Themed Section: Molecular Pharmacology of GPCRs

REVIEW

Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins

Ana C Magalhaes[1,2], Henry Dunn[1,2] and Stephen SG Ferguson[1,2]

[1]*J. Allyn Taylor Centre for Cell Biology, Molecular Brain Research Group, Robarts Research Institute, London, ON, Canada, and* [2]*The Department of Physiology & Pharmacology, the University of Western Ontario, London, ON, Canada*

GPCRs represent the largest family of integral membrane proteins and were first identified as receptor proteins that couple via heterotrimeric G-proteins to regulate a vast variety of effector proteins to modulate cellular function. It is now recognized that GPCRs interact with a myriad of proteins that not only function to attenuate their signalling but also function to couple these receptors to heterotrimeric G-protein-independent signalling pathways. In addition, intracellular and transmembrane proteins associate with GPCRs and regulate their processing in the endoplasmic reticulum, trafficking to the cell surface, compartmentalization to plasma membrane microdomains, endocytosis and trafficking between intracellular membrane compartments. The present review will overview the functional consequence of β-arrestin, receptor activity-modifying proteins (RAMPS), regulators of G-protein signalling (RGS), GPCR-associated sorting proteins (GASPs), Homer, small GTPases, PSD95/Disc Large/Zona Occludens (PDZ), spinophilin, protein phosphatases, calmodulin, optineurin and Src homology 3 (SH3) containing protein interactions with GPCRs.

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Abbreviations

5-HT2AR, serotonin receptor subtype 2; 5HT2CR, serotonin 2C receptor; α1DAR, α1D adrenergic receptor; Arf6, ADP-ribosylation factor 6; ARNO, ARF nucleotide-binding site opener; AT1R, angiotensin II type 1 receptor; β2AR, β2-adrenergic receptor; CAL, cystic fibrosis transmembrane conductance regulator-associated ligand; CASK, calcium/calmodulin-dependent serine protein kinase; CaSR, calcium sensing receptor; cGRP1/cGRP2, calcitonin gene-related peptides; CIPP, channel-interacting PDZ protein; CRF, corticotrophin-releasing factor; CRFR1, corticotropin releasing factor receptor 1; CRLR, calcitonin-like receptor; CXCR2, IL-8 receptor B; CXCR4, chemokine receptor type 4; EBP50, ERM-binding phosphoprotein 50; EEA1, effector early endosome antigen 1; EVH, ENA/VASP homology domain; GABAB, GABA type B receptor; GAPs, GTPase activating proteins; GASP, GPCR-associated sorting protein; GDP, guanosine diphosphate; GEFs, guanine nucleotide exchange factors; GIPC, GIPC PDZ domain containing family member 1; GRB2, growth factor receptor-bound protein 2; GRKs, GPCR kinases; MAGI, membrane-associated guanylate kinase inverted; MAGUK, membrane-associated guanylate kinase; mGluRs, metabotropic glutamate receptors; MINT1, Munc-18-interacting protein 1; MPP3, p55 subfamily member 3; MT1, melatonin type 1 receptor; MUPP1, multi-PDZ-domain protein; Nck, non-catalytic region of tyrosine kinase adaptor protein; NHERF, Na$^+$/H$^+$ exchanger regulatory factor 1; NSF, *N*-ethylmaleimide-sensitive factor; P2Y1, P2Y1 purinergic receptors; PAK, p21-activated kinase; PAR3, Protease activated receptor 3; PDZ, PSD95/Disc Large/Zona Occludens; Pick1, protein interacting with PRKCA 1; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PTB, phosphotyrosine-binding; RacGEF, Rac guanine nucleotide exchange factor; RalGDS, Ral guanine nucleotide dissociation stimulators or exchange factors; RAMPS, receptor activity-modifying proteins; Ras, Rat sarcoma;

RGS, regulators of G-protein signalling; Rho, Ras-homologous; SAP97, synapse-associated protein 97; SH3, SRC homology 3 domain; SSTR5, somatostatin receptor subtype 5; TM, transmembrane regions; VELI3, vertebrate lin-7 homolog 3

Introduction

GPCRs represent the largest family of integral membrane proteins with more than 800 GPCR genes identified in the human genome (Luttrell, 2008). These membrane proteins are comprised of seven transmembrane regions (TM), an extracellular amino-terminus and an intracellular carboxyl-terminal domain. GPCRs not only regulate important physiological processes such as neurotransmission and cardiovascular function, but the dysregulation of GPCR activity also contributes to many different pathophysiological processes (Thompson et al., 2008). The importance of GPCRs for the treatment of disease is highlighted by the fact that at least 30–40% of prescription drugs target these proteins (Overington et al., 2006).

Classically, GPCRs have been thought to transduce the information provided by a diverse assortment of stimuli into intracellular second messengers by functioning as ligand-regulated guanine nucleotide exchange factors for a family of heterotrimeric GTP-binding proteins (G-proteins). In this model, either monomeric or dimeric GPCRs bind agonist and promote the ability of a GPCR to adopt an active conformation that facilitates the exchange of GDP for GTP on the G-protein Gα-subunit, leading to the dissociation of the Gα and Gβγ-subunits (Neer, 1995). Subsequently, the activated G-protein subunits can either positively or negatively regulate a variety of downstream effectors such as phospholipases, adenylyl cyclases and ion channels (Neer, 1995). However, it is now clear that GPCRs can signal via G-protein-independent mechanisms, the best characterized of which is β-arrestin-mediated signalling via Src and mitogen-activated kinase pathways (Ferguson, 2001; Lefkowitz et al., 2006; Luttrell, 2008). However, it is now appreciated that GPCRs also mediate cell signalling by functioning as scaffolds for the recruitment of GPCR interacting proteins (either transmembrane or cytosolic proteins), and through their association with GPCRs, these proteins modulate GPCR function and signal transduction (Bockaert et al., 2010). GPCR interacting proteins play important roles in regulating receptor ligand specificity (e.g. receptor activity modulating proteins), receptor endocytosis (e.g. β-arrestins, Arf6, RalA, phospholipase D2), expression at the cell surface (e.g. post-synaptic density protein 95, PSD95), and receptor recycling (e.g. synapse-associated protein 97, SAP97) (Bhattacharya et al., 2004; Bockaert et al., 2004; 2010; Ferguson, 2007). Thus, by virtue of their ability to modulate the localization of GPCRs to specific intracellular membrane compartments and scaffold multiprotein complexes that have been referred to as 'signalsomes', GPCR interacting proteins fine tune GPCR pharmacology and signal transduction. Recent research in the field has also focused extensively on how biased ligands bind to the extracellular face of GPCRs to selectively activate distinct intracellular signal transduction pathways and how receptor oligomerization modulates receptor pharmacology and signal transduction properties and there are several excellent reviews that overview this topic in detail (e.g. Bouvier, 2001;

Terrillon and Bouvier, 2004; Galandrin et al., 2007; Kenakin, 2007; Rajagopal et al., 2010). Protein interactions with the intracellular face of GPCRs provide additional strategies for the development of novel therapeutic agents that selectively target the inactivation of specific intracellular signalling pathways downstream of the activation of GPCRs. However, the development of these agents has been hampered due to the complexity of introducing drugs into the cell. The current review will overview the role of GPCR interacting proteins in the regulation of the pharmacology, signalling and subcellular localization of GPCRs and will overview new mechanisms by which GPCR interacting proteins regulate GPCR function that might provide additional prospects for drug development.

GRK/arrestins

The first proteins identified as GPCR interacting proteins involved in the regulation of GPCR/G-protein coupling were the GPCR kinases (GRKs) and arrestins (reviewed in Krupnick and Benovic, 1998; Lefkowitz, 1998; Ferguson, 2001; Penela et al., 2003; Gainetdinov et al., 2004; Lefkowitz and Shenoy, 2005; Premont and Gainetdinov, 2007; Luttrell, 2008; Tobin et al., 2008). GRKs play a central role in regulating (i) the desensitization (uncoupling) of GPCR/G-protein signalling, (ii) the endocytosis of GPCRs to endosomes to allow GPCR dephosphorylation and resensitization, and (iii) GPCR signalling via G-protein-independent mechanisms.

