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The Cannabinoid Type-1 Receptor Carboxyl-Terminus, More Than Just a Tail

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Abstract

The cannabinoid type-1 (CB_1) receptor is a G protein-coupled receptor (GPCR) that binds the main active ingredient of marijuana, Δ^9-tetrahydrocannabinol, and has been implicated in several disease states, including drug addiction, anxiety, depression, obesity, and chronic pain. In the two decades since the discovery of CB_1, studies at the molecular level have centered on the transmembrane core. This interest has now expanded as we discover that other regions of CB_1, including the CB_1 carboxyl-terminus, have critical structures that are important for CB_1 activity and regulation. Following the recent description of the three dimensional structure of the full-length CB_1 carboxyl-terminal tail (Ahn *et al.*, Biopolymers (2009) 91: 565–573), several residues and structural motifs including two α-helices (termed H8 and H9) have been postulated to interact with common GPCR accessory proteins, such as G-proteins and β-arrestins. This discourse will focus on the CB_1 carboxyl-terminus; our current understanding of the structural features of this region, evidence for its interaction with proteins, and the impact of structure on the binding and regulatory function of CB_1 accessory proteins. The involvement of the carboxyl-terminus in the receptor life cycle including activation, desensitization, and internalization will be highlighted.

Keywords

cannabinoid receptor; G protein-coupled receptor; carboxyl-terminus; internalization; desensitization; helix 8

Introduction

The plant *Cannabis sativa*, in various forms including marijuana and hashish, has been utilized for its medicinal properties for centuries. The main psychoactive component of marijuana, Δ^9-tetrahydrocannabinol (THC) and its derivatives, are classified as cannabinoids and affect a large number of physiological functions, including pain, body temperature, appetite control, motor coordination, learning and memory, sedation, anxiety, and fear (for review see Porter and Felder 2001). Endogenous cannabinoids, including anandamide and 2-arachidonyl glycerol, have been isolated from brain (Devane *et al.* 1992; Mechoulam *et al.* 1995; Felder *et al.* 1996; Sugiura *et al.* 1995), are highly lipophilic, and mediate cannabimimetic neurological effects (Fride and Mechoulam 1993; Crawley *et al.* 1993; Smith *et al.* 1994).

The cannabinoid receptors that bind THC with high affinity are found as two subtypes, the cannabinoid type-1 (CB_1; Matsuda *et al.* 1990) and the cannabinoid type-2 (CB_2; Munro *et*

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al. 1993) receptors. The CB_1 is predominantly expressed in the central and peripheral nervous systems, and is among the most expressed receptors in the brain (Howlett *et al.* 2002). High-levels of CB_1 expression have been reported in areas of the brain implicated in the actions of marijuana, including the cortex, amygdala, basal ganglia, cerebellum, and brainstem emetic centers (Tsou *et al.* 1998; Galiegue *et al.* 1995; Kumar *et al.* 2001). In contrast, the CB_2 is largely restricted to cells associated with the immune system (Munro *et al.* 1993). Two splice variants of CB_1 with shortened amino-termini have been identified, CB_{1a} and CB_{1b} (Rinaldi-Carmona *et al.* 1996; Shire *et al.* 1995; Ryberg *et al.* 2005), and recently, an orphan GPCR, GPR55, has been proposed as a third member of the cannabinoid receptor family. However, designation of GPR55 as a cannabinoid receptor is still pending (Godlewski *et al.* 2009; Brown and Robin 2009).

The CB_1 is an interesting therapeutic target for a number of disorders, including treatment of anorexia in patients who suffer from AIDS wasting syndrome, reducing nausea and vomiting associated with chemotherapy treatment (Walsh *et al.* 2003), and relief of neuropathic pain in multiple sclerosis (Rahn and Hohmann 2009). To date, synthetic THC and analogues such as Marinol®, Cesamet®, and Sativex® are clinically available in a number of countries; however, the effort to improve efficacy, selectivity, and the therapeutic window of these drugs is ongoing (Walsh *et al.* 2003). CB_1 antagonists/inverse agonists have also received much attention for their potential therapeutic applications such as for smoking cessation, weight loss, and drug addiction; however, CNS side effects have prevented their approval by the FDA (Le *et al.* 2008; Butler and Korbonits 2009; de Kloet and Woods 2009; Beardsley *et al.* 2009). Additional future avenues for CB_1-specific pharmacotherapy may include use of orthosteric ligands and/or ligands that display bias towards the activation of selective cell signaling pathways (i.e. biased ligands); successful development of both will likely require a clear understanding of the CB_1 carboxyl-terminus and its role in CB_1 function.

The CB_1 receptor is a member of the rhodopsin-like class A G protein-coupled receptor (GPCR) superfamily, and like other GPCRs, contains an extracellular glycosylated amino-terminus, seven α-helical transmembrane domains (TMs), with intervening extracellular and intracellular loops, and an intracellular carboxyl-terminus. The cytoplasmic regions are involved in G protein-binding, desensitization, and cellular trafficking of the receptor. For prototypical GPCRs, as depicted in Fig. 1, binding of agonists induces heterotrimeric G-protein activation by exchanging GDP for GTP on the α subunit. The G-proteins then dissociate from the receptor and the α subunit dissociates from the β/γ subunits, allowing these to regulate downstream effectors. Upon prolonged agonist exposure, protein kinases (e.g. G-protein receptor kinases 2/3 (GRK2/3)) can become activated which in turn act to phosphorylate GPCRs, providing a scaffold for arrestins to bind. The G-proteins uncouple from the receptor (desensitization) and the receptors internalize. The carboxyl-terminus, a region found critical for these regulatory events, is the focus of this review. Sequence and structural motifs of the CB_1 carboxyl-terminus will be described, as well as current findings regarding the function of this critical CB_1 receptor domain.

Features of the CB_1 carboxyl-terminus amino acid sequence

The CB_1 carboxyl-terminus has 73 residues (i.e. human CB_1 R400-L472; Bramblett *et al.* 1995; Xie and Chen 2005; Choi *et al.* 2005), a length similar to other GPCRs including the β_2-adrenergic (84 residues; Kobilka *et al.* 1987) and the CB_2 (59; Munro *et al.* 1993) receptors as shown in Fig. 2. Although the human CB_1 and CB_2 receptors only differ in their carboxyl-terminal length by 14 residues, share some ligands, and signal through similar pathways, there is no significant homology between the carboxyl-termini across these receptor subtypes.

Potential functional roles for the CB_1 carboxyl-terminus can be predicted from sequence analysis. The CB_1 carboxyl-terminus contains three cysteine residues, which if palmitoylated may act as membrane anchors, as well as numerous serine and threonine residues (11 and 5 residues, respectively) that may become phosphorylated and play a role in associations with regulatory proteins and secondary signaling molecules (Gurevich and Gurevich 2006). The region also contains aspartate and glutamate residues, whose terminal carboxylic acid groups could mimic phosphorylated serine/threonine residues, as found in other receptors (Gurevich and Gurevich 2006). Nonetheless, defining the three dimensional structure of the CB_1 carboxyl-terminus will help identify structural motifs involved in activities of the receptor. The amino acid numbering system of the human CB_1 carboxyl-terminus has been utilized in this review.

The complexities of analyzing the structure of the CB_1 carboxyl-terminus and strategies utilized to overcome them

Although riddled with their own tribulations, GPCR crystal structures have made significant contributions to the understanding of GPCR structure and function. However, in order to improve the crystal properties, the relatively unstructured and flexible carboxyl-terminus has been truncated in many of the GPCR crystal structures reported to date (Shimamura *et al.* 2008; Rasmussen *et al.* 2007; Murakami and Kouyama 2008). The exceptions include crystal structures of bovine rhodopsin, whose carboxyl-terminus is relatively short and is not appreciably resolved beyond the initial residues due to presumed inherent flexibility (Palczewski *et al.* 2000; Li *et al.* 2004). Therefore, what we know of the structure of the CB_1 carboxyl-terminus has largely evolved from computer modeling studies, circular dichroism (CD) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy.

Hydropathy plot analysis (Kyte and Doolittle, 1982) suggests that the CB_1 carboxyl-terminal tail is not markedly hydrophobic, yet purification of the full-length carboxyl-terminus has been challenging and has lead several investigators to use small peptides corresponding to shorter regions. Only recently has a peptide corresponding to the full-length CB_1 carboxyl-terminus been purified (Ahn *et al.* 2009) which has allowed for observations of additional structures. Because these studies do not examine the carboxyl-terminus within the context of the full-length receptor, interpretation of these studies must be performed with care. However, this approach has provided a good starting point for obtaining structural information.

