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Regulation of G protein-coupled receptor signaling by plasma membrane organization and endocytosis.

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Synopsis: G protein-coupled receptors (GPCRs) have long been known to signal from the plasma membrane via multiple signaling pathways. GPCR organization at the plasma membrane plays a critical role in regulating the signaling consequences of receptor activation by

regulating the interactions of receptors with specific effector proteins. GPCR organization can be regulated by dynamic association with proteins and membrane microdomains. Furthermore, GPCRs also regulate their own endocytic rate in a ligand- and receptor-dependent manner, with consequences for receptor signaling. This review explores our current understanding of basal organization of GPCRs in the plasma membrane and the functional consequences of this organization on GPCR endocytosis and signaling.

Abstract

The trafficking of G protein coupled-receptors (GPCRs) is one of the most exciting areas in cell biology due to recent advances demonstrating that GPCR signaling is spatially encoded. GPCRs, acting in a diverse array of physiological systems, can have differential signaling consequences depending on their subcellular localization. At the plasma membrane, GPCR organization could fine-tune the initial stages of receptor signaling by determining the magnitude of signaling and the type of effectors to which receptors can couple. This organization is mediated by the lipid composition of the plasma membrane, receptor-receptor interactions, and receptor interactions with intracellular scaffolding proteins. GPCR organization is subsequently changed by ligand binding and the regulated endocytosis of these receptors. Activated GPCRs can modulate the dynamics of their own endocytosis through changing clathrin-coated pit dynamics, and through the scaffolding adaptor protein β-arrestin. This endocytic regulation has signaling consequences, predominantly through modulation of the MAPK cascade. This review explores what is known about receptor sorting at the plasma membrane, protein partners that control receptor endocytosis, and the ways in which receptor sorting at the plasma membrane regulates downstream trafficking and signaling.

1 | Introduction

The organization and trafficking of G protein-coupled receptors (GPCRs) at the cell membrane are major regulators of receptor signaling. Since many GPCRs primarily respond to extracellular ligands, a receptor's ability to respond to signals depends on its physical presence at the cell membrane.[1] The spatial organization of receptors at the cell membrane is tightly controlled, as reported by an increasing number of studies. Receptor organization is likely dictated by the biochemical properties of the receptors themselves,[2] lipid composition of the membrane,[3] and the presence of a host of scaffolding proteins,[4] although the mechanisms are still being elucidated. Once receptors bind a ligand, receptors rapidly reorganize to specific domains within the plasma membrane, which could help coordinate spatially restricted signaling.[5,6] GPCRs are further regulated at the cell surface by agonist-mediated endocytosis.[7] After activation, receptors are sorted to endocytic domains through binding to the adaptor protein β-arrestin.[8,9] Many GPCRs continue to signal during endocytosis, making use of β-arrestin as a signaling scaffold.[10,11] Some GPCRs appear to regulate their own endocytic rate through modulation of clathrin-coated pit maturation.[12-14] This regulation, which can differ between ligands acting at the same GPCR, is an additional method by which GPCR signaling can be spatially encoded.

This review explores our currently emerging understanding of how receptors are organized on the membrane, and how this organization could regulate downstream trafficking and signaling. We focus specifically on basal receptor localization and agonist-dependent redistribution, as well as the mechanics of GPCR modulation of receptor-mediated endocytosis of mammalian GPCRs. We also highlight currently open questions in the field relating to how GPCR localization and trafficking at the plasma membrane has physiological significance. Although the principles discussed focus on GPCR signaling specifically, they are applicable to many other signaling receptors or transmembrane proteins whose functions depend on spatial localization.

2 | Basal receptor localization and agonist-dependent redistribution

GPCR organization in the plasma membrane is driven through receptor-receptor, receptor-lipid, and receptor-protein interactions that restrict and regulate receptor movement within the plasma membrane (Figure 1). Most GPCRs begin their signaling lives at the plasma membrane, although some receptors are basally localized to intracellular sites such as the ER or the trans-Golgi network.[15-17] Once delivered to the plasma membrane, the three types of receptor interactions described below help GPCRs localize to specialized membrane domains and to specialized structures such as the neuronal postsynaptic density,[18] primary cilia,[19] and the outer segment of photoreceptor cells.[20]

2.1 Receptor-Receptor Interactions

Despite a great deal of controversy over the past several decades, there is mounting evidence for the existence of semi-stable oligomeric GPCR complexes, as well as receptor-receptor interactions that drive receptor signaling and localization.[21,22] Some of these interactions are stable and long-lasting,[23,24] while some are transient and weak.[25] These receptor-receptor interactions can produce homodimers of the same receptor,[26] or heterodimers of two different receptors.[27] Homodimerization is evolutionarily conserved. The yeast α-factor receptor (Ste2p) shows a significant tendency to dimerize,[28,29] and dimerization of functional receptors might be required for receptor signaling [30]. Dimeric receptor complexes can even couple to a single G protein or arrestin molecule, as demonstrated for the light-activated GPCR rhodopsin.[31,32] Heteromers such as the μ/δ-opioid receptor dimer might couple to different effectors and induce functional effects distinct from their monomers.[33-36] Receptors can oligomerize at multiple steps throughout the biosynthetic trafficking of GPCRs, with the ɣ-aminobutyric acid receptor type B (GABABR) requiring dimerization for ER export [37,38] whereas Ste2p dimerizes only at the plasma membrane.[39]