Receptor desensitization

Mammals express seven GRK (GRK1–GRK7) and four arrestin isoforms, of which GRK1, GRK7, arrestin1 and arrestin4 are specifically localized to the visual system (Premont et al., 1995; Pitcher et al., 1998; Ferguson, 2001). Of the remaining GRK and arrestin proteins, GRK2, GRK3, GRK5, GRK6, arrestin2 (β-arrestin1) and arrestin3 (β-arrestin2) are ubiquitously expressed. GRKs are comprised of three function domains, an amino-terminal regulator of G-protein signalling (RGS) homology (RH) domain, a central catalytic domain and carboxyl-terminal membrane targeting domain. GRKs are targeted to the plasma membrane through multiple mechanisms to phosphorylate serine and threonine residues within the third intracellular loop and carboxyl-terminal tail domains of agonist-activated receptors. GRK2 and GRK3 encode pleckstrin homology domains at their carboxyl-termini that allows them to associate with the Gβγ subunit of hetereotrimeric G-proteins, as well as phosphatidylinositol 4,5-bisphosphate (Koch et al., 1993; Touhara et al., 1994; Pitcher et al., 1995). The light-activated association of GRK1 and GRK7 with the plasma membrane is facilitated by the post-translational farnesylation of its carboxyl-terminal CAAX motif (Inglese et al., 1992), whereas GRK4 and GRK6 are associated with the plasma membrane following palmitoylation (Stoffel et al., 1994; 1998; Premont et al., 1996), and
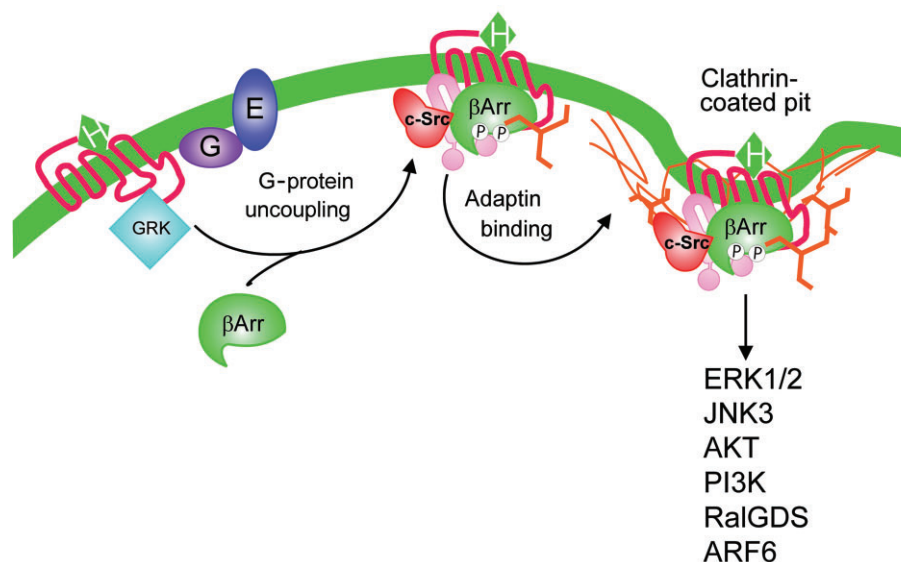
Figure 1

β-Arrestin-dependent endocytosis and signalling of GPCRs. Agonist activation promotes the GRK2-mediated phosphorylation that promotes the translocation and binding of β-arrestins, which serves to uncouple receptors from heterotrimeric G-proteins. β-Arrestins function as adaptor proteins that interact with both clathrin and β2-adaptin promoting the clathrin coated vesicle-mediated endocytosis of many GPCRs. c-Src is recruited to GPCRs as a consequence of its interaction with β-arrestin and couples the receptor to the MAPK pathway. β-Arrestin interactions with a variety of proteins allows for the coupling of GPCRs to a variety of different signal transduction pathways whose activation may be independent of heterotrimeric G-proteins. β-Arr, β-arrestin; P, phosphate.

GRK5 contains a polybasic domain that allows its association with membrane phospholipids (Kunapuli *et al.*, 1994; Premont *et al.*, 1994). For many GPCRs, GRK-mediated phosphorylation on its own is insufficient to mediate the desensitization of many GPCRs. Instead, the recruitment of arrestin proteins to agonist-activated and GRK-phosphorylated GPCRs facilitates the uncoupling of the receptor from hetereotrimeric G-proteins. However, for $G\alpha_{q/11}$-coupled GPCRs, phosphorylation-independent desensitization can be achieved as the consequence of the displacement of $G\alpha_{q/11}$ from the receptor complex by the RH domains of GRK2 and GRK3 (reviewed by Dhami and Ferguson, 2006; Ferguson, 2007). Thus, GRKs regulate GPCR desensitization by both phosphorylation-dependent and -independent mechanisms.

Receptor endocytosis

The endocytosis of many GPCRs appears to be mediated by the same mechanism that is required for GPCR desensitization. GRK-mediated phosphorylation promotes the binding of β-arrestins, which function as endocytic adaptor proteins that facilitate the targeting of receptors for clathrin-mediated endocytosis (Ferguson *et al.*, 1996; Goodman *et al.*, 1996) (Figure 1). Both β-arrestin1 and β-arrestin2 specifically bind to both the clathrin heavy chain and the β2-adaptin subunit of the heterotetrameric adaptor complex to facilitate endocytosis (Goodman *et al.*, 1996; Laporte *et al.*, 1998). In addition, Src-mediated phosphorylation of β2-adaptin regulates the dissociation of the β2-adaptin/β-arrestin complex (Zimmerman *et al.*, 2009). The β-arrestin domain involved in clathrin binding is localized to amino acid residues 373–377 in the carboxyl-terminus of β-arrestin2 (Krupnick *et al.*, 1997). Two

arginine residues (R394 and R396) in β-arrestin2 mediate binding to β2-adaptin *in vitro* (Laporte *et al.*, 2000). β-Arrestins also interact with a variety of other protein complexes that have been implicated in the regulation of clathrin-mediated endocytosis including E3 ubiqitin ligases such as mdm2 (Shenoy *et al.*, 2001). Thus, β-arrestins play an essential role in recruiting proteins that are not only essential for the internalization of GPCRs but also for the regulation of the endocytic machinery.

There are striking differences in the ability of different arrestin isoforms to bind different GPCRs at the plasma membrane (Oakley *et al.*, 2000). Early work indicated that there were two classes of GPCRs: (i) GPCRs that preferentially bind β-arrestin2 with lower affinity and do not internalize as a complex with β-arrestin2 to endosomes and (ii) GPCRs that bind β-arrestins with high affinity and form a stable complex with β-arrestins allowing the receptor to internalize as a complex with β-arrestins to endosomes. This stable association of β-arrestins with many GPCRs is due the phosphorylation of a cluster of serine residues localized a precise distance from the seventh transmembrane domain of the receptor. In general, GPCRs that do not internalize as a complex with β-arrestins are dephosphorylated, resensitized and rapidly recycled to the plasma membrane, whereas receptors that internalize with β-arrestin bound are either inefficiently recycled back to the plasma membrane or are not recycled at all (Ferguson, 2001; Luttrell, 2008). In the case of protease-activated receptors, the carboxyl-terminal tail regulates targeting to lysosomes (Trejo *et al.*, 1998), due to the association of the receptor with sorting nexin 1 (Gullapalli *et al.*, 2006). In addition, the activation of some receptors,
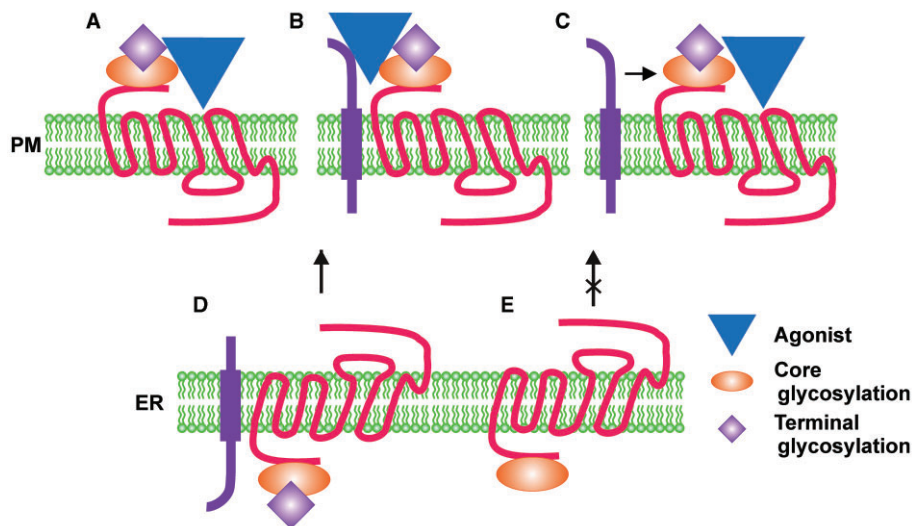
Figure 2

RAMP protein regulation of calcitonin receptor family cell surface expression and pharmacology. (A) RAMP proteins facilitate the terminal glycosylation of calcitonin receptors and glycosylation may contribute to the receptor ligand binding site to regulate ligand binding specificity. (B) RAMP proteins contribute to the calcitonin receptor family ligand binding site to regulate ligand specificity. (C) RAMP proteins interact with and allosterically modulate the ligand binding specificity of calcitonin receptor family members. (D) RAMP proteins facilitate the terminal glycosylation of calcitonin receptors to facilitate their processing in the ER and transport to the cell surface. (E) In the absence of appropriate RAMP protein expression calcitonin receptors are retained in the ER and are not transported to the cell surface.

such as the 5-HT$_{2A}$R, corticotropin releasing factor receptor 1 (CRFR1), and CXCR4 results in the redistribution of β-arrestins to vesicular populations that do not contain the receptor proteins themselves (Barlic *et al.*, 2001; Bhatnagar *et al.*, 2001; Holmes *et al.*, 2006). The physiological consequence of this differential localization of β-arrestin and GPCR proteins is unknown, except that β-arrestin redistribution to azruophilic granules is associated with Src family kinase activation and granule release (Barlic *et al.*, 2001).