The cytoplasmic end of TM7 and the H8 helical segment

In all reported crystal structures TM7 includes an α-helical structure followed by a kink and either a second helix (e.g. squid and bovine rhodopsin and α_2-adrenergic receptors; Murakami and Kouyama 2008; Jaakola *et al.* 2008; Palczewski *et al.* 2000) or an unstructured region (e.g. turkey β_1 and human β_2-adrenergic receptors; Warne *et al.* 2008; Rasmussen *et al.* 2007). In all cases, the highly conserved NPXXY motif, a region at the carboxyl-terminal end of TM7 thought to be critical for receptor activation (Fritze *et al.* 2003), is membrane imbedded. Beyond that, the TM7 carboxyl-terminal end is not universal and data from crystal structures cannot be easily applied to other GPCRs.

Due to the combination of its proximity to TM7 and its defined structure, the most commonly studied region within the carboxyl-terminus of all rhodopsin-like class A GPCRs is the region that encompasses helix 8 (H8). Although the amino acid sequence homology in this region is low, the H8 structure has been identified in all GPCR crystal structures and its existence suggested by numerous receptor modeling and NMR studies, covering a wide-range of GPCR subtypes (e.g. Palczewski *et al.* 2000; Murakami and Kouyama 2008;

Jaakola *et al.* 2008; Warne *et al.* 2008; Rasmussen *et al.* 2007; Li *et al.* 2004; Piserchio *et al.* 2005).

One of the first analyses that predicted the CB_1 H8 employed Fourier transform methods, with the nPRIFT hydrophobicity scale and with a variability profile to calculate the α-helical periodicity in the primary amino acid sequence of the human full-length CB_1 receptor (Bramblett *et al.* 1995). A discrete α-helical segment was identified extending from TM7 and spanning the membrane-cytoplasm interface. The relative orientations of the hydrophobic and variability moment vectors of this TM7 extension suggested that the helix has two faces, one polar and one nonpolar, 180° apart, and indicated that this helical segment is not fully embedded in the plasma membrane (Bramblett *et al.* 1995).

CD spectroscopy has been a useful tool to study the H8 in isolated peptide fragments of the CB_1 carboxyl-terminus. Results indicate that the helicity of this region is dependent on the solvent/detergent utilized. CD studies in phosphate buffer and water have found that low concentrations of a peptide inclusive of the CB_1 H8 region exhibit random coil (Mukhopadhyay *et al.* 1999; Choi *et al.* 2005). However, evidence for a limited number of low energy conformations was provided from molecular dynamics (MD) simulations (Cowsik *et al.* 1997), and taken together with findings from Fourier transform methods (Bramblett *et al.* 1995), suggests that greater helical structure within the carboxyl-terminus is likely. More recently, utilizing a peptide corresponding to the full-length CB_1 carboxyl-terminus, helicity was found to be concentration-dependent in aqueous solution leading the authors to conclude that at higher peptide concentrations amphiphilic helices form by self-association to sequester hydrophobic regions of the peptide (Ahn *et al.* 2009). In contrast, in zwitterionic (DPC) and anionic (SDS and DOC) detergents the peptide exhibits high helical content (36–38% and 48–51%, respectively), at all concentrations of peptide studied. Thus, a mimetic of the membrane leaflet can induce helical formation (Ahn *et al.* 2009). Similar solvent-dependent effects on H8 helicity have been reported by Mukhopadhyay et al (1999) and for other GPCR H8 regions (Choi *et al.* 2002; Mukhopadhyay *et al.* 2000; Bechinger *et al.* 1991; Jung *et al.* 1996; Johnson and Cornell, 1999).

NMR studies utilizing short peptide fragments found little to no evidence for H8 secondary structure in aqueous media (Grace *et al.* 2007; Choi *et al.* 2005); yet using nuclear Overhauser effect spectroscopy, spectra typical of a high degree of α-helix formation in hydrophobic environments were found (Grace *et al.* 2007; Xie and Chen 2005; Tyukhtenko *et al.* 2009; Choi *et al.* 2005; Ahn *et al.* 2009), consistent with CD data. Secondary structural analyses of such small peptides are typically prone to difficulties including underestimates of secondary structure due to the high proportion of peptide constituted by the floppy ends. Consequently, in the intact receptor, H8 is likely to have a very high propensity for adopting a helical domain, comprised of residues S401-F412, in amphipathic environments (Xie and Chen 2005; Ahn *et al.* 2009).

In the GPCR crystal structures reported thus far, the TM7-H8 interface is comprised of 1–2 residues that allow for flexibility of the amino acid backbone, with the TM7 and H8 helices lying perpendicular to each other in three dimensional space and the H8 almost parallel to the membrane surface (Palczewski *et al.* 2000; Cherezov *et al.* 2007; Jaakola *et al.* 2008; Murakami and Kouyama 2008; Rasmussen *et al.* 2007; Shimamura *et al.* 2008; Warne *et al.* 2008). Results from both computer modeling and NMR studies of the CB_1 receptor leave a single residue, R400, as the pivot point in the potential “L-shaped” linker, or flexible hinge, formed between the TM7 and H8 domains (Bramblett *et al.* 1995; Xie and Chen 2005; Ahn *et al.* 2009). Following activation of bovine rhodopsin, it has been suggested that the cytoplasmic end of TM7 undergoes conformational changes exposing the carboxyl-terminus of TM7, as well as the H8 domain via the flexible hinge, to an aqueous environment. Such a

conformational change is believed to unmask critical G protein-binding domains (Abdulaev and Ridge, 1998). Furthermore, a critical interaction between TM7 and H8 (i.e. the $NPXXY(X)_{4,5}F$ motif), which has been found to stabilize rhodopsin in the inactive state, is displaced upon activation (Li *et al.* 2004; Scheerer *et al.* 2008). The location of H8 and its movement upon rhodopsin activation may be assisted by palmitoylation/depalmitoylation of neighboring cysteine residues (Shimamura *et al.* 2008). Consistent with this possibility, NMR-based computations of CB_1 suggest that a cysteine residue just carboxyl-terminal to the H8 (position 415) faces the membrane surface, and if palmitoylated, could stabilize the location of H8 at the membrane surface (Xie and Chen 2005). Thus, the structure of this region seems to be well-suited to be constrained and membrane interactive when the receptor is inactive; yet has the potential for significant movement and exposure of possible regulatory protein binding sites when activated.

The amphipathic nature, membrane association, and potential functional relevance of H8

The amino acid sequence of the CB_1 H8 is interspersed with residues containing polar side chains, yet NMR analyses of H8 in detergent indicate that the nonconsecutive positively-charged residues, including K402, R405, and R409, orient on the same side of the helix (Xie and Chen 2005; Choi *et al.* 2005; Ahn *et al.* 2009; Tyukhtenko *et al.* 2009; Grace *et al.* 2007) while the hydrophobic residues, including L404, F408, and F412, reside on the opposite face of the helix, as depicted in Figs. 3B and 3C. NOE interactions between D403 and H406 are indicative of a salt bridge (2.6 Å long), whose relative distance and orientation supports the helical nature of this region (Tyukhtenko *et al.* 2009; Xie and Chen 2005). MD simulations (at 150-ps) establish that the energetic stability favors helix association with the membrane surface (Ahn *et al.* 2009) and NMR studies find the F408 side chain and L404 amide protons interact with DPC acyl chains and head groups (Choi *et al.* 2005), together supporting a model in which the hydrophobic face of the helix is oriented toward the membrane and sits in proximity to the surface. Moreover, the H8 in all reported GPCR crystal structures is similarly amphipathic and has a similar orientation suggesting this requirement for correct folding, membrane association, or both.

Recently, the impact of specific H8 residues on helicity, ligand binding, and subcellular localization was examined by mutational studies of both a peptide corresponding to the CB_1 carboxyl-terminus and the full-length receptor. In the presence of DPC both a wild type and a K402Q/R405Q/R409Q mutant CB_1 carboxyl-terminal peptide displayed high helical content, whereas a L404A/F408A/F412A mutant peptide displayed substantially reduced helicity (Ahn *et al.* 2010). This suggests that the highly hydrophobic residues are critical for helix formation, likely due to their direct interactions with the membrane mimetic. Moreover, the full-length CB_1 receptor L404A/F408A/F412A mutant, relative to the wild-type CB_1, exhibited aberrant localization in cells and markedly lower B_{max} values indicating that helix formation is needed for proper CB_1 trafficking (Ahn *et al.* 2010). Similar dependence on the hydrophobic H8 residues for proper receptor trafficking was found for the leukotriene BLT2 receptor (Yasuda *et al.* 2009).