Receptor oligomerization regulates the diffusion of the receptors within the plasma membrane. The GABAB receptor, an obligate dimer,[23,24] diffuses slowly within the plasma membrane and primarily exists as dimers and tetramers.[40] The β1-adrenergic receptor (B1AR) exists predominantly as a monomer at the plasma membrane, and the closely related β2-

adrenergic receptor splits its time roughly equally between monomeric and dimeric states.[40] Receptor oligomerization is highly dynamic at physiological concentrations of receptor, as demonstrated by recent studies with the neurotensin receptor NTSR1[41] and rhodopsin[42]. Changes in diffusion rate may serve to change receptor-effector coupling, and GPCR-G protein complexes appear to diffuse much more rapidly than GPCRs alone.[43]

2.2 Receptor-Lipid Interactions

Receptor-lipid interactions can regulate receptor distribution at the plasma membrane, and potentially affect receptor signaling. Fluorescence correlation spectroscopy of the μ- and κ-opioid receptors (μOR, κOR) showed that these receptors are enriched in cholesterol-rich domains and tend to be excluded from ganglioside-rich plasma membrane domains.[3] Some GPCRs contain a cholesterol binding site,[44] and cholesterol has been a necessary additive to GPCR crystallization studies.[45,46] Further evidence suggests that cholesterol is permissive of GPCR GEF activity at G proteins.[47-49] Despite evidence showing a biochemical role of cholesterol in GPCR activity, the exact function of GPCRs residing at cholesterol-enriched membrane sites remains unknown. Since heterotrimeric G proteins can be differentially lipidated via palmitoylation, myristoylation, farnesolyation, and geranylgeranylation,[50] it is possible that GPCR localization to different lipid microdomains could dictate coupling to different G proteins. Indeed, B2AR, which is predominantly coupled to Gαs,[51] can couple to Gαi when restricted to lipid rafts.[52] Receptor-lipid interactions could therefore sort GPCRs to membrane domains where receptors are best situated to signal through different G proteins or even interact with specific effectors or modifying enzymes, although this remains to be tested.

2.3 Receptor-Protein Interactions

GPCR interactions with cytoskeletal and signaling scaffolds regulate receptor organization at the plasma membrane. There are a multitude of known GPCR interacting proteins,[4,53] but only a subset of these have been shown to participate directly in basal GPCR organization. GPCR localization to neuronal synapses has received particular attention. The localization of the metabotropic glutamate receptor 5 (mGluR5) is dependent on the scaffolding protein Homer.[54] Another metabotropic glutamate receptor, mGluR7, is restricted to synapses through

its interaction with the protein PICK1.[55] This interaction is dependent on PICK1's PSD95/Dlg/ZO-1 (PDZ) homology domain, which binds a PDZ ligand at the distal C-terminus of mGluR7. PSD95, another PDZ domain-containing protein, binds to the β1-adrenergic receptor (B1AR) and appears to increase surface expression of the GPCR.[56] Many GPCRs feature PDZ ligands,[57] and as these receptors are explored further in their native context in polarized cells it is likely many similar scaffolds to those described above will be discovered.

GPCR diffusion at the plasma membrane also appears to be regulated by the cortical cytoskeleton. A 'fence and picket' model of membrane organization previously proposed suggests that the diffusion of transmembrane protein 'pickets' is limited within differently sized membrane compartments that are demarcated by actin 'fences'.[58] Single molecule studies of μOR[59] showed that receptors diffused in distinct membrane 'compartments', with straight line barriers that were assumed to be actin filaments. These early findings were recently reaffirmed when single molecule analysis of B2AR and the α2a-adrenergic receptor (A2AR) showed that these receptors avoid actin during their diffusion in the plasma membrane and that their diffusion is restricted to actin-bounded compartments.[2]

2.4 Agonist-Dependent Redistribution

Agonist binding and activation cause substantial reorganization of GPCRs from the basal state. Receptor reorganization includes receptor clustering, as well changes in receptor diffusion kinetics. For example, the μ opioid receptor (μOR) clusters upon activation with the endogenous-like ligand DAMGO, but not after treatment with the exogenous ligand morphine.[6] DAMGO-dependent receptor clustering correlates with downstream signaling through the mitogen activated protein kinase (MAPK) cascade. Because of this MAPK activation, these receptor clusters have been suggested to be a specialized signaling domain. MAPK signaling from these putative signaling domains requires cholesterol at the plasma membrane, though whether receptor clustering independent of signaling requires cholesterol is not clear. Changes in receptor organization after agonist binding has been explored also at a single-molecule level. The Gαs-coupled B2AR and the Gαi-coupled A2AR do not change their diffusive behavior upon agonist activation, but their respective G proteins become more mobile.[2] In this study, only a small fraction of both B2AR and A2AR molecules were shown to rapidly sort into clathrin-coated

pits after agonist addition. In contrast to B2AR and A2AR, the Gαi/o-coupled metabotropic glutamate receptor mGluR3 (a class C GPCR with a much larger extracellular domain compared to the class A B2AR and A2AR) significantly slows its diffusion when bound to an agonist, and sees considerable redistribution into clathrin-coated pits after agonist treatment.[43] The variability between GPCRs suggests that although there may be commonalities to how receptors behave immediately following agonist treatment, receptor redistribution patterns are worth investigating at the level of specific receptor types.

