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Endogenous lipid activated G protein-coupled receptors: Emerging structural features from crystallography and molecular dynamics simulations

Dow P. Hurst, **Marianne Schmeisser**, and **Patricia H. Reggio***
Department of Chemistry and Biochemistry, University of North Carolina Greensboro, Greensboro, NC 27402, United States

Abstract

Class A G-protein coupled receptors (GPCRs) are thought to have a common topology that includes seven transmembrane alpha helices (TMHs) that are arranged to form a closed bundle. This bundle forms the ligand binding pocket into which ligands are commonly thought to enter via the extracellular milieu. This ligand approach direction makes sense for GPCRs that have small positively charged ligands, such as the beta-2-adrenergic or the dopamine D2 receptor. However, there is a growing sub-group of Class A GPCRs that bind lipid-derived endogenous ligands, such as the cannabinoid CB_1 and CB_2 receptors (with endogenous ligands, N-arachidonoylethanolamine (anandamide) and sn-2-arachidonylglycerol (2-AG)) and the $S1P_{1\text{-}5}$ receptors (with endogenous ligand, sphingosine-1-phosphate). Even the widely studied Class A GPCR, rhodopsin, binds a highly lipophillic chromophore (11-cis-retinal). For these receptors, ligand approach from the extracellular milieu has seemed unlikely given that the ligands of these receptors readily partition into lipid or are actually synthesized in the lipid bilayer. The recent X-ray-crystal structure of the sub-type 1 sphingosine-1-phosphate receptor ($S1P_1$) provides important information on the key structural variations that may be the hallmarks for a Class A GPCR that binds lipid-derived ligands. These include an extracellular domain that is closed off to the extracellular milieu and the existence of an opening between transmembrane helices that may serve as a portal for ligand entry via the lipid bilayer. This review examines structural aspects that the cannabinoid receptors may share with the $S1P_1$ receptor based upon sequence homology. This review also examines experimental and simulation results that suggest ligand entry via a lipid portal is quite likely for this emerging sub-group.

Keywords

Cannabinoid; Sphingosine-1-phosphate; GPCR; Crystal structure; Lipid portal

G protein-coupled receptors (GPCRs) are integral membrane proteins that serve as very important links through which cellular signal transduction mechanisms are activated. Class A GPCRs (rhodopsin-like) are thought to have a common topology that includes seven

*Corresponding author. Tel.: +1 336 334 5333; fax: +1 336 334 5402. phreggio@uncg.edu (P.H. Reggio)..

transmembrane alpha helices (TMHs) that are arranged to form a closed bundle. This bundle forms the ligand binding pocket into which ligands are commonly thought to enter via the extracellular milieu. This ligand approach direction makes sense for GPCRs that have small positively charged ligands, such as the beta-2-adrenergic or the dopamine D2 receptor. However, there is a growing sub-group of Class A GPCRs that bind lipid-derived endogenous ligands, such as the cannabinoid CB_1 and CB_2 receptors (Devane et al., 1988; Munro et al., 1993) (with endogenous ligands, N-arachidonoylethanolamine (anandamide) (Devane et al., 1992) and sn-2-arachidonylglycerol (2-AG))(Mechoulam et al., 1995) and the $S1P_{1-5}$ receptors (Chun, 1999, 2005; Chun et al., 1999, 2000; Sanchez and Hla, 2004; Zhang et al., 1999) (with endogenous ligand, sphingosine-1-phosphate) (Choi et al., 2011; Graler, 2010; Hla and Brinkmann, 2011). Even the widely studied Class A GPCR, rhodopsin, binds a highly lipophillic chromophore (11-cis-retinal) (Palczewski et al., 2000). For these receptors, ligand approach from the extracellular milieu has seemed unlikely given that the ligands of these receptors readily partition into lipid or are actually synthesized in the lipid bilayer.

The recent X-ray-crystal structure of the sub-type 1 sphingosine-1-phosphate receptor ($S1P_1$) (Hanson et al., 2012) provides important information on the key structural variations that may be the hallmarks for a Class A GPCR that binds lipid-derived ligands. These include an extracellular domain that is closed off to the extracellular milieu and the existence of an opening between transmembrane helices that may serve as a portal for ligand entry via the lipid bilayer. This review examines structural aspects that the cannabinoid receptors may share with the $S1P_1$ receptor based upon sequence homology. This review also examines experimental and simulation results that suggest ligand entry via a lipid portal is quite likely for this emerging sub-group.

1. Cannabinoid receptors: ligands and signalling

1.1. CB_1 receptor

The cannabinoid CB_1 and CB_2 receptors (see Fig. 1) belong to the Class A (rhodopsin (Rho) family) of G-protein coupled receptors (GPCRs). CB_1 was initially cloned from a rat cerebral cortex cDNA library (Matsuda et al., 1990) and early sequence analyses revealed that this receptor had highest homology with the endothelial differentiation gene (EDG) receptor family (now split into the lysophosphatidic acid (LPA) receptors and the spinghosine-1-phosphate (S1P) receptors) (Bramblett et al., 1995). CB_1 receptors are expressed in the central nervous system (CNS) (Glass et al., 1997; Westlake et al., 1994) and are particularly rich in certain brain areas such as basal ganglia, cerebellum, and hippocampus (Pertwee, 1997). CB_1 receptors are also found in the periphery, including human testis (Gerard et al., 1991), retina (Straiker et al., 1999), sperm cells (Schuel et al., 1999), colonic tissues (Wright et al., 2005), peripheral neurons (Ishac et al., 1996), adipocytes (Roche et al., 2006), and other organs including human adrenal gland, heart, lung, prostate, uterus, and ovary (Bouaboula et al., 1993; Galiegue et al., 1995; Rice et al., 1997).

CB_1 receptors signal via multiple second messenger systems (for a review see Turu and Hunyady, 2009). CB_1 receptor agonists inhibit forskolin-stimulated adenylyl cyclase by

activation of a pertussis toxin-sensitive G-protein (Felder et al., 1995; Howlett et al., 1986). In heterologous cells, CB_1 receptors inhibit N-, P-, and Q-type calcium channels and activate inwardly rectifying potassium channels (Felder et al., 1995; Mackie et al., 1995; Pan et al., 1996). PKA-dependent inhibition of voltage-gated N-type Ca^{2+} channels (N-type VGCCs) has also been detected in neuronal cells (Azad et al., 2008). Inhibition of calcium channels and enhancement of inwardly rectifying potassium currents is pertussis toxin-sensitive, but independent of cAMP inhibition, suggestive of a direct G protein mechanism (Mackie et al., 1995). In pertussis-pretreated cells, CB_1 stimulation has also been shown to lead to adenylyl cyclase activation suggesting that in certain circumstances, CB_1 can couple to Gs proteins (Abadji et al., 1999; Glass and Felder, 1997; Kearn et al., 2005). CB_1 has been reported to induce receptor-mediated Ca^{2+} fluxes, however, the mechanism of this response is unclear. Evidence that such a Ca^{2+} signal may be Gq/PLC dependent in rat insulinoma beta-cells has been reported (De Petrocellis et al., 2007). CB_1 stimulation in vitro and in vivo leads to activation of ERK1/2 kinases in a variety of cell types (Howlett, 2005) and β-arrestins have been reported to play a role in CB_1 desensitization (Breivogel et al., 2008; Kouznetsova et al., 2002).