Although H8 is found in all GPCR crystal structures, and based on sequence alignment, a similar amphipathic motif can be found in a large variety of class A GPCRs (Han *et al.* 2001), it is not yet clear if a common functional role for H8 exists. Numerous studies, including the analysis of crystal structures, peptide crosslinking and peptide competition studies, implicate H8 in productive G-protein coupling (Konig *et al.* 1989; Ernst *et al.* 2000; Cai *et al.* 1999; Swift *et al.* 2006). These studies find that H8 interacts with cytoplasmic loops and TM extensions of the receptor (e.g. Wess *et al.* 2008; Konig *et al.* 1989; Shimamura *et al.* 2008; Murakami and Kouyama 2008), and these interactions become

disrupted upon activation (Sheerer *et al.* 2008; Li *et al.* 2004). However, whether these alterations are due to direct binding of G protein to H8 or are a consequence of indirect binding of G protein remains to be elucidated. Other groups find the carboxyl-terminus necessary for GPCR exit from the endoplasmic reticulum (e.g. Tai *et al.* 1999; Bermak *et al.* 2001; Robert *et al.* 2005; Duvernay *et al.* 2004), with a defective H8 leading to impaired receptor localization and β-arrestin translocation to the plasma membrane (e.g. Ahn *et al.* 2010; Suvorova *et al.* 2009; Yasuda *et al.* 2009). While deletion of H8 of the leukotriene BLT2 receptor led to ER trapping, cell surface expression could be recovered by treatment with ligands that act like pharmacological chaperones (Yasuda *et al.* 2009). These finding suggests that H8 impacts the proper folding of the receptor and, in its absence, ligands can induce receptor assembly into a transport-competent form. Recently, results from a CB_1 model generated by MD simulation suggested that once the helix forms, the hydrophobic residues of H8, TM1, IC1, and TM7 form a strong hydrophobic pocket (Shim 2009). This pocket may directly interact with the hydrophobic tails of membrane lipids, contributing to receptor stabilization during biosynthesis and trafficking. Alternatively, one face of H8 may interact with its counterpart on another CB_1 receptor or other GPCR to promote interactions in a receptor dimer that may be crucial for ER assembly. Indeed, in the crystal structures of photoactivated rhodopsin (Salom *et al.* 2008; PDB code: 2137), the β_1-adrenergic receptor (Warne *et al.* 2008; PDB code: 2vt4), and the β_2-adrenergic receptor (Cherezov *et al.* 2007; PDB code: 2rh1) H8 along with various TM domains (e.g. TM1 and TM2) are found at the dimer interface. Interestingly, the crystal structure of the β_2-adrenergic receptor also reveals a cholesterol-binding pocket in the receptor dimer interface. This is consistent with the observed interactions of CB_1 H8 with lipids (Choi *et al.* 2005; Ahn *et al.* 2009). Regardless of the site of H8 interaction, its importance to receptor assembly is emphasized by the finding that ER-resident chaperones such as calnexin (Free *et al.* 2007) and calreticulin (Duvernay *et al.* 2009) have been found to interact with some GPCRs, including those with substantial H8 mutations, to ensure that only properly folded proteins leave the ER. It is also possible the H8 may directly interact with chaperones or cargo carriers such as Drip 78 (Bermak *et al.* 2001), BiP (Siffroi-Fernandez *et al.* 2002), calmodulin (Labasque *et al.* 2008; Navarro *et al.* 2009), and COPII vesicles (Dong *et al.* 2008), and without a functional H8, receptor maturation and trafficking are unachievable.

H9 and the remainder of the CB_1 carboxyl-terminus

Little structural information regarding GPCR carboxyl-termini beyond the H8 is available. Recently, NMR studies examining the structure of the entire CB_1 carboxyl-terminal tail confirmed the presence and location of the CB_1 H8 and identified an additional helix, termed H9, located towards the end of the CB_1 carboxyl-terminus and encompassing residues A440-M461 (Fig. 3B and 3C; Ahn *et al.* 2009). Unfortunately, the lack of a tertiary fold in the structure prevents mapping of the relative and topological orientation of the H9. However, NMR line-broadening indicates that H9 interacts with DPC micelles, suggesting that the amphiphilic H9, like H8, lies on the inner-membrane surface and is perpendicular to the TM7 bundle. Similar to results from NMR studies, findings from a 150-ps MD simulation of the L374–L472 peptide (TM7 and the full-length CB_1 carboxyl-terminus) fully solvated in a lipid bilayer indicate that the CB_1 H9 (residues A440-M461) energetically favors lying on the membrane surface, with the 28 intervening residues (P413-A439) flexible and fluctuating (Ahn *et al.* 2009). Yet, the distance between residue F412 (H8) and A440 (H9) remains at 26 Å, indicating that despite movement of the intervening regions, the location of the helices remains fixed. Residues carboxyl-terminal to H9 were also found to be unstructured (Ahn *et al.* 2009).

The functional relevance of the CB_1 H9 has yet to be determined and for only a few GPCRs has structure in the carboxyl-terminus beyond the H8 been reported. Although limited, what

we have learned from other H9 regions may provide insight into the significance of the CB_1 H9. For example, the squid rhodopsin H9 indirectly associates with the membrane though interactions with the membrane-anchored H8 and the carboxyl-terminal end of TM6 (Shimamura *et al.* 2008; Murakami and Kouyama 2008; Schertler 2008). Together with the IC2, IC3, and the intracellular end of TM5, these domains tightly fold together, suppressing H9 rotational freedom. The negatively charged H9 residues add an electrostatic potential to the otherwise predominately positively-charged intracellular protein surface. This complex has been postulated to comprise a $G_{\alpha q}$-binding site. NMR studies find that the bradykinin receptor, which also signals through $G_{\alpha q}$, also has an H9 (Piserchio *et al.* 2005). This helix contains a number of hydroxylated (and possibly phosphorylated) residues that were found imperative for $G_{\alpha q}$ signaling. Although the bradykinin H9 is amphipathic, likely membrane-associated, and its interactions with other intracellular structures are unknown, these findings may indicate that like squid rhodopsin, H9 negative charges are important for $G_{\alpha q}$ binding. The CB_1 H9, which is also amphipathic, likely membrane-associated, and contains a number of polar residues on its hydrophilic face (Ahn *et al.* 2010; Fig. 3), could fold similarly with other intracellular components to interact with signaling molecules and regulatory proteins. This possibility is currently under investigation.

It is important to note that what we have learned from peptides corresponding to regions of GPCRs is extensive and informative; however, in all cases the necessary extrapolation of data to the full-length receptor must be weighed carefully. At present we can't predict how the presence of the full-length receptor may alter the structures, orientations, and membrane-interactions identified in the peptides corresponding to regions of the carboxyl-terminal tail. Furthermore, none of these structural strategies utilize biological membranes and thus we can only infer biological information from each model system.

Functional roles of the CB_1 carboxyl-terminus

The carboxyl-termini of GPCRs have been implicated in both the binding of numerous proteins and regulation of receptor activation, signaling, and subcellular localization. The remainder of this review will focus on the role of the CB_1 carboxyl-terminus in these processes and highlight the proteins that are thought to interact with this region of the receptor as summarized in Fig. 4.

CB_1 G-protein coupling

Agonist-induced activation of the CB_1 receptor results in the inhibition of pertussis toxin (PTX)-sensitive inhibition of adenylate cyclase activity and leads to the inhibition of cAMP accumulation, inhibition of N- and P/Q-type calcium channels, and decreases in Ca^{2+} conductance (for review see Howlett 2005). Activation of CB_1 receptors also leads to an increase in G protein-gated inward-rectifying K^+ channel (GIRK) activity and activation of mitogen-activated protein kinases (MAPKs). These effects result from coupling to one of the three subtypes of $G_{\alpha i}$ or either of the two subtypes of $G_{\alpha o}$ proteins (i.e. $G_{\alpha i1,2,3}$ and $G_{\alpha o1,2}$, respectively; Howlett *et al.* 1986). Cannabinoids have also been shown to stimulate cAMP (Bonhaus *et al.* 1998; Maneuf and Brotchie, 1997; Glass and Felder, 1997) as well as activate Ca^{2+} signalling (Lauckner *et al.* 2005; DePetrocellis *et al.* 2007), suggesting the receptors can also couple to $G_{\alpha s}$ and $G_{\alpha q}$ proteins, however the significance of these interactions is unknown.

