Abuse and Alcoholism, National Institute on Drug Abuse) at the US National Institutes of Health and pharmaceutical companies (Sanofi-Aventis, who had developed rimonabant) alike were excited by the potential of rimonabant for the treatment of substance use disorders. Unfortunately, clinical trials of rimonabant for the treatment of obesity proved troublesome. Rimonabant produced adverse events such as increased risk of depression, anxiety, and suicide ideation. Due to these concerns, rimonabant was never approved as an antiobesity drug in the United States, and the marketing of rimonabant in Europe was suspended in 2008.

Although research using rimonabant continues, it is clear that additional development of this and similar compounds to reduce these adverse effects will be necessary before rimonabant-like agents acting at CB_1 receptors can be used in the clinical setting (Le Foll et al. 2009; Di Marzo and Després 2009). In fact, considering our new understanding of the molecular details of how synth-CB interacts with non-CB_1/CB_2 receptors, it is tempting to speculate that some of the adverse events observed with the clinical use of CB_1 receptor antagonists, including rimonabant, may be due to the actions at non-CB_1/CB_2 receptors. Clearly much work needs to be done to define the consequences of cannabinoid actions that are mediated independently of CB_1 or CB_2 receptors on neuronal function and neural circuit activity, in particular to explore the neural substrate specificity of these actions, and seek to associate these actions with specific drug-produced behaviors.

Chronic pain and neuroimmune signaling: Drugs targeting non-CB_1/CB_2 receptors could represent a radically different generation of analgesic drugs. More than 50 million Americans suffer from some form of chronic pain, and many cases of chronic pain cannot be relieved by current therapies highlighting the need for alternative strategies to treat these patients. Furthermore, many analgesics used to treat acute and chronic pain, including opioids and non-steroidal anti-inflammatory drugs (NSAIDs), cause significant side effects, tolerance, and increased risk of addiction with their long-term use. The endo-CB signaling system expressed by neurons may provide a target for the development of pharmacological agents for treating chronic pain. Most active synapses throughout the CNS contain functional elements of the endo-CB signaling system. Furthermore, endo-CB regulate the efficacy of both GABAergic and glutamatergic neurotransmission (Kreitzer and Regehr 2001; Maejima et al. 2001; Wilson et al. 2001; Freund et al. 2003; Lutz 2004; Chevaleyre et al. 2006). Because impaired endo-CB signaling is implicated in several neurological diseases (Lastres-Becker et al. 2001, 2002a, b, c; Ramirez et al. 2005; Kreitzer and Malenka 2007; Katona and Freund 2008; Pazos et al. 2008), the identification of non-CB_1/CB_2 receptors modulating neurotransmission should allow for the development of better tools to understand the intricate role of the endo-CB signaling system in the neuroplasticity involved in the development of chronic pain.

Additionally, some of the atypical actions of cannabinoids appear to be largely confined to microglia and astrocytes, and it is proposed that cannabinoids acting through

non-CB_1/CB_2 receptors on these glial cells might produce either pro-inflammatory and anti-inflammatory responses that mediate or attenuate the generation or sensation of pain. As examples, cannabinoids inhibit lipopolysaccharide (LPS)-induced release of TNFα from microglial cells that is not mediated by either CB_1 or CB_2 receptors (Facchinetti et al. 2003). GPR18 (recently identified as the Abn-CBD or anandamide receptor) directs the chemoattractant properties of microglia thereby guiding their migration and activation (Walter et al. 2003; McHugh et al. 2010; discussed in Chap. 6). Perhaps the strongest evidence comes from the study of GPR55. LPS-induced activation of microglia causes the enhanced release of proinflammatory cytokines. Pietr et al. (2009; discussed in Chap. 7) found that LPS downregulates GPR55 mRNA in primary mouse microglia. Furthermore, $GPR55^{(-/-)}$ mice have a greater sensitivity to inflammatory and neuropathic pain, which was associated with an increased cytokine expression profile (Staton et al. 2008). These data suggest that GPR55 normally plays a protective, anti-inflammatory role that may be suppressed during microglia activation.

Here it is also worth mentioning that the role of neuroimmune signaling in the development of addictive behaviors is an emerging area of research (reviewed by Crews et al. 2011; Coller and Hutchinson 2012). The importance of glia function in substance use disorders is being studied in tandem with the increasing evidence that glia play more than a passive surveillance role in the CNS (Allen and Barres 2005). Early research focused on the ability of drug exposure to elicit a neuroinflammatory response that could contribute to drug-induced neurotoxicity and brain damage. But, as microglia and astrocytes were found to possess receptors for most drugs of abuse, this led to the hypothesis that drug-induced activation of microglia and astrocytes may release factors that modulate neuronal function underlying behavior change. In fact alcohol, opioids, and cocaine activate toll-like receptor 4 (TLR4) and favor a protective inflammatory response (Coller and Hutchinson 2012). Alcohol and other drugs activate microglia and astrocytes, enhancing the release of cytokines, chemokines, reactive oxygen species (ROS), and nitric oxide (NO) known to modulate neuronal function and behavioral outcomes (e.g., the transition from experimentation to addiction following drug exposure: discussed by Crews et al. 2011; Coller and Hutchinson 2012). Neuroimmune signaling within the frontal cortex may alter neuronal excitability that leads to the loss of behavioral control towards drug seeking, and immune signaling within limbic regions may amplify negative effect and bad feelings that are suppressed with drug use. Drug-induced central immune signaling may enhance the engagement of mesolimbic dopamine reward pathways and withdrawal circuitry. During withdrawal, stress, which is a risk factor for relapse, may promote the release of immune factors that contribute to drug seeking and relapse to drug taking, or enhance drug-induced neuroimmune activation (Frank et al. 2011).