1.2. CB_2 receptor

The second cannabinoid receptor sub-type, CB_2 was cloned from a human promyelocytic leukemia cell HL60 cDNA library (Munro et al., 1993). The human CB_2 receptor exhibits 78% homology to the human CB_1 receptor within the transmembrane regions, 64% homology throughout the whole protein (Munro et al., 1993). Unlike the CB_1 receptor, which is highly conserved across human, rat and mouse, the CB_2 receptor is much more divergent. Sequence analysis of the coding region of the rat CB_2 genomic clone indicates 93% amino acid identity between rat and mouse and 81% amino acid identity between rat and human.

The CB_2 receptor is highly expressed throughout the immune system (Galiegue et al., 1995; Howlett et al., 2002) and is expressed in the CNS under both pathological (Benito et al., 2003) and physiological conditions (Van Sickle et al., 2005). Studies suggest that brain CB2 receptors modulate cocaine's rewarding and locomoter-stimulating effects (Xi et al., 2011). The quite specific localization of CB_2, as well as the fact that CB_2 knock-out mice fail to respond to the immunomodulatory effects of classical cannabinoids (Buckley et al., 2000), suggest that CB_2 receptor ligands would have potential therapeutic applications as immunomodulators for the treatment of inflammation and allergy. Several papers report the role of the CB_2 receptor in modulating leukocyte migration (Franklin and Stella, 2003; Jorda et al., 2002; Kishimoto et al., 2003; Massi et al., 2000), activation (Kishimoto et al., 2004), and antigen processing (McCoy et al., 1999). Additional applications could arise from studies on bone physiology, as blockage of CB_2 has been reported to protect from bone loss in ovariectomized mice (Idris et al., 2005). However, others have reported that CB_2 activation is involved in protecting from bone loss (Ofek et al., 2006). Intracellular CB_2-dependent signalling pathways include Gi/o-dependent inhibition of adenylyl cyclase, stimulation of mitogen-activated protein kinase (Bouaboula et al., 1996, 1999), phosphoinositide 3-kinase pathways (Sanchez et al., 2003), and activation of de novo ceramide production or cyclooxygenase-2 (COX-2) induction (Guzman et al., 2001).

1.3. Cannabinoid ligands

The cannabinoid (CB) receptors transduce signals in response to central nervous system-active constituents of Cannabis sativa and their synthetic analogs, such as the classical cannabinoid, (–) trans-Δ^9-tetrahydrocannabinol (Δ^9-THC 1; Chart 1) and to three other structural classes of ligands, the non-classical cannabinoids typified by (1R,3R,4R)-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-4-(3-hydroxypropyl) cyclohexan-1-ol (CP-55940; 2; Chart 1)), the endogenous cannabinoids, typified by N-arachidonoylethanolamine (anandamide, AEA) (3; Chart 1) and sn-2-arachidonylglycerol (2-AG; 4; Chart 1), and the aminoalkylindoles (AAIs) typified by (R)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl](1-naphthalenyl)methanone (WIN55,212-2; 5; Chart 1) (Reggio, 2005). Mutation studies have shown that the classical/non-classical/endogenous cannabinoids bind in the TMH2-3-6-7 region (Kapur et al., 2007; Song and Bonner, 1996), while the AAIs, along with biaryl pyrazole antagonists typified by SR141716A (6; Chart 1) bind in the TMH3-4-5-6 region of CB_1 (Hurst et al., 2006; McAllister et al., 2003).

All known cannabinoid ligands are highly lipophilic and endogenous cannabinoid ligands have been shown to be synthesized in the lipid bilayer. For example, the CB_1/CB_2 endogenous cannabinoid, 2-AG (**4**) (Mechoulam et al., 1995; Sugiura et al., 1995), is synthesized on demand from the lipid bilayer in a two step process in which phospholipase C-β hydrolyses phosphatidylinositol-4,5-bisphosphate to generate diacylglycerol, which is then hydrolyzed by diacylglycerol lipase to yield 2-AG (Di Marzo, 2008; Piomelli, 2003). After 2-AG interaction with the membrane-embedded CB receptor, it is hydrolyzed to arachidonic acid and glycerol by a membrane-associated enzyme, monoacylglycerol lipase (Dinh et al., 2002).

2. S1P receptors: ligands and signalling

Sphingosine 1-phosphate (S1P) (**7**; Chart 2)) is a lipid signaling molecule that regulates the cardiovascular and immune systems and functions in numerous physiological and pathophysiological conditions (for reviews, see Refs. Choi et al., 2011; Graler, 2010; Hla and Brinkmann, 2011). The spinghosine-1-phosphate receptors ($S1P_{1-5}$) are Class A GPCRs that bind the endogenous lipid, S1P. Along with the lysophosphatidic acid (LPA) receptors, the S1P receptors were originally named the endothelial differentiation gene (EDG) family of Class A GPCRs. There are five known S1P receptor subtypes, S1P 1-5, that are expressed on a wide range of cell types, including neural cells and lymphocytes (Chun, 1999, 2005; Chun et al., 1999, 2000; Sanchez and Hla, 2004; Zhang et al., 1999). $S1P_{1-3}$ receptors are found widely distributed in the CNS, immune and cardiovascular systems. $S1P_1$ is found on T and B lymphocytes (Im et al., 2000; Ishii et al., 2004). $S1P_4$ receptor expression is confined to lymphoid and hematopoietic tissues, while $S1P_5$ is found predominantly in the CNS white matter (Sanchez and Hla, 2004; Watterson et al., 2003). The activation and functional status of cells can alter the expression pattern of S1P receptors (Matloubian et al., 2004; Miron et al., 2008).

The five S1P receptor sub-types possess high overall sequence homology that ranges from 33% to 51% (Parrill et al., 2004). Autocrine or paracrine binding of S1P to the $S1P_{1-5}$

receptors activates a variety of G proteins whose downstream signaling accounts for many of S1P's important functions in cancer, inflammation and the cardiovascular system (Choi et al., 2011; Edmonds et al., 2011; Graler, 2010; Hla and Brinkmann, 2011). The fact that S1P action results in inhibition of lymphocyte recirculation (Chun and Hartung, 2010; Mandala et al., 2002) has been the basis for the development of the pro-drug, FTY720 (fingolimod, **8**; Chart 2) which has recently been approved for the clinical treatment of relapsing-remitting multiple sclerosis (Brinkmann et al., 2010). This compound is phosphorylated by sphingosine kinase to produce FTY720P (**9**; Chart 2) which binds to the S1P receptors.