A direct interaction between G-proteins and the CB_1 receptor has been shown following co-immunoprecipitation experiments and utilizing toxins that inhibit G protein-binding (Mukhopadhyay *et al.* 2000; Mukhopadhyay and Howlett 2001). Several studies indicate that the interactions of select G-protein subtypes occur with CB_1 intracellular loops (e.g.

Ulfers *et al.* 2002; Abadji *et al.* 1999), however, discussion of G protein-CB_1 interactions in this review focuses on those associated with the CB_1 carboxyl-terminus.

Evidence for direct G-protein interactions with the CB_1 carboxyl-terminus

Findings from G-protein activation and co-immunoprecipitation studies, utilizing a peptide corresponding to residues R400-E416 of the CB_1 carboxyl-terminus, implicate this region in $G_{\alpha i/o}$ protein binding and activation. In the absence of CB_1 ligands, the R400-E416 peptide stimulated GTPγS binding to rat brain membrane fractions and inhibited adenylate cyclase activity in membrane homogenates from N18TG2 cells (Howlett *et al.* 1998). Peptide activity was also measured in CHO cells lacking CB_1 receptors and was not reversed by co-incubation with a CB_1 inverse agonist (Mukhopadhyay *et al.* 1999), indicating that the R400-E416 peptide can autonomously activate G-proteins. In co-immunoprecipitation studies, co-incubation of high concentrations (0.2–0.5 mM) of the R400-E416 peptide with CB_1-expressing cell membrane homogenates prevents co-immunoprecipitation of the full-length CB_1 receptor with $G_{\alpha i3}$ and $G_{\alpha o}$, but not $G_{\alpha i1,2}$ (Mukhopadhyay *et al.* 2000; Mukhopadhyay and Howlett 2001), suggesting that the peptide can specifically disrupt CB_1-$G_{\alpha i3}$ and CB_1-$G_{\alpha o}$ immunoprecipitable complexes. In contrast, three different peptides of similar length encompassing distinct regions of the IC3 loop (Mukhopadhyay *et al.* 2000), a peptide containing the putative H8 region of CB_2 (Mukhopadhyay and Howlett 2001), and mastoparan (Mukhopadhyay and Howlett 2001), a cationic bee venom peptide that can form an amphipathic α-helix and that can autonomously activate G-proteins (Higashijima *et al.* 1990), were unable to individually compete with the full-length CB_1 receptor for $G_{\alpha i3}$ or $G_{\alpha o}$ subunits. These findings suggest that the R400-E416 region of the CB_1 carboxyl-terminal tail is involved in $G_{\alpha i3/o}$ recognition, binding, and activation (Mukhopadhyay *et al.* 2000).

Structural features of the CB_1 H8 region potentially involved in G-protein recognition, binding and activation

The CB_1 R400-E416 peptide consists of residues corresponding to the “TM7 linker region” (R400), the residues encompassing H8 (S401-F412), and four additional residues carboxyl-terminal to the H8, one of which is a C415S substitution (Howlett *et al.* 1998). Since the bulk of this peptide includes residues corresponding to the H8, it is tempting to speculate that this domain is necessary for the observed interactions of the peptide with G-proteins. As shown in Fig. 3, the H8 contains many potentially bioactive residues, including a cationic patch. Although, charge neutralization of residue R400 with norleucine (which contains similar bulk, yet lacks a charge) and shortening of the peptide by removal of R400 (i.e. peptide S401-E416) results in a significant decrease in affinity and efficacy (5-fold and ~25% loss, respectively), further peptide shortening (i.e. peptides D403-E416, R405-E416, A407-E416) results in no additional losses in peptide activity, underscoring the importance of the R400 residue, rather than the charge or length of the peptide in G-protein activation (Mukhopadhyay *et al.* 1999). Furthermore, charge neutralization of the peptide (i.e. due to acetylation of K402) does not drastically affect R400-E416 peptide activity in adenylate cyclase activity assays (Mukhopadhyay *et al.* 1999) and more recently Ahn *et al.* (2010), reported that substitution of the K402, R405, and R409 residues with glutamine on the full length receptor has wild type-like CB_1 agonist and GTPγS binding, further supporting the conclusion that the particular hydrophilic residues of the H8 do not directly contribute to G-protein activation. Although the above studies indicate that residue R400 is the key residue for G-protein activation, it is still possible that the adjacent H8 region may be involved in G-protein recognition and/or binding as well.

Regulatory roles of the CB_1 carboxyl-terminus in G-protein binding and/or activation

Some evidence suggests that residues carboxyl-terminal to the H8 may play a modulatory role in G-protein binding and/or activity of the full-length receptor. Nie & Lewis (2001a; 2001b) have measured G-protein activation following truncation after the TM7 (i.e. residues following R400) and separately after the H8 (i.e. following G417) domains of the full-length CB_1 receptor. Truncation of the entire carboxyl-terminus was found to eliminate 90% of measured $G_{\alpha i/o}$ activation, whereas truncation following the H8 resulted in only a ~50% loss (Nie and Lewis 2001a; Nie and Lewis 2001b). It is not entirely clear if the observed reduction in G-protein activity is due to a direct loss in G-protein binding sites or to an indirect effect due to structural changes in the receptor that impact G-protein binding (e.g. expression or localization).

The CB_1 receptor has been found to be constitutively active when expressed heterologously in non-neuronal cells (Bouaboula *et al.* 1997; Nie and Lewis 2001a). In CB_1-endogenously expressing neurons, constitutive activity has been measured by some (Pan *et al.* 1998; Hillard *et al.* 1999; Bouaboula *et al.* 1997; Vasquez and Lewis, 1999), but not all (Savinainen *et al.* 2003; Breivogel *et al.* 2004; Shi *et al.* 2003) laboratories. Results from studies performed in the Lewis laboratory utilizing truncated CB_1 receptor mutants suggest that residues carboxyl-terminal to H8 may be involved in regulating CB_1 constitutive activity. For example, when truncated 6 residues carboxyl-terminal to H8 (M1-G417) this CB_1 mutant displays similar surface expression, but exhibits greater reversal of tonic inhibition of voltage-dependent Ca^{2+} current in superior cervical ganglion (SCG) neurons following N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide (SR141716A) treatment (Nie and Lewis 2001a; Nie and Lewis 2001b), suggesting that truncation resulted in higher constitutive activity. Furthermore, co-expression of the α_2-adrenergic receptor with the wild type CB_1 receptor leads to a reduction in UK14304-mediated inhibition of Ca^{2+} currents (Vasquez and Lewis, 1999;Nie and Lewis 2001a), while co-expression with the CB_1 M1-G417 mutant receptor completely abolished these effects (Nie and Lewis 2001a). These studies suggest that the CB_1 M1-G417 mutant constitutively associates with G-proteins to a greater extent than the full-length receptor, confirm that the CB_1 M1-G417 mutant has an enhanced ability to sequester activated G-proteins as compared to the wild-type receptor, and support the hypothesis that residues distal to G417 are involved in attenuating CB_1 constitutive activity. Similar dependence on the carboxyl-terminus to mediate constitutive activity has been found for the dopamine D_5 (Demchyshyn *et al.* 2000), the serotonin 5-HT_4 (Claeysen *et al.* 1999), and the β_2-adrenergic (Parker and Ross, 1991) receptors. However, the extent to which the observations by Nie and Lewis (2001a; 2001b) represent basal activity mediated by endogenous ligands, rather than constitutive activity, requires clarification.