3 | Agonist-mediated receptor endocytosis

The role and regulation of endocytosis - a well-known consequence of receptor activation - is currently being redefined in the field. Activated receptors are phosphorylated by G protein-receptor kinases, after which they recruit the adapter protein β-arrestin.[1,60] β-arrestin binding sorts receptors into clathrin-coated pits – specialized endocytic domains on the plasma membrane.[8,61,62] The traditional view of GPCR endocytosis (excellently reviewed previously[63]) was that it primarily served to desensitize receptors after agonist activation by removing them from the cell surface. Recent work has highlighted several novel aspects of GPCR endocytic trafficking: 1) GPCRs segregate to specialized endocytic domains, 2) GPCRs regulate the maturation of these endocytic domains, 2) this regulation has signaling consequences that differ both between receptors and between ligands acting at the same receptor. These recent advances, discussed below, are summarized in Figure 2.

3.1 Segregation of GPCRs in endocytic domains

Understanding GPCR regulation of endocytosis requires a broad understanding of the steps of clathrin-mediated endocytosis (CME) that GPCR cargo may be able to regulate. Endocytic cargo, including GPCRs, are sorted into nascent clathrin-coated pits (CCPs) through interaction with adaptor proteins possessing both cargo- and clathrin-binding domains. Although adaptor protein 2 (AP2) is the canonical adaptor for a host of CME cargo, there are a variety of endocytic sorting signals and cognate adaptors that bind them.[64] CCP maturation to an internalized vesicle is a highly regulated process.[65,66] A maturing CCP proceeds through

multiple 'checkpoints' before undergoing eventual dynamin-dependent scission.[67,68] Adaptor proteins play a prominent role in implementing these checkpoints during CCP maturation.[69,70]

The diversity in both the number of cargoes internalized via CME as well as the adaptor proteins used for this internalization suggests that multiple routes for biochemically distinct CME pathways may exist. Different cargo sorting signals for CME are differentially saturable, indicating a variety of mechanisms for cargo association with clathrin.[71] Overexpression of distinct cargoes – the transferrin receptor (TfR), the epidermal growth factor receptor (EGFR), and the low-density lipoprotein receptor (LDLR) – can saturate each cargo's respective endocytosis, but do not interfere with the endocytosis of the other cargos.[72,73] A straightforward explanation is that different cargoes recruit distinct adapters and endocytic accessory proteins to nascent CCPs. Consistent with this, the adaptor AP2 is required for the internalization of TfR but not EGFR.[74] But the number of adaptors identified are far fewer than the number of potential cargoes. Alternatively, cargo could change the lipid environment in which CCPs form. For example, EGFR-positive CCPs form preferentially from cholesterol and sphingolipid rich membrane rafts, a phenomenon which does not appear to be conserved by other endocytic cargo.[75]

GPCRs can sort into specific subsets of CCPs. Activated B2AR and μOR are present only in a subset of the CCPs through which TfR endocytoses, both in fixed cell analyses and when visualizing endocytosis at the resolution of single scission events, although the extent of overlap between these cargo in the same CCPs has been variable across studies.[12,76-78] Further, not all GPCRs sort to the same subset of pits. The purinergic receptors $P2Y_1$ and $P2Y_{12}$ localize to different CCP subsets, with $P2Y_{12}$ internalizing in the same CCPs as B2AR while $P2Y_1$ internalizes by a distinct clathrin-dependent pathway.[79] GPCRs and β-arrestin clusters that form at the cell membrane in response to agonist colocalize with preexisting clathrin clusters,[9] suggesting that GPCRs can cluster in a subset of extant CCPs as opposed to exclusively nucleating specialized new CCPs. GPCRs also show different biochemical requirements for endocytosis. Both $P2Y_{12}$ and μOR require phosphorylated clathrin light chain for efficient clathrin-mediated endocytosis, whereas TfR does not.[80,81] B2AR endocytosis is blocked at 16˚C while TfR endocytosis is not.[77] Segregation of different cargo into specific subsets of CCPs could allow individual control over clustering and endocytosis of different receptors.

3.2 Receptor Regulation of Endocytosis

Regardless of the degree to which CCPs specialize based on cargo, it is clear that GPCRs can regulate CCP dynamics [12,13,78]. Although CCPs containing the δ opioid receptor (δOR) last ~40 seconds at the plasma membrane before undergoing dynamin-dependent scission, CCPs containing a chimeric δOR with B1AR's C-terminal PDZ ligand last 3 times as long. CCPs containing B2AR, which has its own PDZ ligand, also last longer than CCPs containing δOR. PDZ ligands extend the duration of clathrin-coated pits ('CCP lifetimes') by delaying dynamin recruitment at the CCPs in which these receptors reside.[12] However, work with μOR has shown that this is not the only mechanism by which GPCRs can regulate CCP lifetimes. μOR promotes long CCP lifetimes by delaying scission after dynamin recruitment.[78] Despite differing mechanisms, both of these studies pinpointed receptor control of CCP lifetimes as being mediated through amino acid motifs in the C-termini of the identified receptors (PDZ ligands 'DSLL' or 'ESKV' for B2AR and db1, a unique 'LENLEAE' motif for μOR). How these sequences regulate CCP dynamics is not known. The dynamin-dependent scission at the end of vesicle maturation could be a point at which CME can be regulated. The protein kinase Src phosphorylates dynamin2 and the actin nucleating factor cortactin to be permissive of TfR endocytosis.[82] The kinase GSK3B also regulates dynamin activity through inactivating phosphorylation of dynamin1.[83] Interestingly, these kinases could be regulated by GPCRs themselves, providing a potential feedback mechanism for precise control of endocytic dynamics at the level of individual CCPs.