3. GPCR X-ray crystal structures

In the dicussion of receptor residues that follows, the Ballesteros–Weinstein numbering system is used (Ballesteros and Weinstein, 1995). In this numbering system, the label .50 is assigned to the most highly conserved Class A residue in each transmembrane helix (TMH). This is preceeded by the TMH number. In this system, for example, the most highly conserved residue in TMH6 is P6.50. The residue immediately before this would be labeled 6.49 and the residue immediately after this would be labeled 6.51.

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) (Li et al., 2004; Okada et al., 2002; Palczewski et al., 2000), 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) (Moukhametzianov et al., 2011; Warne et al., 2008), the adenosine A_{2A} receptor (Jaakola et al., 2008; Lebon et al., 2011), the CXCR4 receptor (Wu et al., 2010), the dopamine D_3 receptor (Chien et al., 2010), the histamine H_1 receptor (Shimamura et al., 2011), the nociceptin/orphanin FQ receptor (Thompson et al., 2012), the μ (Manglik et al., 2012), delta (Granier et al., 2012) and kappa (Wu et al., 2012) opioid receptors and the M2-(Haga et al., 2012) and M3-muscarinic acetylcholine receptors (Kruse et al., 2012). 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 extra-cellularly; and, except for the CXCR4 receptor (Wu et al., 2010), (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 W6.48 of the TMH6 CWXP motif and another residue that interacts with W6.48. 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_1 and CB_2 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 sub-unit of the G protein (Hamm et al., 1988).

3.1. $S1P_1$ receptor X-ray crystal structure

The recently published X-ray crystal structure of the $S1P_1$ receptor fused to T4-lysozyme (T4L) (Hanson et al., 2012) gives us the first opportunity to see the differences between the structures of lipid triggered Class A GPCRs vs those triggered by small aminergic ligands (such as the dopamine or histamine receptors) or peptides (such as the mu-, delta- and kappa-opioid receptors). In the $S1P_1$ receptor crystal structure, the receptor is in complex with the selective antagonist sphingolipidmimic (R)-3-amino-(3 hexylphenyl-amino)-4-oxobutylphosphonic acid (ML056; **10**, Chart 2) (Sanna et al., 2006). The resolution in the $S1P_1$/T4L structure is 3.35Å using traditional X-ray diffraction data processing methods and 2.8Å using an experimental microdiffraction data assembly method to help process data of rapidly decaying micro-crystals (Hanson et al., 2012). The human $S1P_{1-5}$ receptors have very high (62–64%) sequence homology with the human cannabinoid CB_1 receptor in their transmembrane helix (TMH) regions. It is interesting that the $S1P_{1-5}$ family of receptors have such high homology with the cannabinoid CB_1/CB_2 receptors, given that both receptors bind endogenous lipid-derived ligands. With this high sequence homology, it is likely that structural motifs seen in the recent $S1P_1$ receptor X-ray crystal structure will have implications for the structures of the cannabinoid CB_1 and CB_2 receptors.

3.2. Unique and shared structural motifs between $S1P_1$, and the CB_1 and CB_2 receptors

Fig. 1 provides the helix net diagrams for the human CB_1, CB_2 and $S1P_1$ sequences. The human $S1P_1$ receptor sequence has most of the highly conserved residue/sequence motifs found in Class A GPCRs: N1.50, D2.50, TMH3 ERY motif, W4.50, TMH6 motif CWXP (CWAP), and TMH7 motif NPXXY (NPIIY). Like the CB_1 and CB_2 receptors, $S1P_1$ lacks the highly conserved proline in TMH5.

3.3. $S1P_1$ ionic lock

The $S1P_1$ “ionic lock” contains a substitution of an Asn for the negatively charged residue usually found at position 6.30. N6.30 in $S1P_1$ is part of a hydrogen bond network that includes R3.50 and R78 in the intracellular loop-1 (IC-1).

4. $S1P_1$ transmembrane domains

TMH1

TMH1 in the $S1P_1$ structure leans away from the bundle, creating a space between TMH1 and TMH7. Like CB_1 and CB_2, $S1P_1$ has an E at residue 1.49. This residue faces lipid, but is shielded by F1.45 and I7.51.

TMH2

TMH2 in the $S1P_1$ structure has no proline or other helix deforming motif, so the helix is straight and does not lean toward TMH1 and TMH7. CB_1 and CB_2 also lack any helix deforming motif. In CB_2, the accessibility of TMH2 residues to the binding pocket has been characterized with subsituted cysteine accessibility studies (Zhang et al., 2005).

TMH3

TMH3 in $S1P_1$ has the Class A conserved D/ERY motif at residues 3.49-3.51 (E3.49-R3.50-Y3.51). CB_1 and CB_2 also have this motif, but the residue at 3.49 is an aspartate in both (i.e. D3.49-R3.50-Y3.51).

TMH4

The extracellular ends of TMH4 in $S1P_1$ and CB_1 have the GWNC motif (in CB_2 this is GWTC). In this motif, W4.64 forms an aromatic stacking interaction with Y5.39 in the $S1P_1$ structure. CB_1 and CB_2 have Y5.39 as well. This suggests that the extracellular ends of TMH4 and 5 may have an orientation in CB_1 and CB_2 that is similar to the $S1P_1$ structure.

TMH5

In most Class A GPCRs, there is a proline at position 5.50. The $S1P_1$ sequence is unusual in that it lacks a proline in TMH5. The result is that TMH5 in $S1P_1$ is very straight. Both the CB_1 and CB_2 receptors also lack a proline in TMH5. It is interesting to note that if TMH5 in $S1P_1$ had a P5.50, the motif that characterizes the tops of TMH4 and TMH5 (i.e., the W4.64/Y5.39 aromatic stacking interaction) would not be possible (see above).

TMH6

The $S1P_1$ TMH6 has the Class A conserved CWXP sequence motif (CWAP). W6.48 is likely part of the “toggle switch” for ligand activation of $S1P_1$. For this residue to change conformation, F6.52 would need to change conformation first. Thus, it is likely that F6.52 is part of the ligand binding pocket toggle switch. In CB_1 and CB_2, there are no aromatics flanking W6.48, but there is an aromatic residue on TMH3 (F3.36) that has an aromatic stacking interaction with W6.48 in the inactive state of CB_1. Mutation studies suggest that F3.36/W6.48 is the toggle switch interaction in CB_1 (McAllister et al., 2004).

TMH7

THM7 in $S1P_1$ has the Class A conserved NPXXY motif (NPIIY). It is interesting that the CB_1 receptor also has NPIIY, while the CB_2 receptor has a homologous NPVIY motif.

5. $S1P_1$ loops

Many Class A GPCRs have a Cys in the EC-2 loop that forms a disulfide bridge with C3.25. $S1P_1$ lacks a Cys at 3.25, but forms an internal disulfide with a second Cys in the EC-2 loop. This same motif is present in the CB_1 and CB_2 sequences. $S1P_1$ has a second disulfide loop between an EC-3 loop cysteine and C6.61 which is at the end of TMH6/beginning of the EC-3 loop. This causes TMH6 and TMH7 to pack closely. This second disulfide bridge is not possible for the CB_1 and CB_2 receptors, since they lack both the cysteine in the EC-3 loop and the cysteine at position 6.61. This suggests that the packing of TMH6/TMH7 in the CB_1 and CB_2 receptors will deviate from the orientation seen in the $S1P_1$ structure.