G-protein-independent signalling

In addition to functioning as adaptor proteins to facilitate the endocytosis of GPCRs, β-arrestins have been demonstrated to scaffold a wide variety of signalling complexes (Reiter and Lefkowitz, 2006; Luttrell, 2008; Luttrell and Gesty-Palmer, 2010; Rajagopal *et al.*, 2010). Initial studies demonstrated that β-arrestins interact with Src family kinases via a polyproline motif and couple the receptor to MAPK ERK1/2 pathways (Luttrell *et al.*, 1999; Barlic *et al.*, 2001). Subsequently, β-arrestins have been demonstrated to bind to a wide variety of kinases, small GTPases, guanine nucleotide exchange factors, E3 ubiqutin ligases, phosphodiesterases and transcription factors (Lefkowtiz and Reiter, 2006; Luttrell, 2008; Getsy-Palmer and Luttrell, 2008; Rajagopal *et al.*, 2010). There is now extensive evidence indicating that ligands that interact with GPCRs can selectively activate G-protein- versus β-arrestin-mediated signalling pathways by facilitating the formation of distinct receptor conformational states required for receptor association with either heterotrimeric G-proteins or β-arrestins. This concept has been extensively reviewed elsewhere (see Reiter and Lefkowitz, 2006; Galandrin *et al.*, 2007; Gesty-Palmer and Luttrell, 2008; Rajagopal *et al.*, 2010).

Receptor activity-modifying proteins (RAMPS)

RAMPS are single transmembrane spanning GPCR accessory proteins that function to modify the expression, and pharmacology of calcitonin receptor and calcitonin-like receptor (CRLR) (McLatchie *et al.*, 1998). The calcitonin receptor and CRLR interact with several cacitonin family peptides including calcitonin, calcitonin gene related peptides (cGRP1 and cGRP2), amylin and adrenomedulin, which have overlapping but diverse physiological functions (Parameswaran and Spielman, 2006). The first RAMP protein was identified by expression cloning in *Xenopus* oocytes because the heterologous expression of either the calcitonin receptor or CRLR in cell lines failed to recapitulate the calcitonin peptide pharmacology observed in native tissues (McLatchie *et al.*, 1998). RAMP1 is a 148-amino-acid protein that is comprised of a large extracellular domain, a single transmembrane domain and a short intracellular carboxyl-terminus. Database searches revealed the existence of an additional two members of the RAMP family, RAMP2 and RAMP3. RAMPs allosterically interact with the calcitonin receptor and CRLR, and the association of four distinct calcitonin peptides with the calcitonin receptors and RAMPs gives rise to seven distinct receptor phenotypes.

RAMPs appear to allosterically influence the structure of calcitonin family receptors allowing for their terminal glycosylation in the endoplasmic reticulum, thereby facilitating their expression at the cell surface (McLatchie *et al.*, 1998) (Figure 2). In addition, RAMPs influence the pharmacology of calcitonin family peptides by one of three potential mechanism(s). First, RAMPs may allosterically influence the

structure of calcitonin family receptors resulting in alterations in receptor/ligand specificity. Second, RAMPs may contribute to the ligand binding site for calcitonin family receptors, and thus, different receptor and RAMP combinations may dictate ligand binding specificity. Finally, RAMP-regulated terminal glycosylation of calcitonin family receptors may influence the specificity of calcitonin peptide binding. RAMP proteins may also influence calcitonin family receptor trafficking via PSD95/Disc large/Zona Occludens (PDZ) protein interactions with a PDZ domain binding motif localized at the end of the carboxyl-terminal tail of RAMP3. Specifically, RAMP3 interacts with N-ethylmaleimide-sensitive factor (NSF) to regulate the agonist-stimulated recycling of the CRLR (Bomberger et al., 2005a; Kuwasako et al., 2006) and Na$^+$/H$^+$ exchanger regulatory factor 1 (NHERF), which is involved in regulating the endocytosis of CRLR (Bomberger et al., 2005b). Thus, RAMP proteins not only regulate the pharmacology of these GPCRs but also the intracellular trafficking and post-translational modification of the receptors that are essential for the regulation of signal transduction via these receptors.

It is now recognized that the role for RAMPs in the regulation of GPCR signalling may extend beyond the calcitonin receptor family to class C receptors. Bouschet et al. (2005) have demonstrated that the calcium sensing receptor (CaSR) is not transported to the plasma membrane unless either RAMP1 or RAMP3 is co-expressed with the receptor. Similar to what is observed for calcitonin family receptors, RAMP1 and RAMP3 influence the trafficking of the CaSR to the plasma membrane from the endoplasmic reticulum, as well as influences the terminal glycosylation of the receptor. Thus, it is possible that the effect of RAMP proteins on GPCR trafficking and pharmacology may be more diverse than originally envisaged.

Regulators of G-protein signalling (RGS)

RGS proteins comprise a group of proteins that modulates GPCR signalling by acting as GTPase activating proteins (GAPs) and thus accelerates the hydrolysis of GTP bound to the Gα subunit of G_q and $G_{i/o}$ proteins, causing their inactivation. Therefore, RGS can terminate G-protein signalling after agonist stimulation (reviewed in Nunn et al., 2006). Common to all RGS proteins is a highly conserved region, the RGS box, with around 130 amino acids, which is sufficient and critical for GAP activity. The sequence flanking the RGS box is different for each RGS protein and may contain different motifs enabling a variety of protein–protein interactions (reviewed in Sjögren and Neubig, 2010). The presence of different domains other than the RGS box suggests these proteins have other functions that are not related to GAP activity such as the regulation of protein localization and intracellular trafficking as well as receptor selectivity (reviewed in Sethakorn et al., 2010).

The RGS proteins are divided into four classic groups (R4, R7, R12 and RZ) based on sequence homology (Ross and Wilkie, 2000). The group R4 (RGS 1–5, 8, 13, 16, 18 and 21) is represented by small molecules (except for RGS3) with short N- and C-flanking regions. Nonetheless, these small proteins are able to bind other proteins and seem to present functions beyond the modulation of GAP activity (reviewed in Bansal et al., 2007). RGS proteins in this group present a membrane targeting signal at the N-terminus, which is important to localize them in the proximity of the membrane together with their targets (activated Gα-proteins) (Bernstein et al., 2000). Besides its GAP activity, a sequence in the N-terminus of the RGS2 proteins was shown to bind directly to tubulin, enhancing microtubule polymerization in vitro (Heo et al., 2006). Furthermore, overexpression of RGS2 increased neurite outgrowth in PC-12 cells, suggesting that RGS2 may contribute to cell differentiation by regulating tubulin dynamics. It has been also shown that the amino-terminal sequence of some R4 members, such as RGS4 may regulate GPCR signalling independent of its GAP activity. In fact, there is some evidence showing inhibition of G_q-mediated calcium signalling by the RGS4 amino-terminus sequence (Zeng et al., 1998), and consistent with that, recent studies show direct interaction of RGS proteins to the third intracellular loop of GPCRs (Bernstein et al., 2004; Hague et al., 2005).

The R7 group of RGS proteins consists of four highly homologous proteins, RGS 6, 7, 9 and 11, predominantly expressed in the nervous system (Gold et al., 1997). These proteins can be divided in three domains: the RGS domain, a GGL domain capable of recruiting $G_{\beta 5}$ and a DEP/DHEX domain that binds to membrane anchor proteins R7BP and R9AP (reviewed in Anderson et al., 2009; Sjögren and Neubig, 2010). The RGS domain is located at the C-terminus and is the only catalytic domain (GTP hydrolysis) localized within these proteins. The DEP (Disheveled, Egl-10, Pleckstrin) and DHEX (DEP helical extension) domains are located at the N-terminus of the R7 RGS proteins. The DEP domain is important for protein-protein interactions and anchoring RGS proteins at the membrane near their site of action (Drenan et al., 2006; Jayaraman et al., 2009). It also interacts directly with GPCRs and recruits effectors to the receptor, as well as modulates the nature, duration and specificity of GPCR signalling (Ballon et al., 2006). The GGL domain (G-protein gamma like domain) is homologous to the γ subunit of G-proteins and is able to bind a G_β subunit (just like a conventional G-protein γ subunit). However, the R7 RGS GGL domain specifically interacts with the $G_{\beta 5}$ subunit (Snow et al., 1998a; Makino et al., 1999). It is known that the R7 RGS–$G_{\beta 5}$ complex is crucial for R7 RGS proteins expression and stability, and $G_{\beta 5}$ knockout animals show total loss of all R7 RGS proteins (Chen et al., 2003). Accumulated data support the idea that these two molecules exist in a complex and function as a single entity to regulate GAP properties of R7 RGS proteins (He et al., 2000; Skiba et al., 2001).

The R12 family is represented by RGS 12 and 14. They contain additional functional domains such as a PDZ binding motif, a GoLoco motif and Rap binding domain at the very C-terminal end of the proteins. At the amino-terminus, these proteins present a PDZ domain and a phosphotyrosine-binding (PTB) domain, both of which promote protein–protein interactions (Ross and Wilkie, 2000; Martin-McCaffrey et al., 2005). The PTB domain can associate with tyrosine-phosphorylated N-type calcium channels, therefore regulating calcium signalling (Schiff

et al., 2000; Richman *et al.*, 2005). The amino-terminal PDZ domain is reported to associate with either the interleukin-8 receptor B (CXCR2) or its own C-terminal PDZ binding motif (Snow *et al.*, 1998b). The GoLoco motif has a guanine nucleotide dissociation inhibitor activity and when bound to $G_{\alpha i1}$, $G_{\alpha i2}$ and $G_{\alpha i3}$ leads to inhibition of the activation of G-protein by preventing exchange of GDP for GTP (Kimple *et al.*, 2001; Siderovski and Willard, 2005).