Thus, all evidence supports the notion that central immune signaling can modulate the neuronal circuitry underlying pain perception, as well as drug reward and dependence. As we further explore the role of central immune signaling in these behaviors and pathologies, it is tempting to suggest that atypical cannabinoid actions may be used to regulate immune responses within the CNS as a novel target for

treating chronic pain and addiction. This would fit in well with the current efforts towards glial modulation approaches as pharmacotherapies for substance use disorders (Cooper et al. 2012).

Cardiovascular disease: Cardiovascular disease represents a prominent medical burden that could benefit from improved drug treatments for it constitutes the leading cause of death in the United States (over 80 million adults currently suffer from this devastating illness). The growing body of evidence supporting the therapeutic effects of drugs acting on non-CB_1/CB_2 receptors expressed by the vasculature suggests that drugs developed towards these targets could represent the next generation of cardiovascular therapeutics (Bukoski et al. 2002). Considering the rich medicinal chemistry and pharmacological knowledge already available to target various PPAR isoforms that regulate adipogenesis and vasorelaxation, knowing that cannabinoid-based scaffolds are also active at these intracellular targets might help guide the development and optimization of more selective ligands (O'Sullivan et al. 2005, 2006).

Cancer: Compounds that activate or antagonize non-CB_1/CB_2 receptors expressed by tumor cells block some of the most fundamental processes involved in the progression of cancers, namely their migration and proliferation. Ideally, compounds targeting non-CB_1/CB_2 receptors will eradicate malignant cells while sparing healthy cells. Such compounds would also gain therapeutic value if they prevented the accumulation of harmful pro-inflammatory immune cells, while promoting the recruitment of repair immune cells. While these expectations might seem overly ambitious, the data already available for some of the compounds targeting non-CB_1/CB_2 receptors are quite promising. Taking cannabidiol (CBD) as an example, this phyto-CB does not induce the typical psychotropic effects induced by THC in humans (Hollister 1973; Perez-Reyes et al. 1973) and yet it potently inhibits the migration of both astrocytomas and microglia. Accordingly, treating patients afflicted by brain tumors with this compound could reduce the infiltration of the cancer cells into healthy brain parenchyma, and also reduce the recruitment of microglia and the neovasculature towards the tumor mass (for review, see Stella 2010).

12.4 Summary

Demonstration of the existence of non-CB_1/CB_2 receptors activated by cannabinoid compounds has generated a new wave of interest within the scientific community. As you read through these chapters, it is apparent that medicinal chemists have synthesized many synthetic cannabinoid compounds that have been used as tools to identify novel non-CB_1/CB_2 receptors and characterize atypical cannabinoid actions in specific tissues. Cannabinoids have actions at well-defined non-CB_1/CB_2 receptors such as TRP ion channels and PPARs, and at receptors of relatively unknown biological function such as many newly deorphanized GPCRs. However, questions remain

as to the relative sensitivities of non-CB_1/CB_2 receptors towards cannabinoids. Towards this, we still need to develop new ligands (agonists and antagonists) and molecular tools that will enable a more complete pharmacological dissection of cannabinoid binding at atypical receptors.

The evidence covered in each chapter also raises a fundamental question: It is commonly accepted that CB_1 and CB_2 receptors were initially defined by their relatively high-affinity interaction with THC (10–30 nM) and today we know that both THC and cannabidiol (CBD) modulate other proteins with comparable potency. Conversely, some receptors have low potency towards THC, but high affinity for other cannabinoid compounds. Whether or not a particular non-CB_1/CB_2 receptor should be included in the endo-CB signaling system is, in our opinion, quite thought provoking and emphasizes the challenge for providing a thorough definition for the new proteins/components that will be added to the endo-CB signaling system (Pertwee et al. 2010). Questions that need to be considered in identifying a protein as a member of the endo-CB signaling system would include: Does a receptor need to share some amino acid sequence similarity to CB_1 and CB_2? Is a receptor required to interact with endo-CB_s, phyto-CB_s or both? Do these interactions have to be with nanomolar range affinities? Clearly, the development of more selective ligands and the detailed understanding of receptor protein structure and expression profile will guide the answers to these questions. Similarly, in vitro and in vivo models that reliably allow for the testing of the actions of such compounds will be of utmost importance in determining molecular, cellular, behavioral, and pathological responses controlled by non-CB_1/CB_2 receptors.

The curative properties of drugs targeting non-CB_1/CB_2 receptors do not overlap with currently available medicines outlined in the US pharmacopeia, and therefore such drugs represent a new therapeutic venue. Developing such therapeutics will clearly leverage the speed and precision over evolving of drug discovery. It is hoped that soon, moderate and high-throughput screening assays that predict biological responses will be identified and validated, and combined with appropriate disease and behavioral models to undertake a concerted effort towards non-CB_1/CB_2 receptor pharmacological development of medications to treat substance abuse and addiction, pain, cardiovascular disease, cancer, and other biological and behavioral disorders.

These are exciting times for our field of research. The remarkable scientific advances made towards the pharmacological and molecular identification of additional, non-CB_1/CB_2 receptors activated by cannabinoids allow us to thoroughly test the biological function played by these new players in fundamental biological functions and pathophysiology. This will open alternative venues for the development of drugs that act through different mechanisms of action and signaling pathways. This research will certainly fuel exciting research involving multiple disciplines and levels of investigation culminating in the development of novel therapeutics.

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