Different ligands acting at the same receptor have distinct effects on CCP lifetimes, pointing to the physiological relevance of CCP regulation. This was first shown with the cannabinoid receptor 1 (CB1R), where the exogenous ligand WIN 55,212-2 caused shorter CCP lifetimes than the endogenous ligand 2-arachidonoylglycerol (2-AG).[13] Subsequent work with μOR revealed that the clinically relevant ligand morphine produced significantly shorter endocytic lifetimes compared to endomrophin-2, one of the receptor's endogenous agonists.[14] For both CB1R and μOR, longer receptor cluster lifetimes correlated with increased MAPK activation, suggesting that CCPs sustain a signaling complex that links receptors to MAPK, as discussed below.

GPCRs regulate endocytosis through their downstream signaling and interactions with scaffolding proteins. In the case of $P2Y_{12}$ and μOR it is possible that activation of GPCR regulated kinases (GRKs) downstream of these receptors might regulate endocytosis in general by changing the phosphorylation state of clathrin light chain, but it remains unclear whether this phosphorylation plays a role in changing CCP lifetimes.[80] When investigating protein partners that might mediate GPCR control of lifetimes, the PDZ ligands of the β-adrenoreceptors provide tantalizing targets given their requirement for lifetime extension, but no PDZ-domain containing partner has been identified that regulates CCP lifetimes for these receptors. However, a host of other GPCRs have been shown to have PDZ-dependent regulation of their endocytosis, although not specifically though regulating CCP lifetimes. The PDZ ligands of the serotonin 2A receptor (5HT2AR) and the corticotropin releasing factor receptor 1 (CRFR1) both bind the PDZ-domain containing protein synapse associated protein 97 (SAP97), and overexpression of SAP97 slowed global endocytic rate for both of these receptors.[84,85] For CRFR1 alone, expression of the protein PDZK1 increased the receptor's endocytic rate,[86] while PSD-95 expression decreased the receptor's endocytic rate[87] in a manner consistent with the stabilizing effect PSD-95 has on B1AR.[56] Another GPCR, the metabotropic glutamate receptor 1 (mGluR1), interacts with the PDZ-domain containing protein spinophilin and has subsequently decreased endocytosis. The parathyroid hormone receptor (PTHR) interacts with the Na/H-exchanger regulatory factor 1 (NHERF1) through a PDZ-domain dependent interaction, and NHERF1 inhibits internalization of PTHR following agonist treatment.[88] These are only a handful of examples of PDZ-domain containing proteins known to regulate GPCR internalization. For all of these PDZ interactions, it has not yet been investigated whether these effects on endocytosis are mediated through extension of CCP lifetimes or through blocking receptors from sorting into CCPs in the first place, although published results with B2AR's extended CCP lifetimes make this a tantalizing question.

3.3 Signaling Consequences of Receptor-Regulated Endocytosis

The signaling effects of GPCR-mediated extension of lifetimes are primarily mediated through β-arrestins. For CB1R, a receptor mutant that binds β-arrestin1 more strongly than the wild-type receptor also increases CCP lifetimes of the exogenous ligand WIN 55,212-2.[89] These

extended lifetime CCPs produce stronger MAPK activation downstream of β-arrestin as measured by phosphorylation of the MAP kinases ERK 1 & 2.[13] Extended lifetimes downstream of endogenous agonists at μOR also serve to extend the duration of the receptor/arrestin interaction and to increase ERK1/2 activation.[14] Recent work has shown that following activation of B1AR, β-arrestin can translocate to CCPs even in the absence of GPCR translocation. These arrestin-positive CCPs subsequently have significantly prolonged lifetimes and this results in increased ERK1/2 phosphorylation downstream of B1AR agonists.[90] This discovery was extended by showing that GPCRs can act as β-arrestin activators without necessitating a stable interaction between the receptor and β-arrestin.[91,92] This implicates GPCRs as not just cargo, but also regulators of protein trafficking themselves. All of the above findings fit with an emerging model whereby GPCRs affect multiple modes of β-arrestin function through interactions at functionally distinct sites.[93,94] Notably, the dependence of ERK1/2 activation on lengthened endocytic lifetimes has so far been demonstrated only with receptors whose activation of MAPK is dependent on β-arrestin.