The importance of the “L-shaped” arm and the NPXXY motif in CB_1-G protein interactions

The NPXXY motif at the end of TM7 likely participates through aromatic stacking interactions with a relatively conserved phenylalanine in the H8 domain of many GPCRs, playing a key role in switching between the inactive and active states (Prioleau *et al.* 2002; Fritze *et al.* 2003; Ernst *et al.* 2000). The interaction between the NPXXY tyrosine and the H8 phenylalanine in rhodopsin has been proposed to provide structural constraints on the H8 region, driving H8 rearrangement in response to photoactivation and altering the affinity for G-proteins (Fritze *et al.* 2003). In the serotonin 5-HT_{2C} receptor, the tyrosine-phenylalanine interaction has been reported to be important for conformational switching of the receptor

between inactive and active states (Prioleau *et al.* 2002). Substitution of this H8 phenylalanine residue in the 5-HT_{2C} serotonin receptor and in rhodopsin with amino acids lacking aromatic side chains yields results that suggest disruption of the aromatic stacking is detrimental to the receptors' ability to both activate and dock specific G-proteins (e.g. Prioleau *et al.* 2002; Fritze *et al.* 2003). Although H8 sequence alignment (Okuno *et al.* 2005) finds that the phenylalanine residue is relatively conserved across various GPCRs (135/180 GPCRs), the CB_1 receptor contains a leucine (L404, see Fig. 2) at this position, the second most common residue (18/180). Mutation of the full-length CB_1 receptor to restore the more conserved residue (i.e. L404F) results in wild type-like ligand binding affinities and expression profiles; however, CB_1 agonist-induced GTPγS maximal stimulation (i.e. E_{max} values) is reduced (Anavi-Goffer *et al.* 2007). Furthermore, unlike the wild-type receptor (Anavi-Goffer *et al.* 2007; Mukhopadhyay *et al.* 2000), the L404F mutant is not able to co-immunoprecipitate $G_{\alpha i3}$ subunits (Anavi-Goffer *et al.* 2007). MD studies, examining possible CB_1 TM7-H8 interactions in both the wild type and L404F mutant found evidence for π interactions between the tyrosine-phenylalanine residues in the later, but not the former receptor (Anavi-Goffer *et al.* 2007). However, the elbow region, specifically the R400 residue which is thought to play a critical role in CB_1 affinity for G-proteins (Mukhopadhyay *et al.* 1999), is encompassed within a helical region in the L404F mutant, while in the wild-type receptor, it remained uncoiled (Anavi-Goffer *et al.* 2007). Collectively, these data suggest that CB_1 has evolved a somewhat different structural scheme that does not employ aromatic stacking of the tyrosine in NPXXY and a phenylalanine in H8; yet it does retain the elbow motif that appears needed for $G_{\alpha i3}$ binding and activation.

Overall, the structure of the CB_1 carboxyl-terminal tail with its two amphiphathic helices is well-suited for residing at the membrane-cytoplasm interface. This provides a means for association and tight folding of the carboxyl-terminus with the intracellular loops and TM extensions of the receptor. In addition to adding stability to the inactive state of the receptor, this form provides a mechanism by which distinct regions of the receptor could be sequestered, making them accessible to protein binding partners only after critical conformational changes in the receptor occur due, for example, to agonist binding. This could include access to and exposure of specific sites for G protein interactions, whether or not these are directly on the CB_1 carboxyl-terminal tail.

CB_1 internalization, recycling, and desensitization

Prolonged exposure of CB_1 agonists results in rapid attenuation of behavioral responsiveness, also termed tolerance, in human and animal models (Abood and Martin, 1992; Martin *et al.* 1994; Pertwee *et al.* 1993; Martin *et al.* 2004) that has been attributed to both a decrease in the ability of the receptor to activate effector pathways (i.e. desensitization) and in the number of cell surface-expressed receptors (i.e. internalization; Sim *et al.* 1996; Gainetdinov *et al.* 2004; Claing *et al.* 2002; Ferguson *et al.* 1998; Perry and Lefkowitz 2002). In the classical model of desensitization, the agonist-bound GPCR becomes a substrate for GRKs; these kinases phosphorylate serine and/or threonine residues on GPCR cytoplasmic domains, which then become a high affinity target for arrestins. Binding of arrestins uncouples G-proteins (Sim *et al.* 1996) and inhibits additional G-protein associations, as well as stimulates the internalization of the receptor. Following internalization, GPCRs either recycle back to the cell surface or are degraded in lysosomes. This classical model was first described for the β_2-adrenergic receptor and has been found applicable to many other GPCRs (e.g. Inglese *et al.* 1993; Freedman and Lefkowitz, 1996; Kovoor *et al.* 1997; Krupnick and Benovic, 1998); however, the identified protein interactions that mediate these processes vary among GPCRs (Jala *et al.* 2005; Hsieh *et al.* 1999), and likely differ for the CB_1 receptor.

CB_1 internalization

CB_1 localization and the mechanisms for internalization and recycling remain largely unknown. Elucidation of CB_1 trafficking patterns has been complicated by findings that basal CB_1 activity leads to some constitutive internalization and endosomal localization. In model cell lines such as naïve HEK293 cells (Leterrier *et al.* 2004; Bakshi *et al.* 2007; Rozenfeld and Devi 2008; Ahn *et al.* 2009; D'Antona *et al.* 2006), epithelial LLC-PK1 cells, and SHSY-5Y neuroblastoma cells (Leterrier *et al.* 2004), high levels of CB_1 are associated with the endosomal compartment. In cultured hippocampal neurons (Coutts *et al.* 2001; Leterrier *et al.* 2006; McDonald *et al.* 2007) and cortical neurons (Mikasova *et al.* 2008), defining subcellular localization is more complicated; CB_1 receptors are intracellular in the somatodendritic regions, consistent with constitutive internalization, yet the receptors accumulate on the cell surface of axons. One explanation could be that at synapses, but not so much at the cell soma, receptor functions and differences in expression of regulatory proteins inhibit CB_1 internalization and/or promote its rapid recycling.

The carboxyl-terminus has been found to be important for the internalization of some (e.g. Trapaidze *et al.* 1996), but not all (e.g. Liggett *et al.* 1992; Pals-Rylaarsdam and Hosey 1997; Tsuga *et al.* 1998) GPCRs. Studies utilizing CB_1 receptor mutants have identified residues that govern endocytosis at the carboxyl-terminal end of H9. When expressed in AtT20 cells, truncations removing residues carboxyl-terminal to V459 (M1-V459) and V464 (M1-V464) do not alter surface expression levels. However, the M1-V459, but not the M1-V464, mutant receptor fails to internalize following agonist treatment, suggesting that in AtT20 cells residues between V459 and V464 are required for CB_1 internalization (Hsieh *et al.* 1999; Daigle *et al.* 2008b). The CB_1 M1-V459 truncation removed six potential phosphorylation sites. In HEK293 cells, mutation to alanine of two of these residues in the full-length receptor (i.e. T460A/S462A, S464A/T465A, or T467/S468) has no effect, yet mutation of four (T460A-T465A) or all six (T460A-S468A) of these putative phosphorylation sites drastically reduces the extent of agonist-induced internalization of CB_1 receptors (Daigle *et al.* 2008b). In contrast, residues S425 and S429 (which are required for CB_1 desensitization), are not required for endocytosis, as a CB_1 S425A/S429A receptor mutant displays similar agonist-induced internalization as wild-type CB_1 in AtT20 cells. These data implicate the last 14 residues of the carboxyl-terminus in the regulation of CB_1 internalization. Still, the analyses are confounded by a variety of different mutational studies that make clear comparisons difficult. Also, mutations can affect multiple parameters in unknown ways making the analyses more complicated than sometimes are assumed. More in-depth studies of the phosphorylation states and structural requirements for CB1 endocytosis will be valuable for clarifying these issues.

CB_1 trafficking

Recent evidence suggests that the H8 domain can also play a significant role in CB_1 receptor trafficking. The full-length CB_1 receptor with a L404F substitution, a mutation found to affect CB_1 receptor-mediated G-protein activation, displays a faster rate of agonist-induced internalization as compared to wild type (Anavi-Goffer *et al.* 2007). Furthermore, molecular modeling studies of the L404F mutant found that the phenylalanine places structural constraints on the H8 domain, possibly limiting its mobility (Anavi-Goffer *et al.* 2007). Thus H8 flexibility may be crucial for endocytosis. More recently, disruptions of the hydrophobic face of the CB_1 H8 (i.e. L404A/F412A and L404A/F408A/F412A) as well as lengthening the distance between the CB_1 TM7 and H8 regions by the successive addition of the neutral amino acid, glutamine, have been found to decrease maximal agonist binding (i.e. B_{max} values), with no effect on agonist affinity (Ahn *et al.* 2010). Effects on binding were attributed to CB_1 receptor trafficking defects observed via confocal microscopy (Ahn

et al. 2010). Unlike the wild-type receptor, which co-localizes with the late endosome/lysosome marker, LAMP-1, these mutants displayed a more diffuse pattern of CB_1 expression, with significant co-localization with an ER marker (Ahn *et al.* 2010). As the H8 hydrophobic residues are critical for maintenance of helicity (Ahn *et al.* 2010), these data suggest that H8 helical conformation and location are critical for proper CB_1 trafficking. This emphasizes the likelihood of H8 interactions with the membrane and/or other intracellularly-oriented regions of the receptor and that these interactions are key for CB_1 assembly.