6. $S1P_1$ extracellular domain is closed

6.1. N terminus

The most striking aspect of the $S1P_1$ crystal structure is that the N-terminus, which contains a helical segment, is packed across the TMH bundle (from TMH3 to TMH6) with the EC-1 and EC-2 loops packing against the N-terminal helix (see Fig. 2). This arrangement occludes ligand access to the receptor from the extracellular milieu (Hanson et al., 2012). It is likely that this is similar in the CB receptors, particularly in CB_1 since the N-terminus of CB_1 is quite long (112 residues). Mutation and functional studies have shown thus far that the primary function of this long N-terminus in CB_1 is to keep the receptor in the endoplasmic reticulum (ER), diminshing cell surface expression (Andersson et al., 2003).

6.2. Binding site residues

The only positively charged residue facing into the binding pockets of CB_1 and CB_2 is K3.28. In CB_1 this has been shown to be the primary intertaction site for classical and endogenous ligands (Song and Bonner, 1996). $S1P_1$ has a positively charged amino acid facing into the binding pocket at position 3.28 as well (R3.28). However, in contrast to the CB receptors, $S1P_1$ also has a negatively charged residue facing into the binding pocket, E3.29. The presence of these two charged residues suggest that $S1P_1$ should be able to bind negatively charged, positively charged or zwitterionic ligands. In fact S1P, the $S1P_1$ endogenous ligand (see **7**, Chart 2), is a zwitterion.

7. $S1P_1$ antagonist binding

Fig. 3 illustrates the binding pocket for the antagonist, ML056 (**10**, Chart 2) in the $S1P_1$ crystal structure (Hanson et al., 2012). This ligand is likely protonated at physiological pH, which results in a zwitterionic head group. Three charged residues have been commonly thought to bind the zwitterionic head of the endogenous agonist, $S1P_1$. R3.28(120) and R7.35(292) have been proposed to ion pair with the phosphate group, while E3.29(121) has been proposed to ion pair with the ammonium moiety of sphingoshine 1-phosphate (Parrill et al., 2000). In the antagonist bound $S1P_1$ receptor crystal structure, both R3.28(120) and E3.29(121) interact with the phosphonate and primary amine of the antagonist, ML056. However, R7.53(292) does not interact with the antagonist (this of course does not pre-clude that R7.53 may be important for agonist binding). Instead, ML056 has additional polar interactions with N2.60(101), S2.64(105) and with N-terminal residues, Y29 and K34. The aromatic and alkyl chain regions of ML056 are located in a lipophillic region of $S1P_1$. This region includes Y2.57, M3.32, F3.33, L3.36, C5.43,T5.44, F5.47, W6.48, L6.51, F6.52, L6.55, L7.39, V7.43 and L195 on the EC-2 loop. In the binding site (see Fig. 3), the positioning of ML056 (orange) prevents the toggle switch residue, W6.48, from undergoing the χ_1 g+ $\rightarrow$ trans conformational change characteristic of activation. Thus ML056, maintains $S1P_1$ in its inactive state.

8. Ligand entry from lipid

8.1. $S1P_1$ Receptor

Why would there be limited acccess to the ligand binding pocket from the extracellular milieu? The answer seems to be that $S1P_1$ is designed for ligand approach via the membrane bilayer. As suggested by Hanson and co-workers (Hanson et al., 2012), the $S1P_1$ crystal structure shows a gap between TMH7 and TMH1 through which ligands may gain access to the binding pocket (see Fig. 4). This gap is produced by TMH1 leaning away from the TMH bundle, creating a TMH1/TMH7 gap. In the β_2-adrenergic receptor (β_2-AR) (Cherezov et al., 2007; Rasmussen et al., 2007), TMH1 also leans away from the TMH bundle creating space for an opening, however, in the β_2-AR, this opening is filled by the top of TMH2 and by W7.40 and M1.39 which interact with each other and shield the bundle from the lipid bilayer. In $S1P_1$, TMH2 is straight and does help fill the TMH1/TMH7 opening, but residues 7.40 and 1.39 are much smaller (V7.40 and F1.39). The net result is an opening to the lipid bilayer between TMH1 and TMH7 (see Fig. 4). The limited access to the ligand binding pocket from the extracellular milieu may explain why $S1P_1$ ligands, including S1P, show slow saturation of receptor binding in the presence of excess ligand (Rosen et al., 2009).

8.2. Rhodopsin/Opsin

The light activated Class A GPCR, rhodopsin, has an inverse agonist in its ligand binding pocket, 11-cis-retinal (**11**; Chart 3) which is covalently bound by a protonated Schiff base to K296 in TMH7. Light triggered, 11-cis-retinal isomerization (to all-transretinal (**12**; Chart 3)) leads to the Schiff-base deprotonated active Meta II intermediate. With Meta II decay, the Schiff-base bond is hydrolyzed, all-trans-retinal is released from the pocket, and the apoprotein, opsin is reloaded with new 11-cis-retinal (Hildebrand et al., 2009). The ligand free apoprotein opsin crystal structure (Park et al., 2008) and the ligand-free opsin structure stabilized by a high affinity peptide derived from the C terminus of the alpha-subunit of the G protein (Ops*-GaCT) (Scheerer et al., 2008) both exist in activated conformations. Here the R3.50/E6.30 ionic lock is broken due to the movement of the intracellular end of TMH6 away from the bundle. Both of these crystal structures show two lipid bilayer-exposed openings between TMH1 and TMH7 and between TMH5 and TMH6 (see Fig. 5A and B) (Hildebrand et al., 2009; Schadel et al., 2003) that have been proposed to represent entry and exit pathways for 11-cis-retinal/trans-retinal as they are shuttled from the lipid bilayer into or out of the protein (Hildebrand et al., 2009). This entry and exit via the lipid bilayer is consistent with the high lipophilicity of the ligands (**11** and **12**). It is also important to add that the crystal strucures of rhodopsin (Palczewski et al., 2000) and opsin (Park et al., 2008; Scheerer et al., 2008) possess extracellular domains that shield the protein from ligand entrance via the extracellular space. Thus, rhodopsin/opsin represents another case of a GPCR whose lipophilic ligands may gain access to the ligand binding pocket via the lipid bilayer.