Several receptors that rely on PDZ domain-containing proteins to regulate their endocytosis show positive coupling between endocytosis and ERK1/2 activation. Studies with CRFR1 and 5HT2AR show that the PDZ-domain containing protein SAP97 slows the endocytic rate of these receptors while increasing their ligand-dependent ERK1/2 activation.[84,85] However, the effects of PDZ-domain containing protein on receptor trafficking and signaling are not always so stereotyped. For example, PDZK1 overexpression increases CRFR1 ERK1/2 activation while having no effect on CRFR1 endocytosis, but this same overexpression increases 5HT2AR ERK1/2 activation while slowing 5HT2AR endocytosis.[86] The uncoupling of ERK1/2 activation to endocytic lifetimes suggests that, at least for some receptors prolonged endocytic rate may serve a different role.

There is a dearth of evidence directly connecting endocytic lifetimes to specific protein components in the MAPK cascade. Studies exploring PDZ-dependent modulation of endocytosis and ERK1/2 have not demonstrated a direct interaction between any PDZ domain-containing scaffolds and components of the MAPK cascade.[4,84-87,95,96] For some GPCRs, β-arrestin is required for connecting endocytic lifetimes to ERK1/2 activation. But the specific molecular mechanism through which β-arrestin controls ERK1/2 activation is not clear.[13,14,90]

Differences in endocytic lifetimes may also contribute to further downstream spatial encoding of GPCR signaling. The recent explosion in the study of GPCR endosomal signaling (recently reviewed[97]) opens up the exciting possibility that changes in receptor duration on the plasma membrane might affect trafficking at post-endocytic stages of GPCR trafficking.

The changes on the receptors that drive control of endocytic lifetimes are not clear. One potential mechanism through which endocytic lifetimes might regulate downstream trafficking is through regulating the phosphorylation state of GPCRs. GPCR phosphorylation changes in response to agonist, and receptor phosphorylation is a known regulator of GPCR trafficking and signaling. GPCRs are phosphorylated by many kinases including GRKs[98-101] and PKA[101-103]. Receptor phosphorylation begins at the plasma membrane independent of endocytosis[104], and at least some phosphorylation is present on receptors throughout post-endocytic trafficking.[105,106] Receptor phosphorylation is important for post-endocytic trafficking[107,108] and for endosomal receptor signaling[109]. Modulating GPCR ubiquitination is another potential target of endocytic lifetime regulation. Ubiquitination has been implicated in GPCR trafficking and signaling (reviewed by Trejo and colleagues in this same issue). The endocytosis of yeast GPCRs Ste2p and Ste3p depends on ubiquitination, after which they might recruit alpha arrestins or other unique adapters. Ubiquitination plays a prime role in the p38 signaling downstream of the protease activated receptor 1[110]. Ubiquitination is required for effective β-arrestin recruitment at the interleukin-8 chemokine receptor (CXCR2)[111], and for regulating the dynamics of internalization of the μOR.[112] The latter mechanism is mediated through a ubiquitin binding motif in the endocytic accessory protein Epsin1, suggesting that cells proofread receptor modification states before allowing the receptor to internalize.

In summary, GPCRs are not passive components in endocytic trafficking. Rather, they can control the dynamics of endocytic components. This control might modulate downstream signaling pathways including the MAPK pathway. It is possible that the endocytic lifetimes indirectly regulate the phosphorylation or ubiquitination states of receptors themselves, which in turn could regulate interactions of receptors with components of the endocytic pathway, although there is little evidence to support this model at present. The interplay between signaling and endocytic control is an area with tremendous potential that still needs to be understood better.

4 | Concluding Remarks and Future Directions

In the past decade, we have learnt much about GPCR organization at the plasma membrane, but much still remains to be learned both with regards to the nature of basal organization, the mechanism of endocytic regulation, and the physiological effects of both. For example, while targets such as PDZ proteins and β-arrestins have been confirmed, we do not understand how these interactions modify CME. Further, ERK1/2 is the main signaling output that has been measured downstream of CCP regulation, but the physiological consequences of ERK1/2 activation remain unknown. Nevertheless, current work has uncovered the potential physiological and translational impact of spatial organization of signaling receptors and has underlined the importance of studying receptor trafficking. Another interesting aspect that still needs to be addressed is the contribution of GPCR reorganization and endocytic control on how different ligands acting at the same receptor can bias downstream signaling to different effector-driven pathways – a phenomenon termed biased agonism or functional selectivity.[113] Although the correlation of endocytic lifetimes and arrestin-mediated activation of MAPK suggest a mechanism through which different agonists may produce bias, the exploration of how membrane organization relates to bias is still in its infancy. Learning how receptor partitioning into lipid domains regulates signaling, or how receptor oligomerization is regulated, may also be key to understanding the pleiotropy of signaling and mechanisms of bias.

The next frontier is to validate the importance of the mechanistic findings discussed to receptor physiology *in vivo*. Model cells, where receptors can be heterologously expressed, receptors and effectors specifically mutated or modified, and signaling outputs isolated, have been indispensable in understanding the fundamental principles of receptor organization and trafficking. Nevertheless, as we continue to use these models to tease out mechanistic details, a concomitant step is to move the study of receptor localization and function into physiologically relevant systems expressing endogenous receptors. Some of the receptor-lipid, receptor-receptor, and receptor-protein interactions have been demonstrated directly in primary cells of interest. At present, the degree of endocytic specialization in primary cells and the role of endocytic regulation is still not well understood. Newer advances in imaging and profiling receptor location and signaling and in inducible stem cells, as well as using animal models with cell-specific expression and gene-editing tools, provide exciting avenues for addressing the role

of spatial organization in receptor physiology *in vivo*. As we continue to validate findings in specific physiological systems, we anticipate that this will open a new druggable proteome, allowing pharmaceutical targeting of trafficking factors to regulate the endogenous signaling of GPCRs that are important in physiology and disease.