CB_1 desensitization

Results from mutational studies, performed to determine residues involved in CB_1 desensitization, find that truncation at residue 417, but not at 438 and 459 causes a dramatic attenuation of desensitization, without affecting agonist activation (Jin *et al.* 1999). This led researchers to further examine the residues between H8 and H9 helices as potential residues critical for GRK3/β-arrestin 2-mediated desensitization. Like the receptor truncated at position 417, a deletion mutant with residues 417–438 removed fails to exhibit agonist-induced desensitization in oocytes (Jin *et al.* 1999). Two putative GRK3 phosphorylation sites exist within this region, at residues S425 and S429, of the CB_1 carboxyl-terminus. Point mutations that remove these potential phosphorylation sites (i.e. S425A/S429A), yield CB_1 receptors with reduced levels of agonist-induced desensitization as measured through activation of GIRK channels (Jin *et al.* 1999) and ERK1/2 phosphorylation (Daigle *et al.* 2008a), yet have levels of agonist-induced internalization (Daigle *et al.* 2008a; Jin *et al.* 1999) and recruitment of β-arrestin to the plasma membrane comparable to wild type (Daigle *et al.* 2008a). These later studies highlight the role of phosphorylation in desensitization and distinguish separate mechanisms for CB_1 receptor desensitization and internalization. Therefore, these specific serine residues could be involved in GRK and/or β-arrestin binding, or act as a regulatory region mediating the binding and/or activities of these proteins.

Interactions of the CB_1 carboxyl-terminus with other accessory proteins

Arrestin interactions

Of the four known arrestin subtypes, only β-arrestin 1 and 2 (also known as arrestin 2 and 3, respectively) have been shown to interact with non-visual GPCRs under physiological conditions. β-arrestins play a key role in modulating the duration and amplitude of signal transduction by promoting desensitization and/or internalization of the receptors. Unlike most effector proteins, β-arrestins do not recognize a unique well-defined consensus sequence across GPCRs and thus mapping these binding sites has been difficult. In general, arrestin binding sites contain at least two phosphorylated residues (or mimicks of phosphorylated residues; e.g. aspartic or glutamic acid) within close proximity to each other (see Gurevich and Gurevich 2006). However, it has been proposed that arrestin binding sites are more-likely dependent on topological structure (Chen *et al.* 1993), and although GPCRs are predominately phosphorylated prior to β-arrestin interactions, for some GPCRs phosphorylation is not a prerequisite (Gurevich and Gurevich 2006). Class A GPCRs have been proposed to have a higher affinity for β-arrestin 2 as compared to β-arrestin 1, while class B GPCRs do not distinguish a preference for either β-arrestin subtype (Oakley *et al.* 2000), inferring that the CB_1 receptor is more likely regulated by β-arrestin 2.

The CB_1 carboxyl-terminus contains residues that regulate β arrestin-mediated desensitization and internalization. Potential phosphorylation sites at S425 and S429 have been proposed to be critical for β arrestin-mediated desensitization in AtT20 cells (Jin *et al.* 1999), but not internalization (Jin *et al.* 1999; Daigle *et al.* 2008a), differentiating β-

arrestin's role in these processes. In contrast to desensitization studies, the last 14 residues of the CB_1 receptor have been implicated in CB_1 internalization, yet effects are dependent on the cellular expression system studied. Truncation at V459 in AtT20 but not HEK293 cells results in attenuated agonist-induced β-arrestin translocation to the plasma membrane (Daigle *et al.* 2008a). However in HEK293 cells, mutation of more than 2 serine/threonine residues carboxyl-terminal to V459 results in a receptor unable to alter β-arrestin 2 subcellular distribution. This discrepancy in the required residues for β arrestin 2-mediated internalization is intriguing and once clarified, may help explain CB_1 receptor trafficking inconsistencies not only across cell lines, but also between neuronal soma and axons.

Few studies have examined direct binding of arrestin to the CB_1 receptor and the sequence and structural features involved. Recently, alternative cell-based screening assays for $G_{\alpha i/o}$ protein-coupled GPCRs have been examined, utilizing CB_1 as the prototype (van der Lee *et al.* 2009; Vrecl *et al.* 2009), and have provided some findings that suggest direct binding of β-arrestins to the CB_1 receptor. In one set of studies, the imaging-based Redistribution assay (Thermo) and two non-imaging based assays, Tango (Invitrogen) and PathHunter (DicoverRX), were utilized to examine CB_1 agonist-induced binding of β-arrestin 2 to CB_1 receptors labeled on their carboxyl-termini (van der Lee *et al.* 2009). In these studies, β-arrestin 2 was found to redistribute and co-localize with CB_1 in an agonist-dependent manner (van der Lee *et al.* 2009), suggesting that CB_1 and β-arrestin 2 directly interact. However, care must be taken when interpreting these data, as the CB_1 carboxyl-terminus was altered by the addition of a tag in all three assays, possibly impacting the association; the assays themselves measure close localization, but not necessarily direct binding. In contrast, a second set of studies utilizing a bioluminescence resonance energy transfer (BRET) approach to measure association of GFP-β-arr2 and CB_1-Rluc, found little agonist-induced BRET signals, indicating these proteins interact with low affinity and/or do not associate under physiological conditions (Vrecl *et al.* 2009). In order for the authors to obtain signals high enough to analyze CB_1 agonist-dependent β-arrestin 2 translocation, chimeric structures were required, where the CB_1 receptor carboxyl-terminal tail after the H8 region (i.e. after residue G417) was replaced with the carboxyl-terminus of the vasopressin V_2 receptor (Vrecl *et al.* 2009). This chimera bound β-arrestin 2 much more efficiently (~8-fold) as compared to wild-type CB_1-GFP receptor. The authors proposed that the reduced activity of the full-length CB_1 receptor for β-arrestin 2 associations as compared to other class A GPCRs may be indicative of class B-like arrestin interactions (i.e. the CB_1 receptor may prefer β-arrestin 1; Vrecl *et al.* 2009). Another interpretation is that the GFP tag placed on the CB_1 receptor interfered with β-arrestin binding. More detailed study of the β-arrestin binding site is required for full interpretation.

The only study to date that reports direct association of β-arrestin binding to the CB_1 receptor utilized an NMR approach, studying the association of purified human β-arrestin 1 with a diphosphorylated peptide (phosphorylated at S425 and S429) corresponding to CB_1 residues T418-N437 (Bakshi *et al.* 2007). Broadening of the NMR signal, as well as small changes in chemical shifts occurred following the addition of β-arrestin 1 to the CB_1 peptide, indicative of an exchange between free and bound peptide. Furthermore, an increase in the number and intensity of NOE peaks was indicative of complex formation. The CB_1 peptide was found to undergo a conformational change following interaction with β-arrestin 1, forming two helical segments (L423-G428 and D429-L433), with residues amino terminal and carboxyl-terminal to these residues exhibiting random coil. Glycine, an amino acid known to introduce flexibility in α-helices, acts as a hinge at position 428, providing flexibility to the relative orientation of the helical regions. Similar structural changes were observed for a peptide of rhodopsin corresponding to the region carboxyl-terminal to H8 when bound to arrestin 1 (Kisselev *et al.* 2004a; Kisselev *et al.* 2004b). These data indicate that a direct binding event occurs between β-arrestin 1 and a portion of the phosphorylated

carboxyl-terminus of CB_1 (Fig. 4). Further study is required to determine if 1) β-arrestin 2 also binds to the CB_1 receptor, and if so, where and how, 2) if additional β-arrestin binding sites exist on the CB_1 receptor, and 3) the role of putative regulatory regions in β-arrestin 1,2 binding and affinity.

Cannabinoid Receptor Interacting Protein (CRIP1a/b)

CRIP1a and CRIP1b are newly identified proteins that are alternatively-spliced variants of the same gene, generating two mRNAs that encode for proteins 164 (CRIP1a) and 128 (CRIP1b) amino acids in length (Niehaus *et al.* 2007). These CB_1-interacting proteins were discovered utilizing a yeast two-hybrid assay of a human brain cDNA library, with a peptide encompassing the last 55 residues of the CB_1 carboxyl-terminal tail as bait. Both CRIP1a and b co-immunoprecipate with CB_1 from CHAPS solubilized rat brain membrane homogenates and can be isolated in a pull-down assay using a GST-CB_1 carboxyl-terminal tail construct, further supporting a direct binding event between these proteins. Furthermore, both CRIP1a and CRIP1b are found to co-localize at the plasma membrane with, and trafficked to, the same subcellular compartment as the CB_1 receptor, suggesting that spatially these proteins can interact in vivo (Niehaus *et al.* 2007).