8.3. Cannabinoid receptors

Experimental and computational studies of the CB receptors have also suggested a lipid portal for cannabinoid ligand entry. Isothiocyanate covalent labeling studies have suggested that a classical cannabinoid, (–)-7′-isothiocyanato-11-hydroxy-1′,1′ dimethylheptyl-

hexahydrocannabinol (AM841), enters the cannabinoid CB_1 receptor via the lipid bilayer at the level of C6.47 (Picone et al., 2005) (a CWXP motif residue that faces lipid), forming a covalent bond with this residue. Similar results were found for the CB_2 receptor (Pei et al., 2008). Microsecond timescale molecular dynamics simulations of the interaction of the endogenous cannabinoid, 2-AG (**4**, Chart 1) with the CB_2 receptor in a palmitoyl-oleoyl-phosphatidylcholine (POPC) lipid bilayer have suggested that (1) 2-AG first partitions out of bulk lipid at the TMH6/7 interface; (2) 2-AG then enters the CB_2 receptor binding pocket by passing between TMH6/7; (3) the entrance of the 2-AG head group into the CB_2 binding pocket is sufficient to trigger activation of CB_2 (Hurst et al., 2010). Fig. 6 illustrates the opening that forms between TMH6 and TMH7 in CB_2 as 2-AG is poised to enter the receptor from lipid. Fig. 7 illustrates the progress that 2-AG makes into the CB_2 binding site over the microsecond long molecular dynamics simulation. Taken together, these studies suggest that the lipid portal for cannabinoids is TMH6/7.

A recent paper from the D.E. Shaw group (Dror et al., 2012) that explored ligand entry into the β_1- and β_2-AR, reported the existence of a “vestibule” in the EC regions of the β_1- and β_2-ARs to which ligands bound before entering the binding pocket. An analogy of this appears to exist for “lipid binding pathway” receptors like the cannabinoid receptors as our simulations have suggested that 2-AG initially partitions out of bulk lipid and associates with the lipid face of TMH6/TMH7 first, before entry into CB_2 (Hurst et al., 2010).

9. Conclusions

Although they all share a common topology, every Class A GPCR has sequence dictated conformational differences in their transmembrane helix domains that make each receptor structure unique. Having said this, the $S1P_1$ X-ray crystal structure has two important features that would appear to be important for CB_1 and CB_2 structures: (1) a closed extracellular domain and (2) an opening between transmembrane helices that allows ligand to pass from the lipid bilayer and into the ligand binding pocket.

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Abbreviations

MD	molecular dynamics
2-AGPI	2-arachidonoyl-sn-glycero-3-phosphoinositol
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
AEA	N-arachidonoylethanolamine
TMH	transmembrane helix
GPCR	G protein-coupled receptor
EDG	endothelial differentiation gene

LPA	lysophosphatidic acid
S1P	spinghosine-1-phosphate

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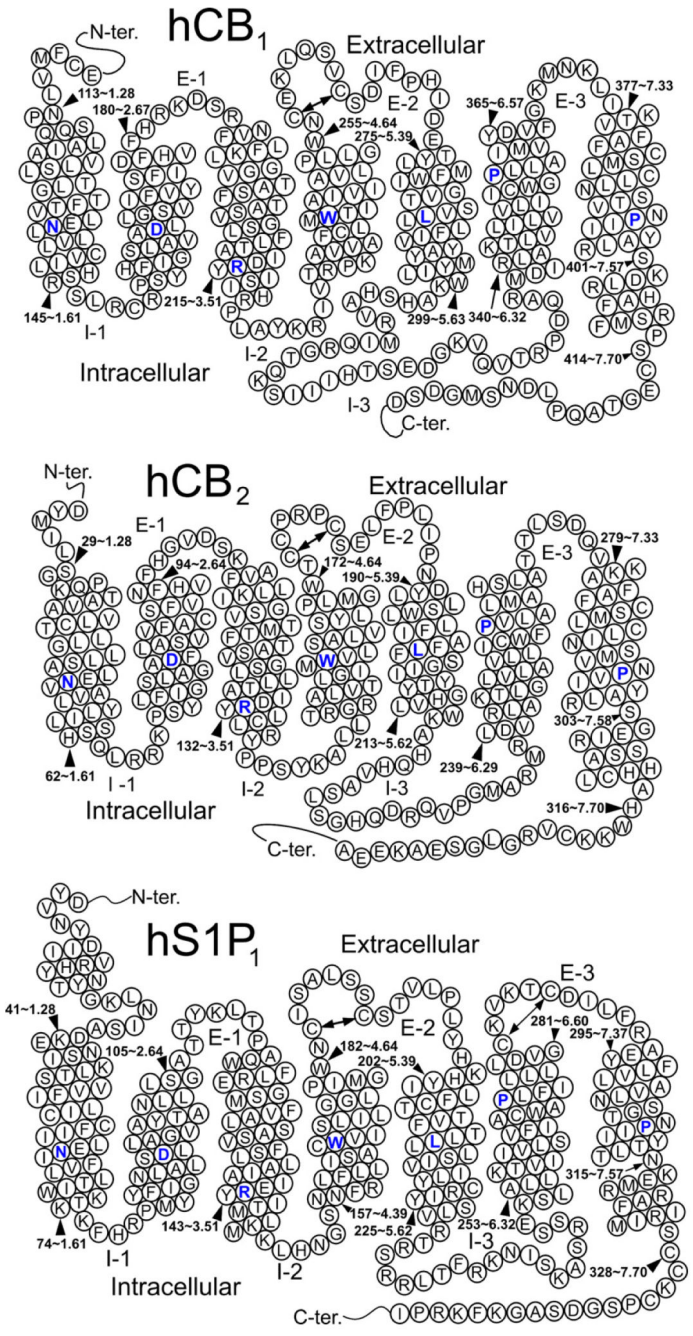

Fig. 1.

The sequences of the human CB_1, CB_2 and $S1P_1$ receptors are illustrated here in helix net diagrams.

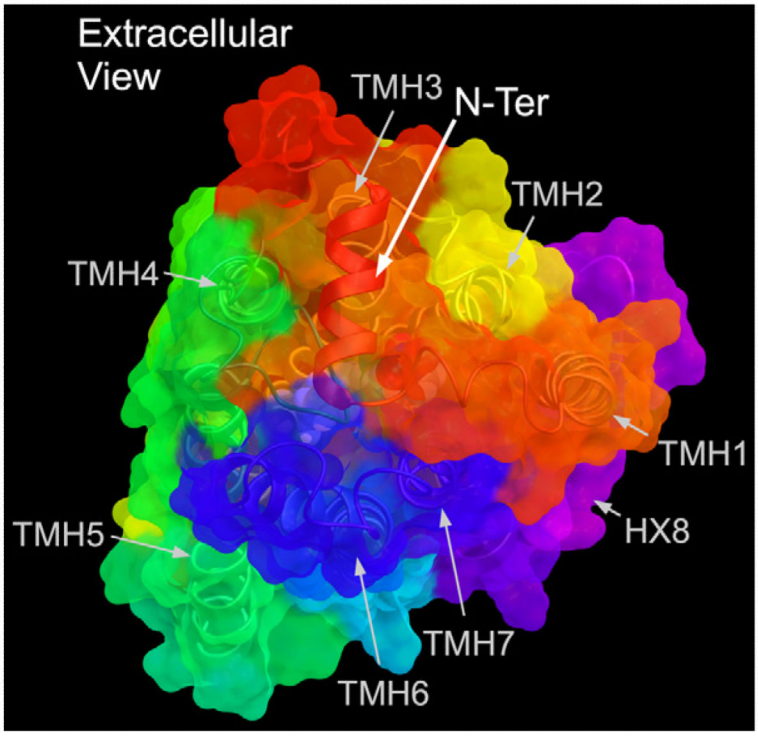

Fig. 2.