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Figure legends:

Figure 1: GPCR organization at the plasma membrane is dynamic and regulated. **a)** Before agonist addition, many GPCRs exist at the plasma membrane as monomers. **b)** Through receptor-receptor interactions, some receptors dynamically exchange between monomeric and oligomeric states, with the degree of time a receptor spends in each of these states varying between different types of receptors. Receptors are enriched at cholesterol rich regions (darker blue) through receptor-lipid interactions, although they can diffuse between these domains and the surrounding membrane. **c)** After agonist addition, receptors cluster in clathrin coated pits (CCPs), regardless of their oligomeric state, but the rate at which a given receptor is sorted into CCPs can be variable. The gray bars denote the clathrin coat. **d)** Some receptors are obligate homodimers or heterodimers or higher order oligomers, existing always in these states. **e)** Receptor diffusion in the plasma membrane is restricted by actin and microtubule 'fences' (red rods) which confine receptors. **f)** Receptors can also cluster tightly together into domains that could mediate signaling after agonist addition prior to localizing to CCPs. **g)** Scaffolding proteins associate with and restrict the localization of certain GPCRs. Receptors bound to scaffolding proteins may be protected from endocytosis.

Figure 2: GPCRs modulate endocytosis at distinct phases of the endocytic process. **a)** After ligand binding to a given receptor, β-arrestin is recruited to the receptor at the plasma membrane. **b)** In the case of B1AR, an interaction between β-arrestin and the B1AR core region causes β-arrestin to sort to clathrin-coated pits (CCPs) independent of the receptor. Other GPCRs sort with arrestin to CCPs. **c)** $P2Y_{12}$ and μOR regulate clathrin light chain (CLC) phosphorylation through the activation of GPCR related kinases (GRKs) which is permissive of endocytosis continuing. **d)** After receptors are sorted into nascent CCPs, μOR is 'proofread' by Epsin1 to ensure that it is ubiquitinated before CCP maturation continues. At about the same phase, the PDZ ligand of B2AR delays recruitment of the GTPase dynamin through an unknown protein partner. **e)** After dynamin recruitment, μOR can delay dynamin-dependent scission through an unknown protein interacting with its C-terminal LENLEAE motif. CB1R, through an arrestin interaction mediated by two serines on its C-terminal tail, can also delay CCP lifetimes.

f) Through as yet unknown mechanisms, GPCR interactions with PDZ domain containing proteins can globally upregulate (e.g. CRFR1 & PDZK1) or downregulate (e.g. mGluR1 & spinophilin) receptor internalization.

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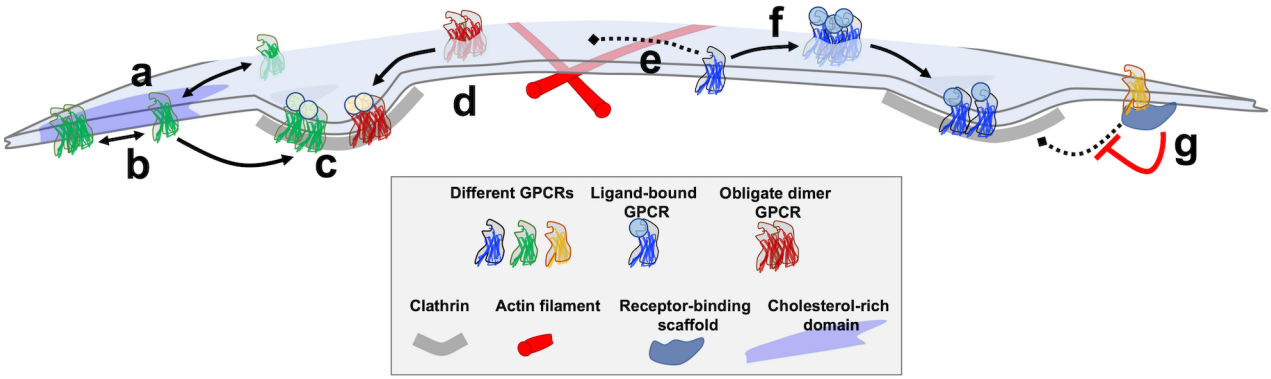