Currently the functional relevance of the CRIP1b protein is unknown. In contrast, in SCG neurons coexpressing both CRIP1a and the CB_1 receptor, the effects of SR141716A-induced increases in Ca^{2+} current are attenuated relative to expression of CB_1 alone, suggesting that CRIP1a plays a role in regulating CB_1-mediated tonic/constitutive inhibition of voltage-gated Ca^{2+} channels (Niehaus *et al.* 2007) and thus is involved in the downregulation of CB_1 receptor function. Although the exact binding site for CRIP1a has not been determined, when coexpressed in SCG neurons with a CB_1 receptor mutant containing a deletion of the last 9 residues of the CB_1 carboxyl-terminus, SR141716A effects on Ca^{2+} currents are restored (Niehaus *et al.* 2007). With the finding that the last 9 amino acids of CB_1 are necessary for CRIP1b interaction in yeast two-hybrid assays, these data suggest that CRIP1a binds to a motif within residues 464–472 of the CB_1 carboxyl-terminal tail (Fig. 4).

G protein-coupled receptor-associated sorting protein (GASP1)

GASP1 binds to the carboxyl-terminus of various GPCRs, modulating their post-endocytic sorting (Abu-Helo and Simonin 2010; Heydorn *et al.* 2004; Moser *et al.* 2010). Initial studies suggest GASP1 also regulates CB_1 degradation, and in animal models is necessary for the development of tolerance (both behaviorally and in the population of surface receptors; Martini *et al.* 2007; Martini *et al.* 2010; Tappe-Theodor *et al.* 2007). However, the CB_1-GASP1 binding site remains elusive. A direct interaction was proposed following the successful co-immunoprecipitation of GASP1 with the full-length CB_1 receptor from HEK293 membrane homogenates (Martini *et al.* 2007; Tappe-Theodor *et al.* 2007). Attempts at isolating the residues involved in binding, however, have yielded seemingly conflicting results. Studies utilizing a construct of GST fused to the last 14 amino acids of the human CB_1 receptor and in vitro translated GASP1, found that GASP1 and the GST-CB_1 construct could be co-isolated in GST pull-down assays (Martini *et al.* 2007), implicating the last 14 residues in GASP1 binding. In contrast, a CB_1 receptor mutant lacking the last 13 residues was found to co-immunoprecipitate with cGASP1 (a dominant negative mutant containing the last 459 residues of GASP1; Tappe-Theodor *et al.* 2007) suggesting additional binding domains on the CB_1 receptor. These studies indicate that although the last 14 residues may be sufficient for GASP1 binding, these residues may contain a component of a single GASP1 binding site that is comprised of additional CB_1 residues or is one of multiple individual GASP1 binding sites.

Factor associated with neutral sphingomyelinase activation (FAN)

Cannabinoids have recently been found to initiate growth arrest and apoptosis in transformed neuronal and nonneuronal cells as well as serve a protective role in healthy neurons exposed to toxic insults. These effects of cannabinoids are thought to be mediated, at least in part, through activation of a non-G protein-mediated signal transduction pathway, the less-explored sphingomyelin metabolic pathway (reviewed in Guzman *et al.* 2002; Velasco *et al.* 2005). Activation of the sphingomyelin metabolic pathway leads to ceramide generation, either through sphingomyelin hydrolysis or through ceramide synthesis de novo (Guzman *et al.* 2002; Velasco *et al.* 2005). In primary astrocytes and C6 glioma cells, THC has been found to induce the breakdown of sphingomyelin and intracellular ceramide accumulation in both a time and dose-dependent manner (Sanchez *et al.* 1998a; Sanchez *et al.* 1998b; Blazquez *et al.* 1999; Galve-Roperh *et al.* 2000). These effects of CB_1 agonist are blocked by SR141617A, but not the CB_2 antagonist SR144528 or PTX (Sanchez *et al.* 2001), indicating CB_1-specificity and confirming that ceramide accumulation is not $G_{\alpha i/o}$-mediated. Although little is known about the mechanism by which CB_1 receptor activation leads to ceramide accumulation, successful co-immunoprecipitation studies with the CB_1 receptor have implicated the adaptor protein factor associated with neutral sphingomyelinase activation (FAN) as a key mediator of this signaling cascade (Sanchez *et al.* 2001), and thus association with FAN allows the CB_1 receptor to function through a non-G protein-mediated signalling pathway. Transfection of dominant-negative FAN into the CB_1 endogenously-expressing cell line ECV304 results in a reduced level of CB_1 agonist-induced sphinogomyelin hydrolysis (Sanchez *et al.* 2001), providing further evidence that FAN acts as an adaptor protein in CB_1-mediated ceramide accumulation.

To date, a direct interaction between FAN and CB_1 has not been reported, nor has FAN activity been identified as being mediated specifically by the CB_1 carboxyl-terminus. However, examination of FAN interactions with other receptors suggests a putative CB_1 binding site. For example, the domains of the tumor necrosis factor (TNF) receptor that couple to sphingomyelinase activation have been identified (Kolesnick and Kronke, 1998; Adam-Klages *et al.* 1998), and are composed of a stretch of nine amino acids including residues DSAHK (Adam-Klages *et al.* 1998). The CB_1 carboxyl-terminal tail contains a highly homologous region comprised of a DCLHK sequence from residues 431–435 (Figs. 3, 4). This sequence is highly conserved across rat, human, mouse, and cat CB_1 receptors. Further study of this motif remains to be performed to assess its potential as a FAN-binding site.

Concluding remarks

Recent advances have found significant structure within the CB_1 carboxyl terminus and interactions of accessory proteins with this region have been found to be critical for mediating key points of the receptor life cycle. Nonetheless, an understanding of the extent to which the CB_1 cellular fate is governed by the carboxyl-terminus is still in its infancy. In the future it will be important to elucidate additional protein binding partners and their involvement in CB_1 receptor function. For instance, a plethora of additional proteins in CB_1 receptor trafficking, desensitization, and recycling have been implicated including clathrin, dynamin, rab 4, rab 5, esp15, caveolin-1, and AP-3 (Hsieh *et al.* 1999; Daigle *et al.* 2008b; Leterrier *et al.* 2004; Leterrier *et al.* 2006; Bari *et al.* 2008; Rozenfeld and Devi 2008), yet the specific CB_1-binding domains or motifs to which they interact have not been identified. Other future studies include delineating the pharmacological and physiological relevance of the H8 and H9 regions, as well as to identify interactions of these domains with potential binding partners and the remainder of the receptor. It will also be interesting to see if, like β-arrestin 1 (Bakshi *et al.* 2007), other accessory proteins induce or alter carboxyl-terminal

structure upon binding, and how the binding of one protein affects the interactions with others. Further insight into this critical region and its accessory proteins will advance our understanding of CB_1 receptor function and potentially identify novel drug targets for CB_1-mediated diseases.

List of abbreviations

BRET	Bioluminescence resonance energy transfer
CB_1	cannabinoid type-1 receptor
CB_2	cannabinoid type-2 receptor
CD	circular dichroism
FAN	factor associated with neutral sphingomyelinase activation
GPCR	G protein-coupled receptor
GIRK	G protein-gated inward-rectifying K^+ channel
GRK	G-protein receptor kinase
H8	helix 8
H9	helix 9
MAPK	mitogen-activated protein kinase
MD	molecular dynamics
SR141716A	N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide
NMR	nuclear magnetic resonance
SCG	superior cervical ganglion
THC	Δ^9-tetrahydrocannabinol
TM	transmembrane domain