The investigation of RGS protein activity suggests that their functions go beyond GAP activity and accumulating data in the past 15 years show the importance of RGS proteins for regulation of GPCR signalling. Moreover, the amino-termini of these proteins represents potential sites for interaction with both GPCRs and other signal transduction proteins, suggesting the formation of protein complexes that could contribute to the regulation and generation of GPCR-generated signals. The development of drugs targeting those interactions has a great potential and may produce a new class of therapeutic agents likely to be more specific since RGS proteins regulatory mechanisms may be specific for different cellular environments.

GPCR-associated sorting proteins (GASPs)

The GASP family is comprised of 10 proteins with significant sequence similarity, particularly in the C-terminus where they share a conserved 250-amino-acid residue domain. GASP proteins interact with GPCRs. For example, GASP-1 interact with the C tails of more than 30 GPCRs including dopamine D2 and D4, δ opioid and mGluR1a receptors (Whistler *et al.*, 2002; Heydorn *et al.*, 2004; Bartlett *et al.*, 2005). GASPs have also been shown to interact with proteins that are not GPCRs, but the physiological relevance of these interactions is still to be determined. For example, GASP-1 is shown to interact with Period-1, which is one of the proteins involved in the transcription/translation-based auto-regulatory loop of the endogenous master clock (Cermakian and Sassone-Corsi, 2000). This interaction may be relevant, since Period-1 causes translocation of GASP-1 to the nucleus in cultured cells (Matsuki *et al.*, 2001). GASP-2 interacts with huntingtin and four other partners including a nucleocyto-plasmatic protein involved in DNA repair and regulation of transcription (Goehler *et al.*, 2004; Irminger-Finger and Jefford, 2006). The first evidence that GASPs regulate GPCR function came from the study of Whistler *et al.* (2002) showing that GASP-1 is responsible for the rapid degradation of δ opioid, but not μ opioid receptors, following agonist stimulation. This observation suggests that GASPs function to regulate the post-endocytic sorting of GPCRs. The antagonism of D2 dopamine receptor/GASP-1 interactions using neutralizing antibodies results in the attenuation of agonist-stimulated D2 receptor desensitization (Bartlett *et al.*, 2005). These studies suggest that the blockage of GPCR-GASP interactions may lead to increased GPCR signalling in response to sustained agonist stimulation as a consequence of reduced receptor degradation. However, chronic cocaine treatment of a GASP-1 knockout mouse still results in the down-regulation of dopamine and muscarinic receptor responses (Boeuf *et al.*,

2009). This result supports the idea that GASP proteins are involved in post-endocytic sorting, although alternative mechanisms of GPCR sorting in endosomes may be employed in their absence. For example, sorting nexins are likely to play an important role in regulating GPCR trafficking to lysosomes (Marchese *et al.*, 2008). The interactions between these proteins and GPCRs indicate that GPCR trafficking between intracellular compartments is not a passive process but involves a series of protein/protein interactions that can be expected to be highly regulated.

Homer proteins

Homer proteins share a 120-amino-acid amino-terminal domain and are the products of three genes in mammals and are highly expressed in neurons and skeletal muscle (Bockaert *et al.*, 2010). Homer1 exists as three alternative splice variants (Homer1a, b, c), and Homer1a was first identified as an immediate early gene that was up-regulated in response to seizure-induced synaptic activation (Brakeman *et al.*, 1997; Kato *et al.*, 1998; Xiao *et al.*, 1998). The amino-terminus of all Homer proteins encodes an ENA/VASP homology (EVH) domain, which binds to a PP-x-x-F motif found in the carboxyl-terminal tails of both metabotropic glutamate receptors (mGluRs) 1 and 5, as well as the α_{1D} adrenergic receptor (Tu *et al.*, 1998). The long Homer isoforms encode a carboxyl-terminal coiled-coiled domain that is organized into two separate regions, CC1 and CC2 (Hayashi *et al.*, 2006). The Homer EVH domain also interacts with a variety of mGluR effector proteins including inositol 1,4,5 triphosphate receptors, ryanodine receptors, transient receptor channels 1 and 4, P/Q type Ca^{2+} channels, Shank, phosphoinositide 3-kinase enhancer long, dynamin III, the actin cytoskeleton and activated Rho family GTPases (Tu *et al.*, 1998; 1999; Shiraishi *et al.*, 1999; Kammermeier *et al.*, 2000; Feng *et al.*, 2002; Gray *et al.*, 2003; Rong *et al.*, 2003; Yuan *et al.*, 2003).

The long isoforms of Homer are constitutively expressed and function as scaffolds that regulate their substrates by cross-linking them in the post-synaptic density via the dimerization of the Homer coiled-coiled domains (Hayashi *et al.*, 2006). Homer1a lacks a carboxyl-terminal coiled-coiled domain, and in response to synaptic activity, its up-regulation is thought to disrupt protein complexes that are scaffolded by the Homer long forms (Kammermeier and Worley, 2007). The overexpression of Homer1b leads to the retention of mGluRs in the endoplasmic reticulum and cooperates with Shank to accumulate mGluRs at synapses in a co-cluster with PSD-95 and GKAP (Roche *et al.*, 1999; Tu *et al.*, 1999) (Figure 3). Moreover, Homer/Shank interactions play a role in regulating synaptic spine morphology (Sala *et al.*, 2001). Homer1c also encodes a leucine zipper following the coiled-coiled domains that is involved in the clustering of mGluRs (Tadokoro *et al.*, 1999). Homer1b/c facilitates the localization and clustering of mGluRs in dendrites, whereas overexpression of Homer1a disrupts the clusters and leads to the myslocalization of mGluRs to axons (Ango *et al.*, 2000). Homer1b/c proteins have also been implicated in coupling mGluR5 to extracellular signal-regulated protein kinase cascades in neurons (Mao *et al.*, 2005a). Homer3 is unique in
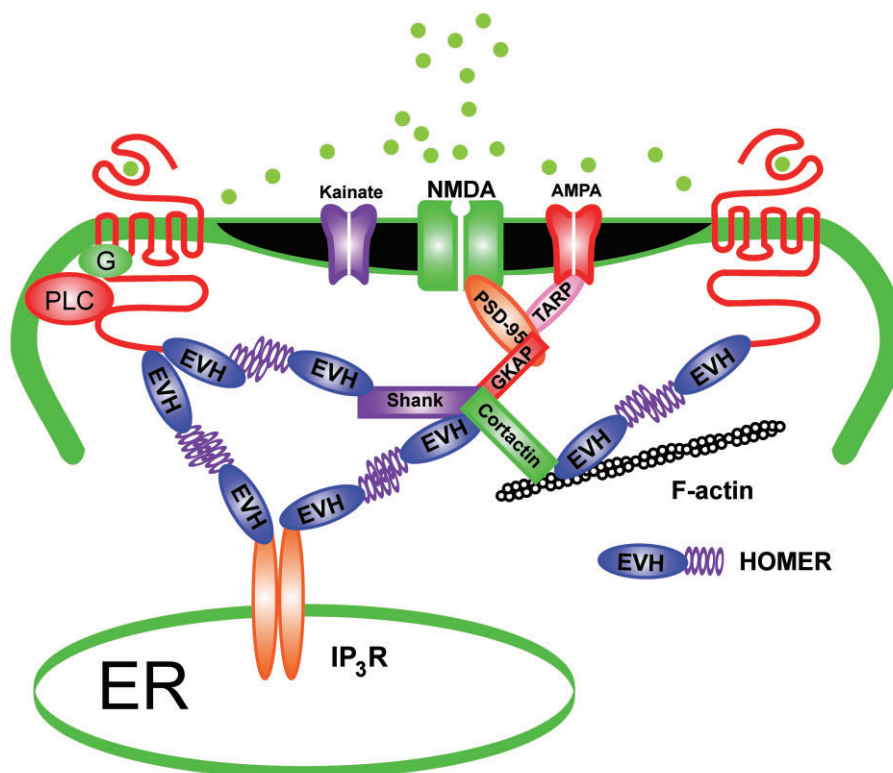
Figure 3

Homer proteins function to regulate the association of protein complexes in the post-synaptic density. The Homer Ena/Vasp homology (EVH) domain as a consequence of the dimerization of their coiled-coiled domains couple mGluR1 and mGluR5 to inositol 1,4,5 triphosphate receptors and NMDA receptors via their interaction with SHANK which interacts with a protein complex involving GKAP and PSD95. The AMPA receptor is coupled to PSD95 via an interaction with TARP. The Homer EVH domain also couples mGluRs to the regulation of the actin cytoskeleton.

that it binds to the carboxyl-terminal tails of group I mGluRs and functions to antagonize their intrinsic constitutive activity (Ango *et al.*, 2001). Consistent with the role of Homers in regulating the activity and clustering of mGluRs at synapses, disruption of mGluR/Homer complexes is associated with impaired synaptic plasticity and is observed following chronic cocaine administration (Swanson *et al.*, 2001; Szumlinski *et al.*, 2004; Ronesi and Huber, 2008). Consequently, the disruption of mGluR/Homer complexes has been implicated in Fragile X, schizophrenia, anxiety, addiction and epilepsy (Polese *et al.*, 2002; Potschka *et al.*, 2002; Szumlinski *et al.*, 2004; Giuffrida *et al.*, 2005; Ronesi and Huber, 2008; Spellmann *et al.*, 2011).