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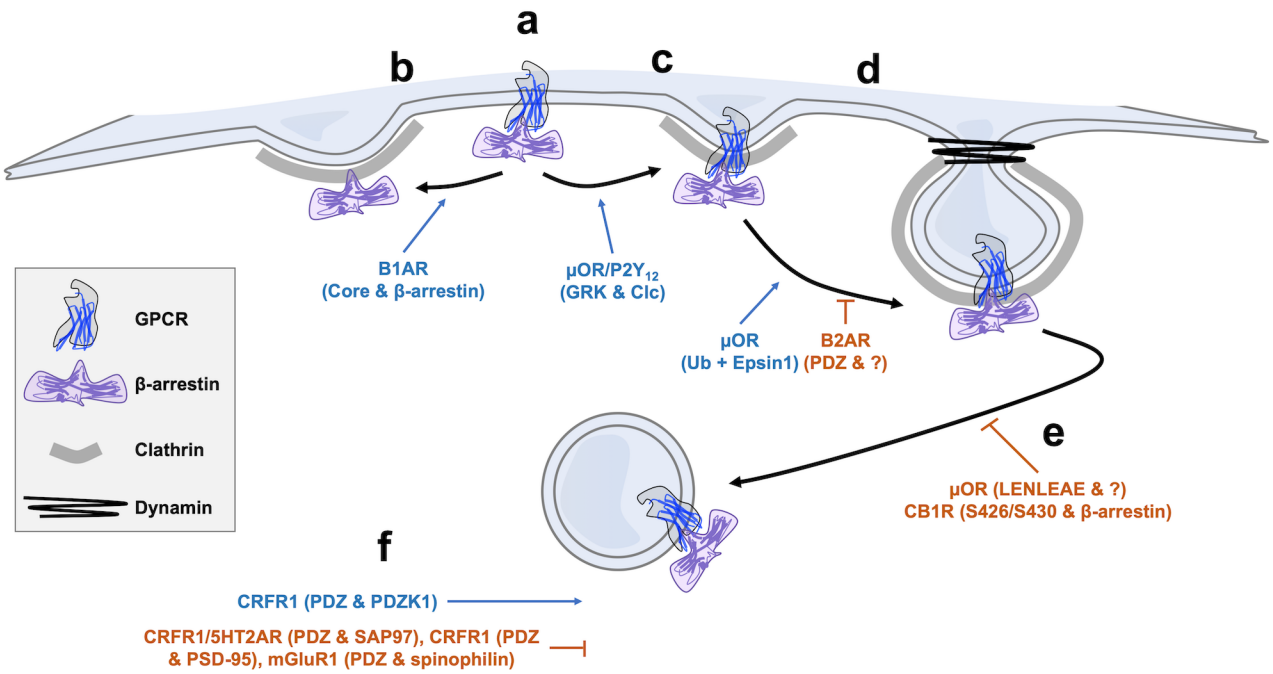

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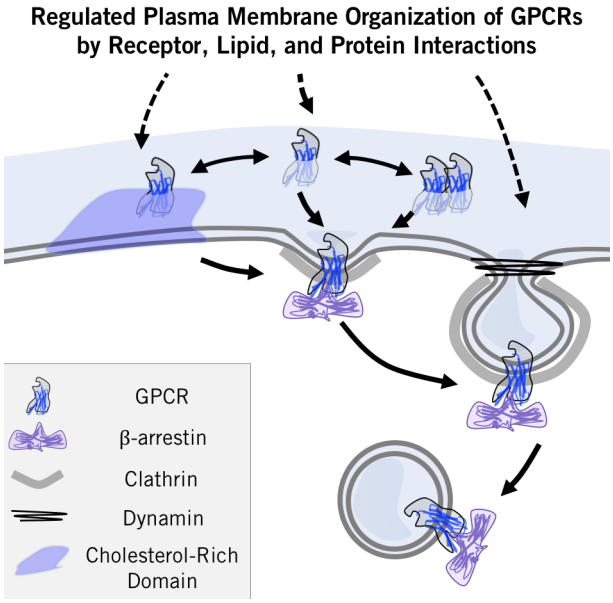

tra_12628_AbstractFigure.tiff

Title: **Regulation of G protein-coupled receptor signaling by plasma membrane organization and endocytosis**

Authors: Zara Y. Weinberg and Manojkumar A. Puthenveedu

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Decision and Reviews

Dear Manoj,

Thank you for taking the time to write your review "Organization and trafficking of G protein-coupled receptors at the plasma membrane" for Traffic. I asked an expert in the field to review the paper and his/her verbatim comments are appended below. I share the view of the referee that this is a timely review that will be of interest to the readers of Traffic. The referee has made a number of suggestions for revisions that I agree will strengthen this review. I have added a few additional comments below as "Referee #2". With these changes I would be pleased to accept this paper for publication in Traffic. Thanks so much again for supporting Traffic!

Sincerely yours,

Mickey

Michael S. Marks, Ph.D.
Co-editor, Traffic

Referee's Comments to the Authors

Referee: 1

Comments to the Author
The current review on GPCR organization and trafficking at the plasma membrane is well written, thorough and an extensive review of the subject matter -GPCRs in mammalians systems. However, the review can be improved by addressing the following.

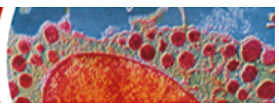
1. Provide some discussion about what is known about organization and trafficking of yeast Ste2, Ste3 receptors at the plasma membrane

2. Provide general comment about what is known about the influence of bias agonism on the organization and trafficking of mammalian GPCR receptors at the plasma membrane

3. In section 2, the inclusion of Rhodopsin in the discussion of GPCR dimerization, oligomerization is needed

4. Section 3 "Agonist-mediated receptor endocytosis" is very long and detailed and could be improved by breaking up this section into several other smaller succinct sections

5. The authors should include a discussion of the role of actin and microtubule fences shown to confine localization



of GPCRs at the plasma membrane in the main text

6. There are several typos in the main text that need to be corrected.

Referee #2

The authors should consider the following minor comments:

1. The abstract would benefit from rewriting for clarity and logic. For example, in the first sentence, "The trafficking of G protein coupled-receptors" is not a question.