The N-terminus of the $S1P_1$ crystal structure (red) contains a helical segment that is packed across the TMH bundle (from TMH3 to TMH6) with the EC-1 and EC-2 loops packing against this N-terminal helix. This arrangement occludes ligand access to the receptor from the extracellular milieu (Hanson et al., 2012).

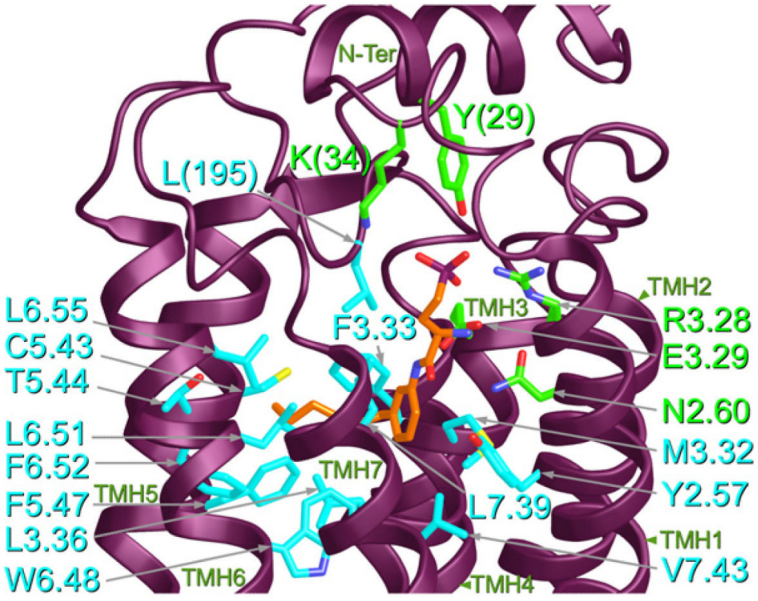

Fig. 3.

This figure illustrates the binding pocket for the antagonist, ML056 (**10**) in the S1P1 crystal structure (Hanson et al., 2012).

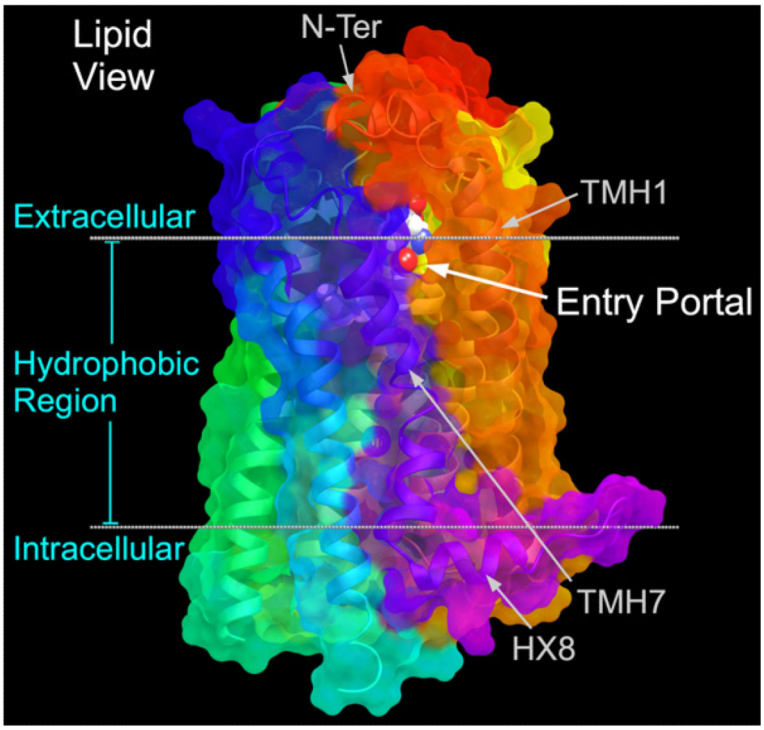

Fig. 4.

The $S1P_1$ crystal structure shows a gap between TMH7 and TMH1 through which ligands may gain access to the binding pocket (Hanson et al., 2012).

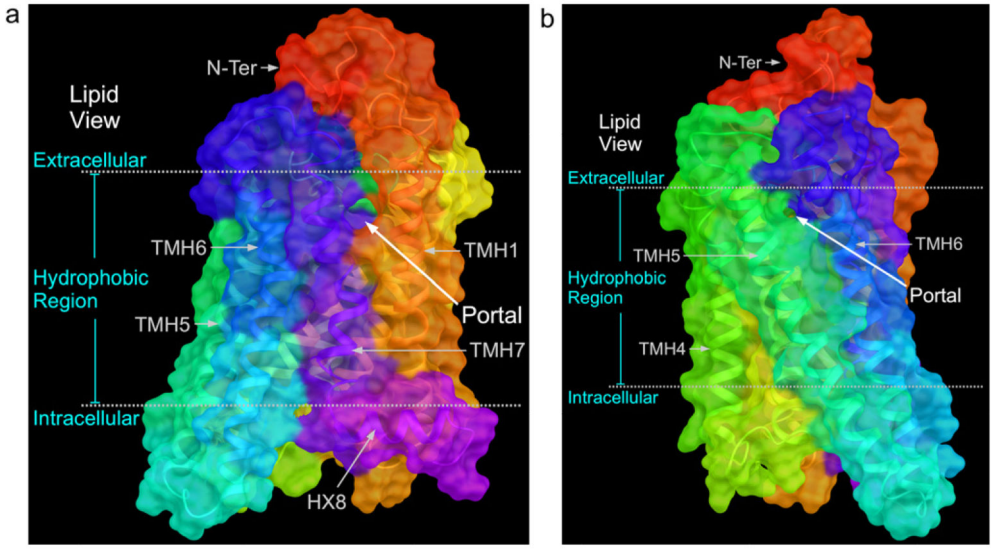

Fig. 5.

The ligand free apoprotein opsin crystal structure (Park et al., 2008) is illustrated here. In (A), the view point is from the lipid bilayer looking towards TMH7 and TMH1. Here one can see that an opening between TMH7 and TMH1 exists. In (B), the view point is from the lipid bilayer looking towards TMH5 and TMH6. Here one can see that an opening between TMH5 and TMH6 also exists The openings illustrated in (A) and (B) have been proposed to be portals such that ligand movement from the lipid bilayer into opsin would be possible (Hildebrand et al., 2009; Schadel et al., 2003).