Small G-proteins

The small GTP-binding protein superfamily contains over 100 members that are generally classified by structural similarity into five subfamilies: Ras family GTPases (e.g. Ras, Rap and Ral), Rho family GTPases (Rho, Rac and cdc42), Arf family GTPases (Arf 1–6, Arl 1–7 and Sar), Rab family GTPases (>60 members, e.g. Rab5) and Ran family GTPases (Takai *et al.*, 2001). Rho family GTPases function as regulators of the actin cytoskeleton and can also influence gene transcription,

whereas Rab and Arf family GTPases control the formation, fusion and movement of vesicular traffic between different membrane compartments of the cell. The activation of small GTPases results in their conversion from a GDP-bound 'inactive' state to a GTP-bound 'activated' state. This conversion requires the dissociation of GDP from the small G-protein a process that is facilitated by guanine nucleotide exchange factors (GEFs).

Arf/PLD2

The first evidence that small GTPases might interact directly with GPCRs comes from a study by Mitchell *et al.* (1998) where Arf1/3 and Rho were shown to co-immunoprecipitate with m3 muscarinic acetylcholine receptors in response to agonist activation. GPCRs that form a complex with Arf1/3 and Rho exhibit the capacity to activate phospholipase D (PLD) in a manner that is independent of $G_{q/11}$ and $G_{i/o}$ heterotrimeric G-proteins. In addition, Arf1 and Arf6 are implicated in the regulation of GPCR endocytosis (Claing *et al.*, 2001; Koch *et al.*, 2003; Houndolo *et al.*, 2005). Arf6 plays an essential role in regulating the endocytosis of the β_2-adrenergic receptor (β_2AR), angiotensin II type 1 receptor (AT_1R) and vasopression receptor (Claing *et al.*, 2001; Houndolo *et al.*, 2005). The activation of the β_2AR has been demonstrated to result in the formation of a complex between
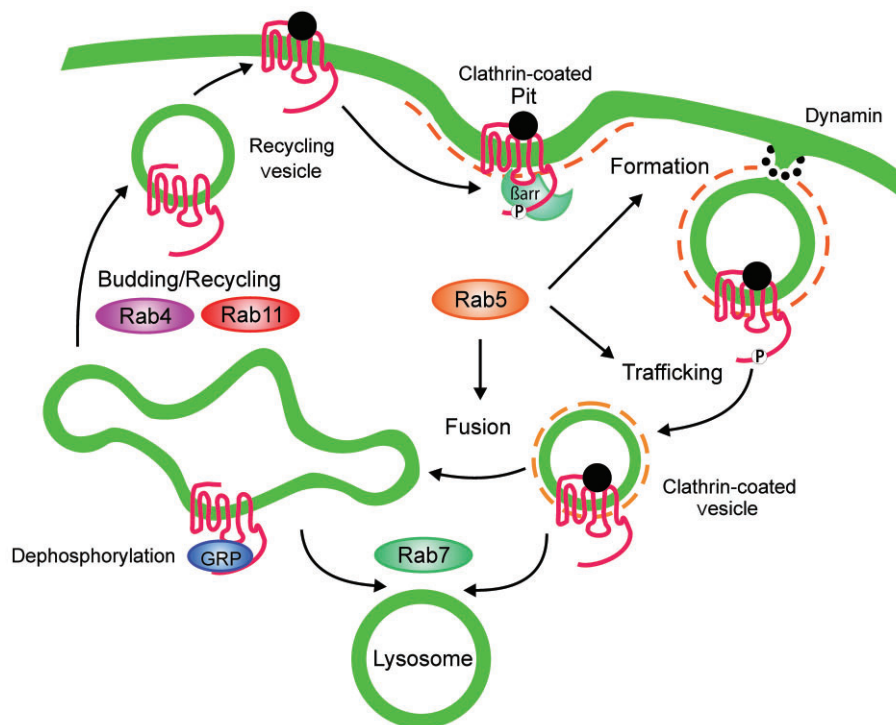
Figure 4

Role of Rab GTPases in regulating GPCR trafficking. Rab5 plays a role in the formation, trafficking and fusion of endocytic vesicles with the early endosome. Rab5 interacts with the AT_1R, which regulates Rab5 activity. Rab4 regulates the rapid recycling of vesicles containing GPCRs to the cell surface, whereas Rab11 regulates slow vesicular recycling. Rab7 plays a role in targeting GPCRs to the late endosome and lysosomes. Each of these Rab GTPases exhibits the capcity to interact with GPCR cargo proteins.

β-arrestin, Arf6 and the Arf6 GEF, ARNO, and that the activation of Arf6 is essential for $β_2AR$ internalization (Claing et al., 2001). More recently, in studies examining the role of Arf6 in the regulation of AT_1R endocytosis, Poupart et al. (2007) demonstrated that Arf6 plays a role in regulating the recruitment of clathrin and β2-adaptin to activated AT_1Rs during the endocytic process. Although Arf1 dominant-negative mutants have no effect on the internalization of the $β_2AR$, there is evidence linking Arf1 to the regulation of μ-opioid receptor endocytosis (Koch et al., 2003). Yeast two-hybrid screening with the C-tail of the μ-opioid receptor identified PLD2 as a μ-opioid receptor interacting protein that could also be co-immunoprecipitated with the receptor in a complex with Arf1. μ-Opioid receptor-mediated activation of PLD2 is also dependent on Arf1 activity. PLD2 activation results in the activation of the p38 MAPK pathway, resulting in the phosphorylation of a Rab5 effector, effector early endosome antigen 1 (EEA1), which is essential for μ opioid receptor internalization (Yang et al., 2010a). PLD2 activity also regulates AT_1R internalization (Du et al., 2004). Thus, GPCR endocytosis is both mediated and regulated by protein interactions that are both dependent and independent of β-arrestins.

Ral/PLD2

Bhattacharya et al. (2004) have shown that the constitutive endocytosis of mGluR1a and mGluR5 is mediated by a RalA/

PLD2-mediated pathway. Group I mGluRs scaffold a constitutive protein complex that contains Ral, Ral guanine nucleotide dissociation stimulator (RalGDS) and PLD2. This complex regulates the constitutive internalization of group I mGluRs in both heterologous cell cultures and primary cortical neurons. Constitutive mGluR internalization is dependent upon PLD2, but not PLD1, activity and requires PLD2-dependent phosphatidic acid formation. Recently, it has been demonstrated that phosphatidic acid plays a regulatory role in clathrin-coated vesicle formation and is involved in receptor mediated endocytosis (Antonescu et al., 2010). RalA has also been shown to interact with the AT_1R receptor by bioluminescence energy transfer and provides a novel mechanism for the coupling of this receptor to the activation of phospholipase C (PLC) δ1 (Godin et al., 2010). Thus, PLD and RalA not only contribute to the regulation of GPCR endocytosis, they may also function as GPCR effector proteins.

Rab GTPases

Multiple Rab GTPases, such as Rab1, Rab4, Rab5, Rab7 and Rab11, have been identified to regulate ER–Golgi transport as well as the endocytosis and trafficking of GPCRs between early, late and recycling endosomes and lysosomes (Figure 4). Several groups have investigated the role of Rab GTPases in regulating the endocytosis and recycling of GPCRs, and it is now recognized that many Rab GTPases interact directly with the carboxyl-terminal tails of a number of GPCRs (Seachrist

et al., 2002; Hamelin *et al.*, 2005; Parent *et al.*, 2009; Reid *et al.*, 2010; Esseltine *et al.*, 2011). The first indication that Rab GTPases could interact directly with a GPCR came arose from the identification of Rab5a as a protein that interacts with the $AT_{1A}R$ C-tail by yeast two-hybrid (Seachrist *et al.*, 2002). Rab5 plays a role in regulating the formation, trafficking and fusion of clathrin-coated vesicles in the early endosome (Zerial and McBride, 2001). Rab5 can be co-immunoprecipitated with the AT_1R and agonist activation leads to the exchange of GDP for GTP on Rab5, suggesting that the receptor can function as a Rab5 GEF. The interaction between Rab5 and the AT_1R results in the retention of the receptor in homotypic endosomal vesicles. The AT_1R Rab5 binding site is bipartite and involves amino acid residues within both the membrane proximal region of the AT_1R carboxyl-terminal tail and the last 10 amino acids of the carboxyl-terminal tail. More recently, it has been reported that Rab4, Rab5, Rab7 and Rab11 all compete for binding to the AT_1R, and that proline residue 354 and cysteine residue 355 represent important residues involved in Rab protein binding (Esseltine *et al.*, 2011). Rab4, a Rab GTPase involved in the rapid recycling of vesicles from the endosome to the plasma membrane promotes AT_1R dephosphorylation and the overexpression of a constitutively active Rab4 mutant, enhances AT_1R resensitization. Several other GPCRs are reported to interact with Rab GTPases (Hamelin *et al.*, 2005; Parent *et al.*, 2009; Reid *et al.*, 2010). However, the residues that are identified to be essential for Rab GTPase binding to the AT_1R are not conserved in any of these GPCRs. For example, residues 335–345 within the central region the thromboxane A2 receptor carboxyl-terminal tail are required for Rab11 binding, whereas α-helix 8 at the membrane proximal end of the prostacyclin receptor binds Rab11 (Hamelin *et al.*, 2005; Reid *et al.*, 2010). In contrast, Rab11 binding to the β_2AR involves a bipartite binding motif, with arginine 333 and lysine 348 representing the essential amino acid residues mediating Rab11 binding to the receptor (Parent *et al.*, 2009). To date, there is no clearly defined consensus sequence for Rab GTPase binding to GPCRs. Thus, multiple Rab GTPases are able to associate with their cargo GPCRs, and the activity of these receptors can be differentially regulated via their association with these Rab GTPases.