2. It is rather odd that the Introduction lacks citations.

3. The structures in Figure 1 need to be defined in the legend. For example, the purplish blobs need to be defined as cholesterol-enriched "rafts", the gray bar as clathrin, the red rods as actin, and the differences between green, red and blue receptors need to be indicated. Similarly, components of Figure 2 need to be defined (I presume clathrin, beta-arrestin, GPCR and dynamin?).

4. At bottom of page 2, where else to GPCRs reside at basal state?

5. End of second to last paragraph on page 3 - is there evidence that different G proteins accumulate in different plasma membrane microdomains? How might such microdomains select for different G proteins? Might inclusion or exclusion from such domains also affect interactions with beta-arrestin or with ubiquitin ligases?

6. Second paragraph of page 4 - what is meant by "specialized signaling domains"? How are they defined? Later in that paragraph, more should be stated regarding the "actin fence" concept, its origins, and whether the evidence really supports this for stimulated B2AR and A2AR.

7. In the third paragraph on page 5, saturation of different receptor internalization was also shown by Marks et al., 1996 JCB 135:341 (don't snub the editor!). Proof that distinct binding events are mediated by different adaptors was provided by Motley et al., 2003, JCB 162: 909. In the next paragraph the authors present conflicting statements regarding the overlap of GPCR- and TfR-containing CCPs; can they reconcile why the results of the two studies differ? Did they differ regarding analyses of endogenous vs. exogenously expressed cargoes?

8. The paragraph on the bottom of page 6/ top of page 7 is very confusing regarding what "shorter lifetimes" refers to (is this residence time at the plasma membrane?) and the link between CCP lifetime duration and MAPK signaling (is increased duration or decreased duration correlated with MAPK signaling?). Should this section be more closely linked to the paragraph that begins at the bottom of page 7 in which these associations are better described? There, beta-arrestins do not need to be reintroduced, as they were introduced earlier in the review. The following paragraph on page 8 also aligns better with an earlier section on PDZ interactions.

9. GRK needs to be defined when first introduced.

10. There is no citation in the paper to Figure 2.

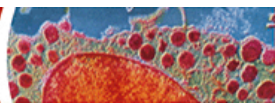
11. Given the focus of the review, would a more appropriate title be "Regulation of G-protein coupled receptor signaling by plasma membrane organization and endocytosis"? "Trafficking" to me implies more post-endocytic routing.

Author Rebuttal

Dear Dr. Marks,

We appreciate the insightful feedback and suggestions of the referees to improve the scope and accuracy of our review. We have addressed all the comments, as noted below in the response to reviewers.

The additional time has also allowed us to include a few recently published papers that further highlight and elucidate



several of the points we make throughout the review. We also accepted your gracious suggestion and have changed the title of the article.

We hope that these changes adequately address the referees concerns and we hope that this article now meets the quality of work published in the journal.

Thank you again for your consideration,

Sincerely
Dr. Zara Weinberg
Dr. Manoj Puthenveedu

RESPONSE TO REVIEWERS:

Reviewer 1

We are happy that the referee found the manuscript well written, thorough and extensive. We have addressed all the points noted by the referee. Specifically, as requested, we have:

1. Added discussion of yeast Ste2p specifically in the section of dimerization. We feel that an exhaustive discussion of non-mammalian GPCRs is outside the scope of this review, so we also added a phrase to clarify that we have focused primarily on mammalian GPCRs.
2. Discussed, in the conclusions section, how GPCR organization and endocytosis could affect agonist bias.
3. Discussed Rhodopsin in the section of receptor oligomerization.
4. Broken up both topics (organization and endocytosis) into succinct sections
5. Added a discussion of the 'fence and picket' model of how actin and microtubules restrict membrane diffusion of GPCRs
6. Fixed the typographical errors in the manuscript.

Reviewer 2

We thank the referee for the comments. We have addressed all the questions and comments. Specifically, we have:

1. Rewritten the abstract, including the first sentence.
2. Added citations to the introduction
3. Revised the legend and the figure in Figure 1 to clarify the features of the cartoon.
4. Revised the section on basal organization of GPCRs.
5. Expanded our discussion on microdomains and ubiquitination.
6. Clarified the section on signaling domains and elaborated on the role of actin fences in restricting GPCR diffusion
7. Rewritten the section on saturation of cargo internalization to include a key reference that was inadvertently overlooked, and the other relevant references. We have also revised the section on CCP subsets to clarify that the main disparity between studies was in the extent of overlap.
8. Rewritten the sections linking endocytic lifetimes to MAPK signaling to avoid redundancy and streamline the logic better.
9. Defined GRKs
10. Added a citation to Figure 2
11. Changed the title to "Regulation of G protein-coupled receptor signaling by plasma membrane organization and endocytosis."

We thank the reviewers again for reading the manuscript and offering these comments and suggestions. We will be happy to address any further changes that need to be made.