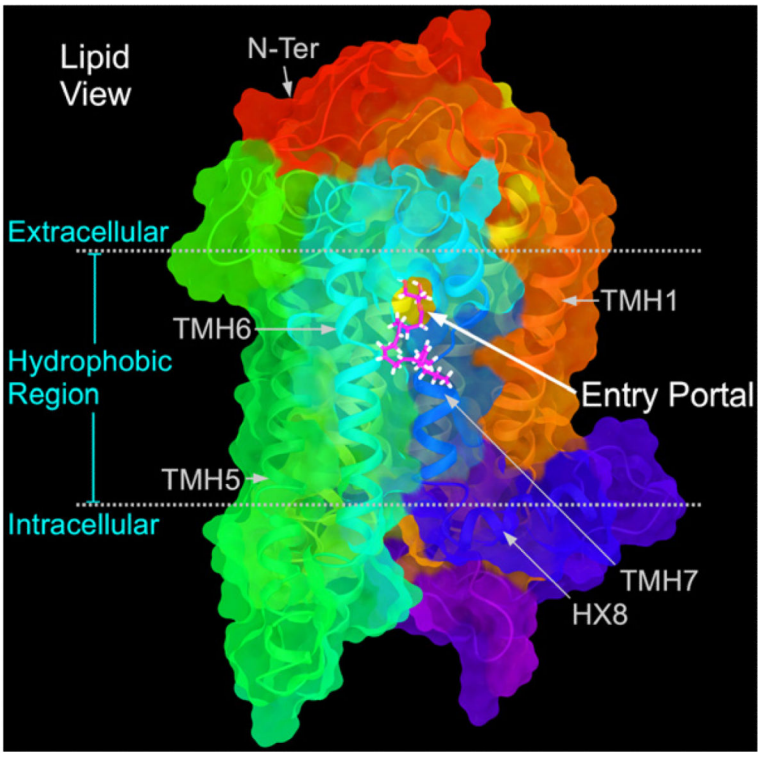

Fig. 6.

This figure illustrates the result of molecular dynamics simulations of endogenous cannabinoid, 2-AG (**4**) binding to the membrane embedded CB_2 receptor. Here an opening forms between TMH6 and TMH7 in the CB_2 receptor as 2-AG (magenta) is poised to enter the receptor from the lipid bilayer (Hurst et al., 2010).

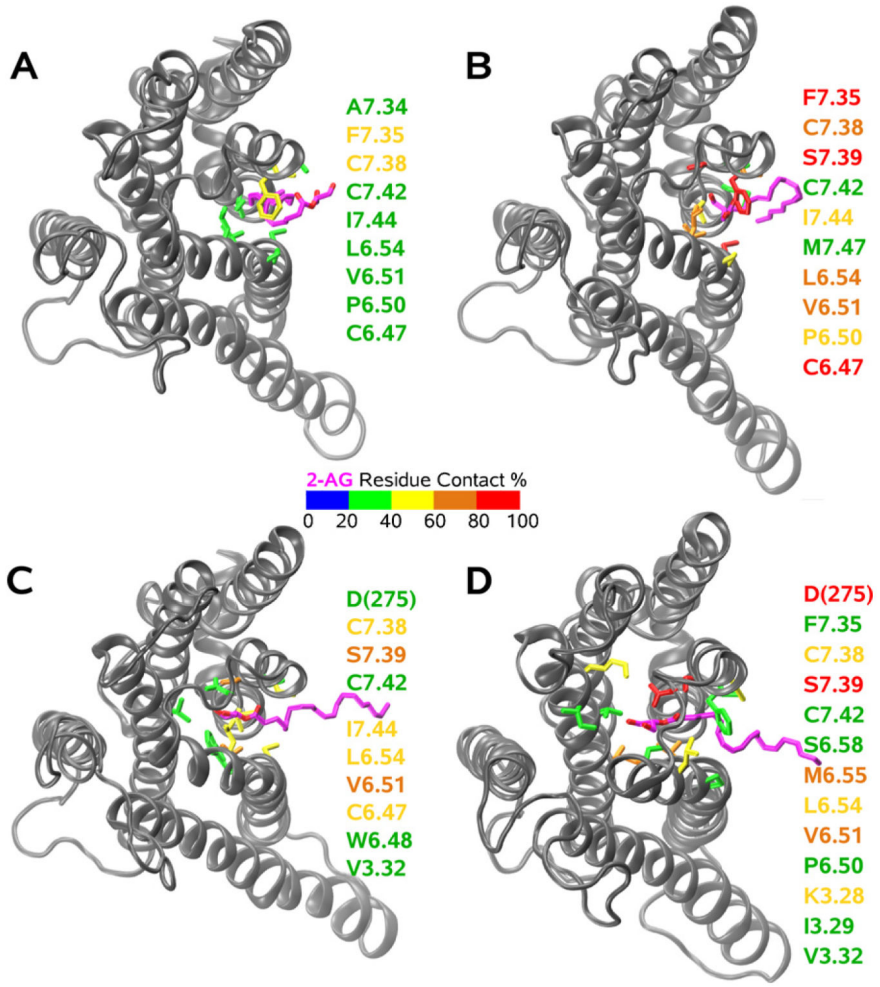

Fig. 7.

This figure illustrates the progress of 2-AG into the CB_2 binding pocket. The color scale represents the percentage of the trajectory in which any portion of 2-AG is within 4Å of residues on CB_2 (defined here as within contact distance). Residues within contact distance are listed on the right and are color coded according to this scale. The view is from the extracellular side of the receptor. (A) 2-AG has partitioned out of bulk lipid and contacts residues in or near the TMH6/7 interface. Highest contact is with F7.35(281) and C7.38(284). (B) 2-AG interaction with residues in the TMH6/7 interface increases with greater than 80% contact ocurring with F7.35(281), S7.39(285) and C6.47(257). (C) 2-AG begins to contact binding pocket residues on TMH3 (V3.32(113)), TMH6 (W6.48(258)), TMH7 (C7.42(288)) and the EC-3 loop (D(275)). (D) Subsequent to protonation, 2-AG contacts multiple residues on TMH3/6/7 and the EC-3 loop with formation of hydrogen bonds with D(275) in the EC-3 loop and to a lesser extent with S7.39(285) (Hurst et al., 2010).