PDZ domain protein interactions

PDZ proteins contain multiple PDZ domains that consist of an 80–90 amino acid sequence folded in a globular structure, and PDZ domains bind to specific sequences at the very carboxyl-termini of their interacting protein called PDZ binding motifs. These motifs are constituted of three to four amino acids and represent the minimal sequence that binds to PDZ proteins. The PDZ binding motif can be divided into classes I, II and III depending on their sequence. Class I motifs show a -S/T-x-V/I/L, class II motifs present a -Φ-x-Φ- and class III motifs present a -ψ-x-Φ- sequence where Φ represents an hydrophobic and ψ represents an acidic amino acid (Sheng and Sala, 2001). Many GPCRs contain a PDZ binding motif at their C-terminus enabling PDZ proteins to associate and scaffold multiprotein complexes, which can modulate different receptor properties such as trafficking, signalling, receptor stability and cell distribution (Table 1).

PDZ proteins and GPCR signalling

$GABA_B$ receptors can associate with the PDZ protein MUPP1, and this interaction enhances receptor signalling either by affecting G-protein coupling or by affecting the receptor interaction with other proteins (Balasubramanian *et al.*, 2007). MUPP1 association with $GABA_B$ receptors results in fine tuning of receptor signalling with potential relevance to diseases such as epilepsy. MUPP1 also binds to the melatonin $(MT)_1$ receptor, destabilizing receptor-G_i protein interaction, and abolishing G_i-mediated signalling of MT_1 receptor (Guillaume *et al.*, 2008). It has also been shown that PLC-β can interact with PDZ proteins, and NHERF2 physically connects the lipophosphatidic acid receptor 2 to the activation of PLC-β3, while PAR-3 connects bradykinin receptor to PLC-β1 to generate specific and efficient signalling (Oh *et al.*, 2004; Choi *et al.*, 2010). These findings suggest that each subtype of PLC-β can be selectively coupled to a receptor through a PDZ protein in a given cell type and lead to a specific signalling pathway. The PDZ protein NHERF-2 also interacts with mGluR5 to regulate the calcium signalling of this receptor (Paquet *et al.*, 2006). Both mGluR5 and NHERF-2 are localized in populations of astrocytes and neurons in the CNS, and this interaction may be relevant for the regulation of cellular responses to glutamate. It has also been demonstrated that $P2Y_1$ purinergic receptors ($P2Y_1Rs$) can bind NHERF-2, and this association prolongs $P2Y_1R$-mediated calcium signalling (Fam *et al.*, 2005). The β_1-adrenergic receptor (β_1AR) is known to bind to several PDZ proteins such as CAL, PSD-95, MAGI-2, GIPC and MAGI-3 (He *et al.*, 2006). The interaction of this receptor with MAGI-3 abolished β_1-AR-mediated ERK1/2 activation with no effect on agonist dependent receptor internalization or agonist-stimulated cAMP formation.

PDZ proteins and GPCR trafficking

The β_2AR interacts with the PDZ protein NHERF/EBP50 via its C-terminus PDZ binding motif, and this interaction is required for efficient plasma membrane recycling of the receptor (Hall *et al.*, 1998; Cao *et al.*, 1999). However, receptor recycling can be mediated by multiple PDZ domain containing proteins and is not unique to a single receptor as a β_2AR chimera containing the β_1AR C-tail, which contains a PDZ binding motif that does not bind to NHERF, still effectively recycles (Gage *et al.*, 2005). Thus, modulation of GPCR trafficking by PDZ proteins may be a general mechanism of PDZ protein interactions. In contrast, Wente *et al.* (2005) have demonstrated that the somatostatin receptor subtype 5 (SSTR5) associates with the PDZ protein cystic fibrosis transmembrane conductance regulator-associated ligand (CAL), and this interaction inhibited SSTR5 recycling. In addition, NHERF1 inhibits type I parathyroid hormone receptor (PTH1R) desensitization, and knock down of NHERF1 with shRNA restores normal receptor desensitization (Wang *et al.*, 2009). The serotonin 2C receptor ($5-HT_{2C}R$) contains a carboxyl-terminal PDZ binding motif class I (SSV) and binds to MUPP1 (MUlti PDZ domain Protein 1). This interaction seems to facilitate receptor phosphorylation and resensitization (Ullmer *et al.*, 1998; Becamel *et al.*, 2001; Parker *et al.*, 2003). CAL is a Golgi-associated PDZ protein, and its binding to β_1AR results in the retention of the receptor within the cell (He *et al.*,

Table 1

Functional role of known PDZ protein interactions with GPCRs

PDZ protein	GPCR	Reported effect on GPCR	Reference
CAL	β_1AR	↓ membrane expression	He et al., 2004
(PIST, GOPC, FIG)	mGluR1a	Agonist-induced intracellular co-localization with receptor, ↓ ERK activation	Zhang et al., 2008
	mGluR5a	↑ mGluR5a protein expression, ↓ receptor ubiquitination	Cheng et al., 2010
	SSTR5	Targets receptor to Golgi apparatus (agonist-independent), ↓ membrane-targeting/recycling	Wente et al., 2005
GIPC	β_1AR	↓ ERK activation, no effect on cAMP signalling	Hu et al., 2003
(TIP-2, Synectin)	D_2	Targets receptor to Golgi region	Jeanneteau et al., 2004
	D_3	↓ signal transduction through G_i, targets receptor to Golgi region, ↓ D_3 degradation	Jeanneteau et al., 2004
	hLHR	Maintains receptor cell surface expression during hormone internalization	Hirakawa et al., 2003
MAGI-2	β_1AR	↑ agonist-induced internalization, no effect on cAMP signalling	Xu et al., 2001
(S-SCAM, ARIP-1)	VPAC1	↓ cAMP signalling, ↓ agonist-induced internalization	Gee et al., 2009
MAGI-3	β_1AR	↓ ERK activation	He et al., 2006
	β_2AR	↓ ERK activation	Yang et al., 2010b
	LPA_2	↑ ERK activation, ↑ RhoA activation	Zhang et al., 2007
MPP3	5-HT2c	↑ membrane stability, ↓desensitization	Gavarini et al., 2006
MUPP-1	5-HT2a	↑ localization to cell surface	Jones et al., 2009
	$GABA_B$	↑ Ca^{2+} signalling	Balasubramanian et al., 2007
	MT_1	↑ coupling with G_i protein, ↑ inhibition of adenylyl cyclase activity	Guillaume et al., 2008
	OR2AG1	Modulates Ca^{2+} signalling	Dooley et al., 2009
NHERF1	5-HT4a	Targets receptor to microvilli to interact with ezrin (potential role in cytoskeletal remodelling)	Joubert et al., 2004
(EBP50)	β_2AR	Agonist-induced co-localization with receptor	Hall et al., 1998
	β_2AR	Regulates receptor sorting through ERM-binding domain interactions	Cao et al., 1999
	hKOR	↓ receptor down-regulation, ↑ recycling	Li et al., 2002
	PTH1R	↓ agonist-induced internalization, negligible effect on recycling and cAMP signalling	Wang et al., 2007
	PTH1R	↑ coupling to, and activation of, G_q	Wang et al., 2010
	PTH1R	↓ interaction with β-arrestin-2, ↓ desensitization	Wang et al., 2009
	PTH1R	Targets receptor to membrane regions in close proximity to cytoskeleton, ↑ cAMP signalling	Wheeler et al., 2008
	TPβ	↓ internalization	Rochdi and Parent, 2003
NHERF2	LPA_2	↑ interaction with PLCβ3	Choi et al., 2010
(E3KARP, SIP-1, TKA-1)	LPA_2	↑ IP_3 signalling, ↑ ERK activation	Oh et al., 2004
	mGluR5a	↑ agonist-induced Ca^{2+} signalling	Paquet et al., 2006
	$P2Y_1R$	↑ interaction with PLCβ, ↑ agonist-induced Ca^{2+} signalling	Fam et al., 2005
	PTH1R	↑ interaction with, and activates, PLCβ	Mahon et al., 2002
	PTH1R	↓ adenylyl cyclase activity through stimulation of inhibitory G-proteins ($G_{i/o}$ proteins).	Mahon et al., 2002
	PTH1R	↑ coupling to, and activation of, G_q and G_i; ↓ coupling to, and activation of, G_s	Wang et al., 2010
PAR-3	BK	↑ interaction with PLCβ1	Choi et al., 2010
PDZ-GEF	β_1AR	Required for agonist-induced Ras activation via G_s-mediated cAMP signalling	Pak et al., 2002
(CNrasGEF, RA-GEF, Rap GEP)			
PICK1	mGluR7a	↓ PKCα-mediated receptor phosphorylation	Dev et al., 2000
	PrRP (GPR10)	↑ intracellular clustering of receptor	Lin et al., 2001
PSD-95	5-HT2a	↓ agonist-induced internalization, ↑ IP_3 signalling	Xia et al., 2003b
(DLG4, SAP90)	5-HT2c	↑ constitutive and agonist-induced internalization, ↑ desensitization	Gavarini et al., 2006
	β_1AR	↓ agonist-induced internalization, no effect on cAMP signalling	Hu et al., 2000
	D_1	↑ constitutive internalization, ↓ cAMP signalling	Zhang et al., 2007
	D_1	↑ recycling, ↑ resensitization, no effect on G_s activation or cAMP signalling	Sun et al., 2009
SAP97	β_1AR	↑ PKA-mediated receptor phosphorylation, ↑ recycling	Gardner et al., 2007
(DLG1)			
Tamalin	mGluR1a	↑ membrane expression, ↑ complex formation with cytohesin-2 (GEF)	Kitano et al., 2002
(GRASP)			

2004). PSD-95 can compete with CAL for the binding to β_1AR and function to both promote receptor trafficking to the cell surface and antagonize β_1R endocytosis (Hu *et al.*, 2000; He *et al.*, 2004). β_1AR also strongly binds MAGI-2, and this interaction promotes the internalization of the receptor (Xu *et al.*, 2001).