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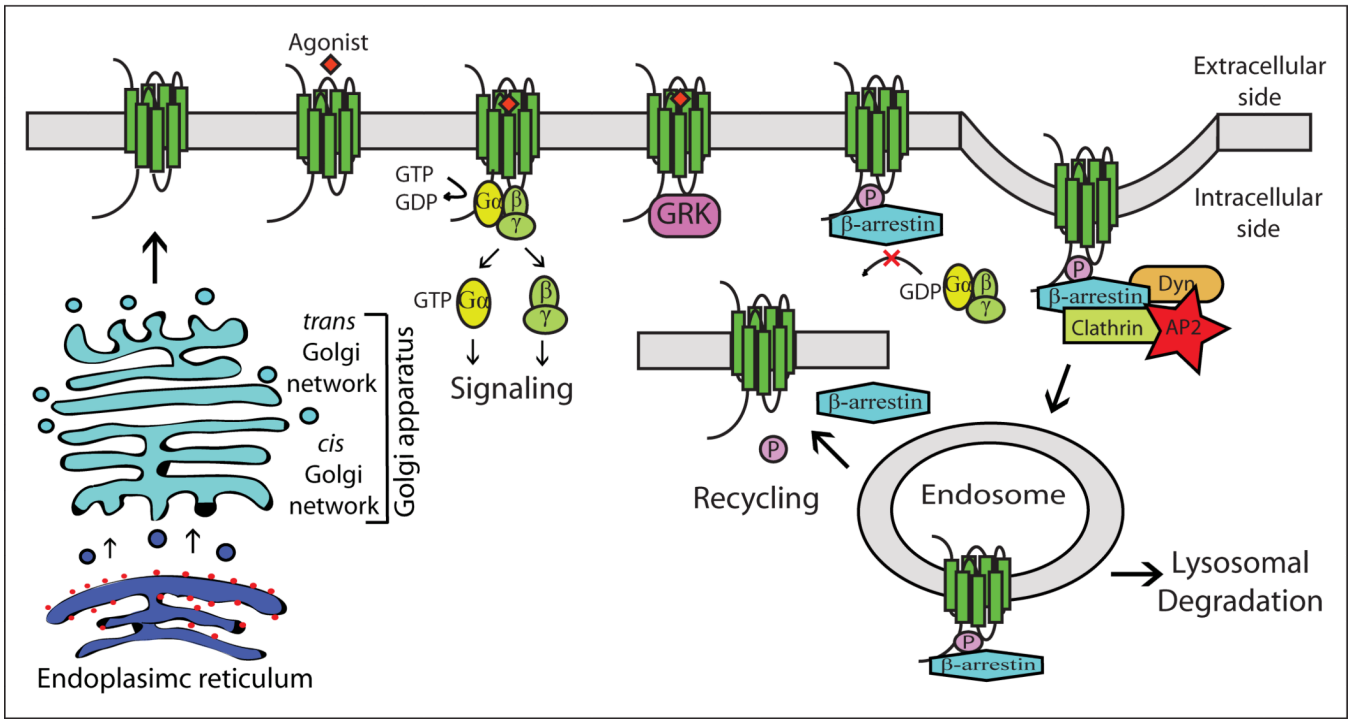

Fig. 1.

Model of the GPCR life cycle. GPCRs are synthesized, folded, and assembled associated with the endoplasmic reticulum (ER). Properly folded receptors are transported from the ER through the Golgi complex to the plasma membrane by passing a quality control process and undergoing post-translational modifications (e.g. glycosylation, methylation, and palmitoylation). Upon agonist stimulation, GPCRs activate their associated G-protein, which in turn dissociates to impact downstream signaling pathways. Prolonged exposure to agonist results in a rapid loss of responsiveness (desensitization) and removal of the receptors from the cell surface (internalization) by phosphorylation (e.g. via GRK) and subsequent arrestin recruitment. Internalized receptors are either targeted to lysosomes for degradation, or recycled back to the cell surface (resensitization).

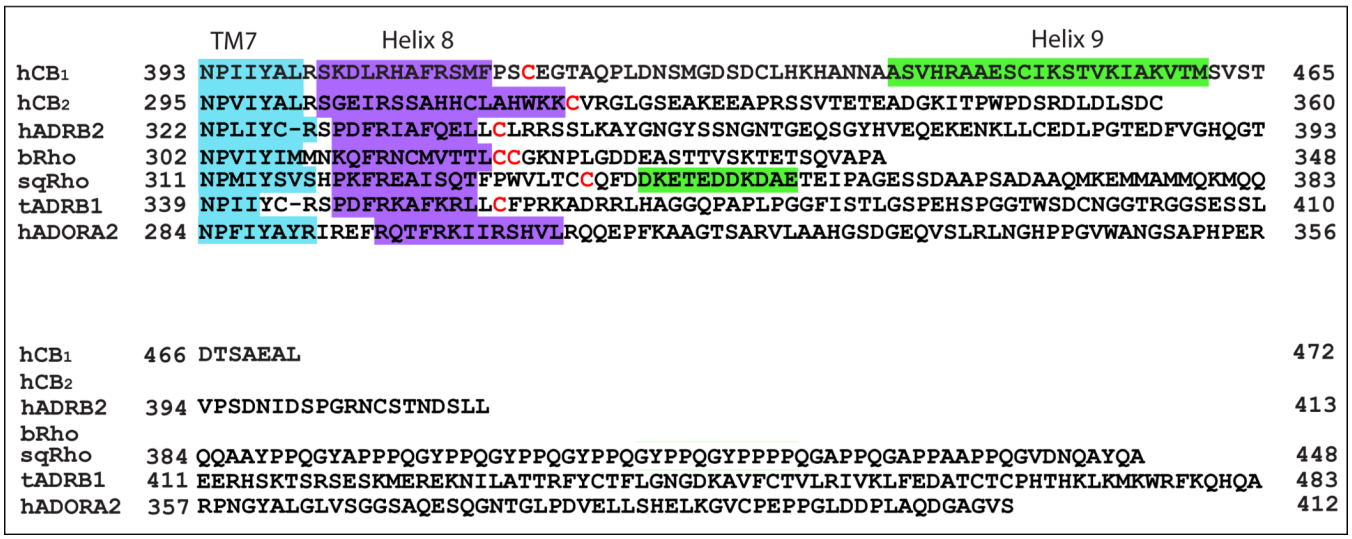

Fig. 2.

Sequence alignment of the carboxyl-termini of human CB_1 and other selected GPCRs. The human CB_1 carboxyl-terminus is presented with human CB_2 and other rhodopsin-like family A GPCRs including hADRB2, human β_2-adrenergic receptor (PDB code: 2RH1); bRho, bovine rhodopsin (PDB code: 1U19); sqRho, squide rhodopsin (PDB code: 2Z73); tADRB1, turkey β_1-adrenergic receptor (PDB code: 2VT4); hADORA2, human adenosine 2A receptor (PDB code: 3EML). The carboxyl-terminal end of TM7, helix 8, and helix 9 are highlighted in cyan, purple, and green, respectively. The carboxyl-terminal sequences are aligned in two rows. Numbers correspond to the starting and ending residues in each line.

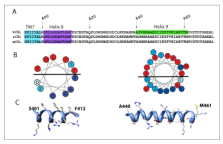

Fig. 3.

CB_1 carboxyl-terminal sequences and structural representations of two helical motifs within the carboxyl-terminus. (A) Amino acid sequence alignment of the carboxyl-terminus of CB_1 from different species; hCB_1, human CB_1 receptor, rCB_1, rat CB_1 receptor, mCB_1, mouse CB_1 receptor. Two helical motifs within the CB_1 carboxyl-terminus were defined based on the NMR structure (Ahn *et al.* 2009). Helix 8 and helix 9 are highlighted in purple and green, respectively. Residues are numbered according to their gene-specific position in the protein sequence. (B) Helical wheel projections of CB_1 helix 8 (left) and helix 9 (right). Hydrophobic and positively charged residues are colored red and blue, respectively. Negatively charged residues and serines/threonines are colored light blue and turquoise, respectively. The black bar through each helical wheel highlights the amphipathic nature of the helix and the nonpolar and polar faces. (C) Illustration of the amphipathic nature of the two α-helices observed for the carboxyl-terminus of the human CB_1 receptor. The helical domains CB_1 (401–412) (left) and CB_1 (440–461) (right) identified in Ahn *et al.* (2009) are shown as ribbons.

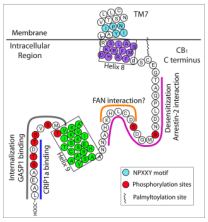

Fig. 4.

Schematic diagram of the carboxyl-terminus of the human CB_1 showing the proposed structural and functional domains. The relevant portion of TM7 is shown. The NPXXY motif, helix 8, and helix 9 are highlighted in cyan, purple, and green, respectively, as described in Fig. 2. Fuschia, orange, grey, and blue lines indicate potential interaction domains with β-arrestin 1, FAN, GASP1, and CRIP1a, respectively. A putative palmitoylated cysteine at position 415 is depicted and potential phosphorylation sites are indicated by red filled circles. Two domains implicated in desensitization (Jin *et al.* 1999;Bakshi *et al.* 2007) and internalization (Heieh *et al.* 1999) are indicated.