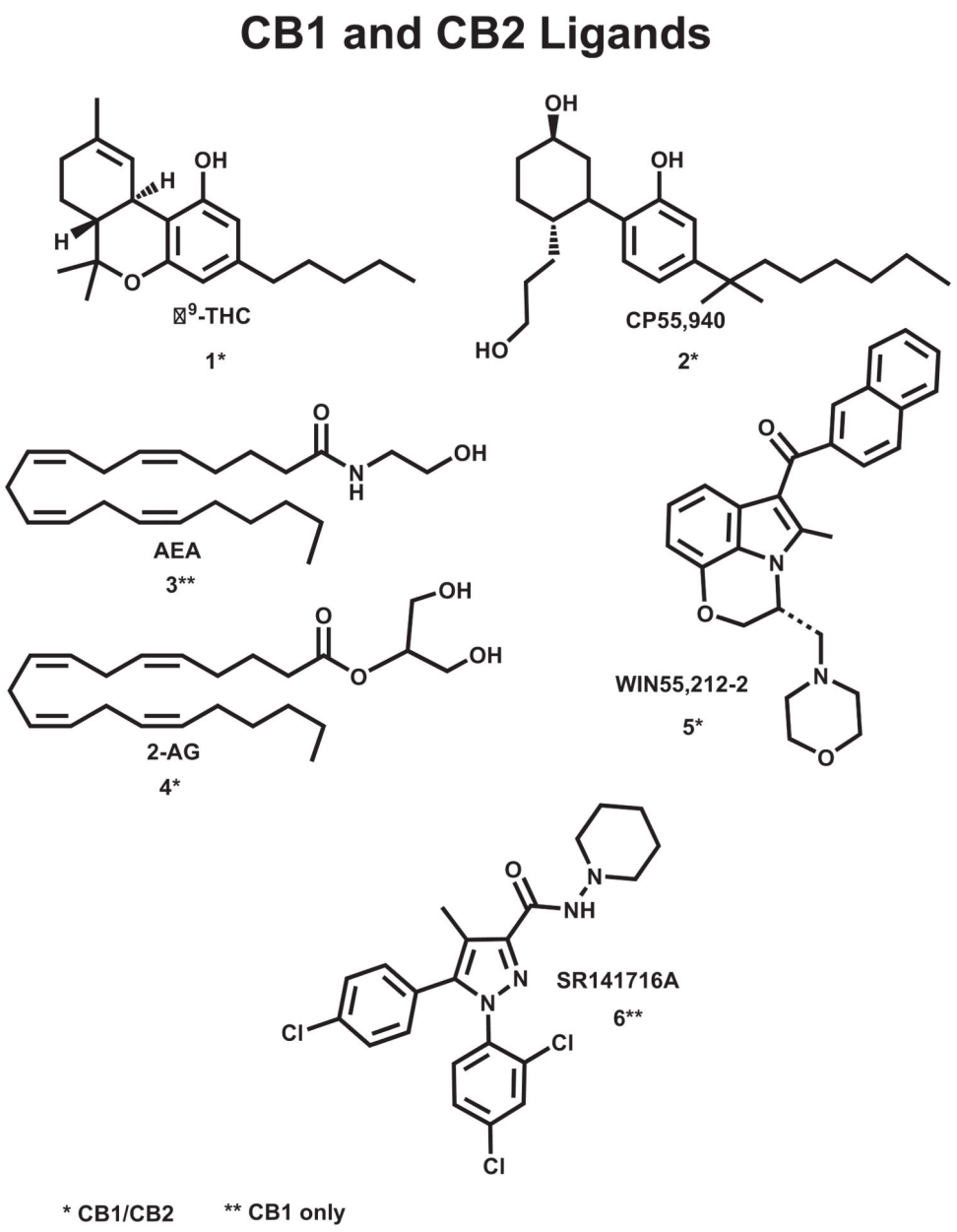

Chart 1.

This figure illustrates the structures of ligands that bind to the CB_1 and CB_2 receptors.

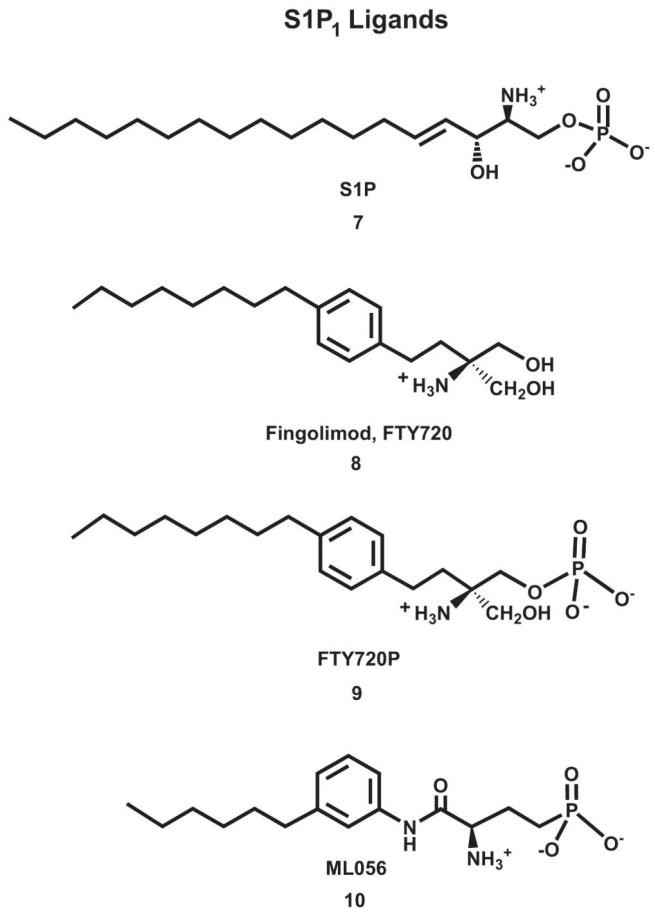

Chart 2.

This figure illustrates structures of agonists, prodrugs and antagonists that bind to the $S1P_1$ receptor.

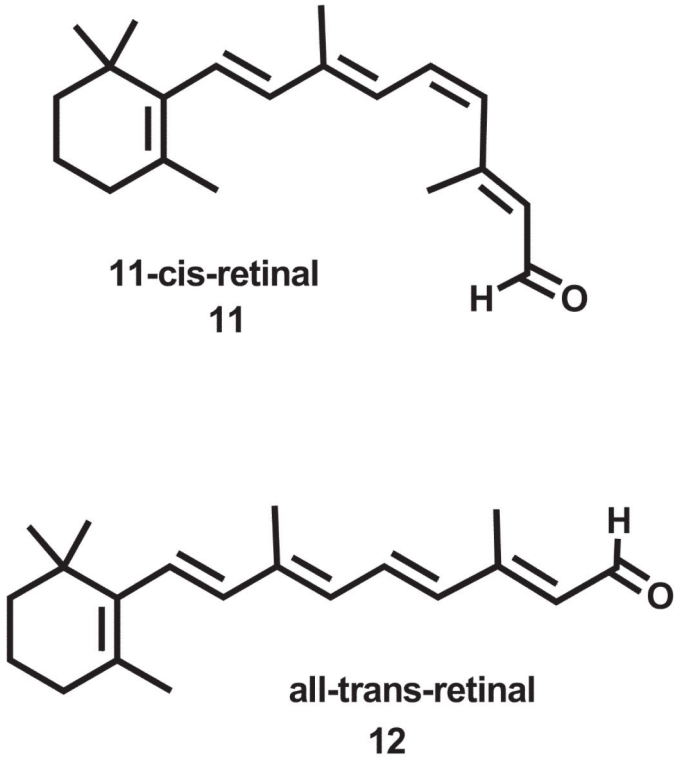

Chart 3.

This figure illustrates the structure of the chromophore for rhodopsin, 11-cis-retinal (**11**), and the structure to which this chromophore is converted by light, all trans-retinal (**12**).