PDZ proteins and GPCR subcellular distribution

In the mammalian cerebral cortex, $5-HT_{2A}Rs$ are enriched in pyramidal neurons and preferentially distributed in dendrites instead of axons (Jakab and Goldman-Rakic, 1998). Disruption of PDZ binding motif on $5-HT_{2A}R$ receptor greatly decreases targeting of those receptors to dendrites, indicating that the PDZ binding motif is a critical signal for the sorting of $5-HT_{2A}R$ to dendrites in pyramidal neurons (Xia *et al.*, 2003a). The $5-HT_{2A}R$ is present in a subset of dendritic spines and shafts and is colocalized with MUPP1 (Jones *et al.*, 2009). $5-HT_{2A}R$ agonist treatment induces a transient increase in dendritic spine size and phosphorylation of p21-activated kinase (PAK), which is downstream of the neuronal Rac guanine nucleotide exchange factor (RacGEF) Kalirin-7. Kalirin-7 is involved in dendritic spine morphogenesis. And its activity is regulated via its association with PDZ proteins such as PSD-95 and afadin/AF-6. Kalirin-7 interference peptides prevent $5-HT_{2A}R$-mediated PAK phosphorylation and spine morphogenesis, indicating that $5-HT_{2A}R$/PDZ protein interactions contribute to the modulation of spine morphogenesis. PSD-95 is also crucial for $5-HT_{2A}R$ and $5-HT_{2C}R$ cell surface expression, and PSD-95 promotes $5-HT_2R$ sorting to apical dendritic spines and stabilizes receptor turnover *in vivo* (Abbas *et al.*, 2009). Similarly, Tamalin (GRASP) increases the cell surface expression of mGluR1 (Kitano *et al.*, 2002). The interaction of the $\alpha_{1D}AR$ with the PDZ domain containing protein α-syntrophin also regulates $\alpha_{1D}AR$ protein stability, indicating that multiple PDZ domain containing proteins may regulate the life span of GPCRs (Chen *et al.*, 2006). Pick1 interactions with mGluR7 are essential for the pre-synaptic clustering of the receptor (Boudin *et al.*, 2000). Taken together, these observations indicate that PDZ interactions play an important role in the subcellular localization of GPCRs to membrane microdomains.

Specificity of GPCR/PDZ protein interactions

$5-HT_{2A}R$ and $5-HT_{2C}R$ exhibit very similar pharmacological properties and signalling pathways, and both express very similar class I PDZ binding motif at their carboxyl-termini (Bécamel *et al.*, 2002; 2004). However, using a proteomic approach, Bécamel *et al.* (2002; 2004) demonstrated that these receptors interact with an overlapping, yet distinct, set of PDZ proteins. The $5-HT_{2C}R$, but not the $5-HT_{2A}R$, forms a complex with Veli3–CASK–MINT1 complex and to SAP102, whereas both receptors bind to PSD-95, MAGUK and p55 subfamily member 3 (MPP3). In contrast, $5-HT_{2A}R$ effectively interacts with channel-interacting PDZ protein (CIPP), whereas $5-HT_{2C}R$ does not appear to associate with CIPP. Thus, despite similarities in the PDZ binding motifs for $5-HT_{2A}R$ and $5-HT_{2C}R$, these proteins exhibit differences in their substrate specificity, suggesting that amino acid residues upstream of the S/T-x-V/I/L motif may influence the selectivity of PDZ domain interactions.

Many interesting concepts regarding the physiological importance of GPCR/PDZ protein interaction arise within the studies described above. It is well known that a single GPCR has the capacity to associate with multiple different PDZ proteins, and that each of these proteins may modulate different intracellular events that often have opposing functions with regards to trafficking and signalling. However, how the physiological context and cell type favour interactions between specific GPCRs and specific PDZ proteins remains unknown. It is tempting to speculate potential scenarios. It is likely that the association PDZ domain proteins with other accessory proteins may drive the specificity of GPCR/PDZ domain interactions. Alternatively, the activation of GPCRs may modulate changes in the transcription regulation of specific PDZ proteins and may stimulate post-translational modifications of either the GPCR or PDZ domain-containing protein that may dictate the specificity of interaction. Finally, it is possible that different GPCR conformational states might contribute to the regulation of PDZ protein interactions with the receptor. However, most studies regarding PDZ protein interactions with GPCRs are performed in over-expression systems, and the assessment of endogenous proteins with GPCRs will be required to determine how interaction specificity is determined.

Physiological role of GPCR/PDZ protein interactions – 'heterologous GPCR sensitization'

Recently, Magalhaes *et al.* (2010) demonstrated that the activation of the CRFR1 results in the 'heterologous sensitization' of $5-HT_2R$ signalling in both heterologous cell cultures and primary prefrontal cortical neurons. Specifically, pre-stimulation of the CRFR1 for 30 min leads to a significant potentiation of $5-HT_2R$ signal transduction. This CRFR1-mediated sensitization of $5-HT_2R$ signalling is dependent upon CRFR1 endocytosis and receptor recycling via Rab4 positive endosomes that results in the recruitment of an intracellular pool of $5-HT_2R$ to the cell surface (Figure 5). Moreover, the CRFR1-mediated sensitization of $5-HT_2R$ signalling and increase in $5-HT_2R$ cell surface expression are dependent upon intact PDZ protein interactions with both CRFR1 and $5-HT_2R$. However, the identity of the PDZ protein mediating the interaction remains unknown. Both CRFR1 and $5-HT_2Rs$ are associated with stress and anxiety responses, and the pre-administration of corticotrophin-releasing factor (CRF) into the prefrontal cortex of mice enhanced 5-HT2R-mediated anxiety behaviours in response to 2,5-dimethoxy-4-iodoamphetamine. Thus, PDZ protein interactions with GPCRs may serve to regulate functional crosstalk between GPCRs.

Other GPCR interacting proteins

Spinophilin

Spinophilin is a modular protein that is comprised of two F-actin domains, three putative Src homology 3 (SH3) domains, a receptor- and PP1-binding domain, a PDZ domain, three coiled-coiled domains and a potential leucine/isoleucine zipper motif (Sarrouilhe *et al.*, 2006). Spinophilin has been demonstrated to interact with more than 30 differ-
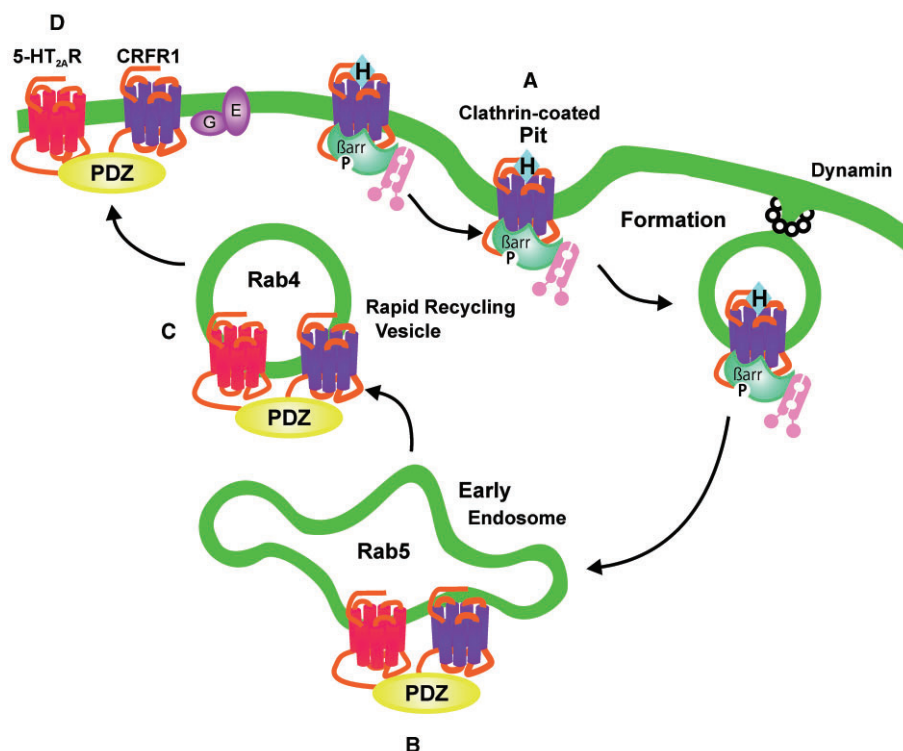
Figure 5

Schema depicting proposed mechanism of CRFR1-mediated sensitization of 5-HT$_2$R signaling. (A) Agonist activation of CRFR1 leads to the β-arrestin and clathrin-dependent endocytosis of CRFR1. (B) CRFR1 is internalized to Rab5-positive endosomes, where it is colocalized with constitutively internalized 5-HT$_2$R. (C) The PDZ binding motifs of both CRFR1 and 5-HT$_2$Rs are required for the Rab4-positive rapid recycling endosome-dependent and CRFR1-facilitated recycling of internalized 5-HT$_2$R to the cell surface via a putative interaction with a PDZ domain containing protein. (D) Due to increased 5-HT$_2$R cell surface expression mediated by CRFR1 activation 5-HT$_2$R signalling is enhanced. H, hormone.

ent proteins including GPCRs and ion channels. Spinophilin also interacts with and regulates the activity of protein phosphatase (PP1). Spinophilin interacts with several GPCRs including the IL3 of the D2 dopamine, α2-adrenergic (α2AR) and μ-opioid receptors (Smith *et al*., 1999; Richman *et al*., 2001; Charlton *et al*., 2008). Following agonist treatment, spinophilin competes with GRK2 for binding to the α2AR, which prevents both β-arrestin recruitment and α2AR endocytosis (Wang *et al*., 2004). Spinophilin also regulates α2AR Ca^{2+} signalling by scaffolding RGS2 in a complex with the receptor (Wang *et al*., 2005). In contrast, spinophilin promotes the endocytosis of the μ-opioid receptor (Charlton *et al*., 2008). Surprisingly, no spinophilin PDZ domain interactions with GPCRs have been reported. Moreover, it has not been reported whether GPCR/spinophilin interactions may contribute to the regulation of spine morphology at synapses.

Protein phosphatases

The resensitization of many GPCRs is dependent upon receptor dephosphorylation in endosomes (Pippig *et al*., 1995; Seachrist *et al*., 2000). One candidate phosphatase for mediating the dephosphorylation of the β$_2$AR is protein phosphatase 2A (PP2A), which has been demonstrated to co-immunoprecipitate with the receptor (Krueger *et al*., 1997). In addition, PP2A binds to the carboxyl-terminal tail

of mGluR5 and functions to antagonize mGluR5-dependent activation of ERK1/2 phosphorylation (Mao *et al*., 2005b). PP1γ is also scaffolded on the carboxyl-terminal tail of mGluR1/5 via a PP1γ binding motif (Croci *et al*., 2003). However, the functional consequence of this interaction remains to be determined. In addition, PP2C binds to the carboxyl-terminal tail of mGluR3 and mediates the dephosphorylation of the receptor (Flajolet *et al*., 2003). However, our overall understanding of how the dephosphorylation of desensitized GPCRs is regulated by protein phosphatase is limited.

Calmodulin

Calmodulin has been shown to bind to the third intracellular loop of the μ-opioid receptor and reduces both constitutive and agonist-stimulated G-protein coupling (Wang *et al*., 1999; 2000). Moreover, single nucleotide polymorphisms within the G-protein coupling domain of the μ-opioid receptor are associated with altered calmodulin binding and increase basal μ-opioid receptor activity (Wang *et al*., 2001). Turner *et al*. (2004) demonstrated the first evidence that calmodulin interacts with the 5-HT$_{1A}$ receptor in intact living cells by BRET. They identified two calmodulin binding sites in the third intracellular loop of the receptor, which also happen to be a protein kinase C (PKC) binding

site. Their results showed that calmodulin and PKC binding to the receptor were antagonistic *in vitro*, suggesting a potential role for calmodulin binding in the modulation of PKC-dependent (heterologous) desensitization of 5-HT$_{1A}$ receptor. Calmodulin was also shown to bind 5-HT$_{2A}$ receptors at two additional sites, one within the second intracellular loop and the other within the C-terminus (Turner and Raymond, 2005). The calmodulin binding to the 5-HT$_{2A}$ receptor C-terminus also antagonized PKC binding, whereas calmodulin binding to the second intracellular loop attenuates 5-HT$_{2A}$ receptor G-protein coupling. Calmodulin also binds to the C-terminus of the 5-HT$_{2C}$ receptor (Bécamel *et al.*, 2002; Labasque *et al.*, 2008). Data obtained with over-expressed protein system (HEK 293 cells), as well as endogenously expressed receptors in the choroid plexus demonstrated that calmodulin binding to the 5-HT$_{2C}$ receptor stabilized a 5-HT$_{2C}$/β-arrestin complex leading to G-protein-independent ERK1/2 signalling (Labasque *et al.*, 2008). mGluR5 also presents a calmodulin binding site in the carboxyl-terminal tail that overlaps a PKC phosphorylation site, and PKC-mediated phosphorylation and calmodulin binding appear to be antagonistic with one another (Minakami *et al.*, 1997). Lee *et al.* (2008) has shown that mGluR5 surface distribution is stabilized by calmodulin binding. The authors showed that PKC dependent phosphorylation of mGluR5 C-terminus abolishes calmodulin binding and causes receptor internalization (desensitization); on the other hand, calmodulin binding prevented receptor phosphorylation and increased cell surface distribution. These data suggest that calmodulin may be a modulator of glutamate receptor-induced synaptic plasticity. Calmodulin also interacts with the carboxyl-terminal tails of several class B receptors and modulates the agonist-stimulated activity of the parathyroid hormone, vasoactive intestinal peptide, pituitary adenylate cyclase activating corticotrophin releasing hormone, calcitonin and glucagon-like peptide receptors (Mahon and Shimada, 2005). Therefore, calmodulin binding to receptors seem to affect both G-protein-dependent and -independent GPCR signalling as well as receptor trafficking.

Optineurin

Optineurin is a protein linked as a causative factor for open angle glaucoma and has been identified as a huntingtin-interacting protein (Rezaie *et al.*, 2002; Harjes and Wanker, 2003). Optineurin binds to the second intracellular loop domain and carboxyl-terminal tail domains of group I mGluRs and functions to inhibit mGluR1/5 signalling (Anborgh *et al.*, 2005). Polyglutamine expanded mutant huntingtin, but not wild-type huntingtin protein functions synergistically with optineurin to further uncouple mGluR1/5 signalling, and mGluR5 signalling is selectively uncoupled in mice that express polyglutamine expanded huntingtin protein (Ribeiro *et al.*, 2010). Thus, additional phosphorylation-independent mechanisms for the antagonism of GPCR signalling exist.

SH3 domain adaptor proteins

Several SH3 domain proteins have been identified as potential GPCR interacting proteins. The D4 dopamine receptor, as

well as other catecholamine receptors, have putative polyproline binding sites for SH3 domain contain proteins (Oldenhof *et al.*, 1998). The SH3 binding motifs within the third intracellular loop domain of the D4 dopamine receptor strongly interact with both Grb2 and Nck. The removal of these SH3 binding motifs results in a D4 dopamine receptor that is unable to activate either adenylyl cyclase or ERK1/2 phosphorylation, but that exhibits normal G-protein interactions. The removal of these motifs also results in a receptor that is constitutively internalized, which may account for the deficit in cell signalling. Similarly, the SH3 domain containing protein endophilin interacts with polyproline motifs localized within the third intracellular loop of the β$_1$AR (Tang *et al.*, 1999). The over-expression of endophilin results in impaired G$_s$ coupling efficacy and enhanced β$_1$AR internalization. In addition, Src can be co-immunoprecipitated with the β$_3$AR in the absence of β-arrestin recruitment and allows the receptor to couple to the activation of the MAPK pathway (Cao *et al.*, 2000). Thus, the interaction of SH3 domain containing proteins with GPCRs not only modulates the G-protein-coupling, and trafficking of GPCRs, but these proteins may function as adaptors coupling GPCRs to signal transduction pathways that are regulated independently of heterotrimeric G-proteins.

Summary

The present review has provided an extensive, but not exhaustive, review of proteins that have been identified to interact with GPCRs. While the prototypic role of GPCRs is G-protein signalling and the generation of second messenger signals that alter the activity of downstream effector enzymes, GPCRs also exhibit the capacity interact with a wide variety of accessory proteins. The interaction of GPCRs with these accessory proteins not only serves to modulate G-protein coupling, desensitization, endocytosis and the subcellular localization of GPCRs, they also allow for the formation of novel signal transduction complexes that have the capacity to alter cellular function. Strategies to either selectively block or promote the formation of GPCR scaffolded complexes may have the potential for the design of novel drugs that target GPCR signalling that is independent of G-protein activation. By targeting specific GPCR interactions, it may be possible to design pharmaceutical agents that are clinically effective but spare the undesired side effects of antagonizing agonist binding to GPCRs. A further understanding of the nature of the protein complexes that are scaffolded by GPCRs and the physiological consequence of these interactions will be required before such an aim is realized.

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Conflict of interest

The authors declare no competing financial interests.

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