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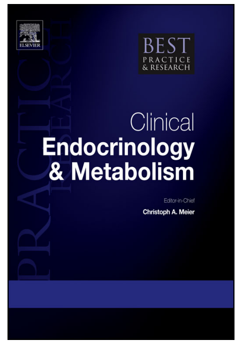

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Internalization of GPCRs: implication in receptor function, physiology and diseases

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Abstract

G protein-coupled receptors (GPCRs) are the largest family of membrane receptors and mediate the effects of numerous hormones and neurotransmitters. The nearly 1,000 GPCRs encoded by the human genome regulate virtually all physiological functions and are implicated in the pathogenesis of prevalent human diseases such as thyroid disorders, hypertension or Parkinson´s disease. As a result, 30 to 50% of all currently prescribed drugs are targeting these receptors. Once activated, GPCRs induce signals at the cell surface. This is often followed by internalization, a process that results in the transfer of receptors from the plasma membrane to membranes of the endosomal compartment. Internalization was initially thought to be mainly implicated in signal desensitization, a mechanism of adaptation to prolonged receptor stimulation. However, several unexpected functions have subsequently emerged. Most notably, accumulating evidence indicates that internalization can induce prolonged receptor signaling on intracellular membranes, which is apparently required for at least some biological effects of hormones like TSH, LH and adrenaline. These findings reveal an even stronger connection between receptor internalization and signaling than previously thought. Whereas new studies are just beginning to reveal an important physiological role for GPCR signaling after internalization and ways to exploit it for therapeutic purposes, future investigations will be required to explore its involvement in human disease.

Keywords

GPCR, cAMP, receptor internalization, TSH, PTH, LH, endosomal signaling.

Abbreviations

G protein-coupled receptor (GPCR), protein kinase A (PKA), cyclic adenosine monophosphate (cAMP), mitogen-activated protein kinase (MAPK), thyroid stimulating hormone (TSH), parathyroid hormone (PTH), protein kinase A (PKA), neurokinin (NK), clathrin-mediated endocytosis (CME), clathrin-coated pits (CCPs).

Introduction

G protein-coupled receptors (GPCRs), with a share of almost 4% of the human genome [1], constitute the largest family of receptors that allow cells to sense extracellular stimuli [2, 3]. These external stimuli range from sensory cues like light, odorants and tastants to small-molecule neurotransmitters, peptides and hormones [2, 3]. This high diversity underscores the fundamental role that GPCRs play in the function of the endocrine, nervous, cardiovascular, sensory and immune systems.

The main initial steps of GPCR activation and signaling have been elucidated in detail [2, 4]. These events are initiated by binding of an agonist to a receptor, which triggers a series of conformational changes in the receptor that culminate in its activation. The activated receptor, in turn, binds to and activates heterotrimeric G proteins, which are composed of an α, β and γ subunit and exist in different isoforms. The α and βγ subunit finally modulate the activity of membrane-localized effectors, including ion channels and enzymes like phospholipase Cβ (PLCβ) and adenylyl cyclase.

A classic example of the role of these receptors in physiology is their involvement in the regulation of heart contractility. β-adrenergic receptors located on the surface of cardiomyocytes mediate the positive ionotropic and chronotropic effects of adrenalin and noradrenalin, released upon sympathetic activation. Binding of adrenalin or noradrenalin to these receptors, which are coupled to the G_s protein, activate adenylyl cyclases to produce cAMP, which stimulates protein kinase A (PKA). PKA, in turn, phosphorylates different molecules involved in cardiac contractility, including L-type Ca^{2+} channels, phospholamban and troponin I, ultimately leading to enhanced cardiomyocyte contractility [5]. In addition, cAMP directly promotes the opening of pacemaker (HCN) channels in the conductive tissue, thus increasing heart rate [6, 7]. Parasympathetic activation counteracts these effects via release of acetylcholine, which binds to muscarinic (M2) receptors coupled to $G_{i/o}$, thus inhibiting adenylyl cyclase activation. In addition, the βγ subunits released upon $G_{i/o}$ activation stabilize the membrane potential via activating potassium (GIRK) channels in the conductive tissue [8-12]. In the endocrine system, GPCRs play an essential role as receptors for several hormones, hypothalamic releasing factors and local modulators. All major known hypothalamic releasing (TRH, GnRH, CRH, GHRH) and inhibiting (somatostatin, dopamine) hormones act via specific GPCRs [13-17]. With the exception of GH and PRL, anterior (TSH, LH, FSH, ACTH, MSH) and posterior (vasopressin, oxytocin) pituitary hormones also signal through activation of GPCRs [18]. For an extensive discussion of the specific roles of GPCRs and G proteins in human physiology we refer the reader to the comprehensive review by Wettschureck and Offermanns [19].

Mechanisms of GPCR internalization

Like for other types of receptors, prolonged agonist stimulation often leads to GPCR internalization, which can occur via different pathways [2, 20-23]. Of these pathways, clathrin-mediated endocytosis (CME) is the best characterized and arguably most relevant one (Figure 1) [2, 20-23]. The first molecular event involved in GPCR internalization is the binding of a family of G protein-coupled receptor kinases (GRKs) to an agonist-occupied receptor, which phosphorylate multiple intracellular serine and threonine residues located in the 3rd intracellular loop or at the C-terminus of the receptor [24-27]. This is followed by binding of arrestins to the phosphorylated receptor, which plays a major role in both fast signal desensitization and receptor internalization [24, 26]. On the one hand, arrestins compete with G proteins for binding to the receptor, thus leading to signal desensitization. On the other hand, they promote receptor internalization via interacting with key proteins involved in the assembly of clathrin-coated pits (CCPs) such as the clathrin heavy chain and the clathrin adaptor protein AP2 [28, 29]. This leads to the recruitment of GPCRs into CCPs, which detach form the plasma membrane in a process that requires the small GTPase dynamin [30]. Receptors are then rapidly transferred to early endosomes, from where they can follow either of two main trafficking pathways [21, 31]. Some GPCRs are sorted out in the endosomal compartment, where they are dephosphorylated, to be then recycled back to the plasma membrane. Others are directed to lysosomes where they are degraded, leading to receptor downregulation [24, 26].

Role of receptor internalization in MAPK signaling

While rapid desensitization was shown to occur before receptor internalization and be mediated by receptor phosphorylation and β-arrestin recruitment, it also began to emerge that β-arrestin recruitment and receptor internalization might also exert other functions. In experiments using a dominant-negative dynamin mutant, Daaka et al. showed that receptor internalization is required for efficient ERK activation in response to β_2-adrenergic receptor stimulation [32]. Subsequently, it was shown that β-arrestins can bind several components of mitogen-activated protein kinase (MAPK) pathways [33, 34], thus promoting G protein-independent MAPK signaling. Since some GPCR are found on early endosomes in complex with β-arrestins, it has been suggested that these events result in endosomal MAPK signaling (Figure 1) [35]. Intriguingly, the activation of arrestin-bound ERK has been shown to favor cytoplasmic vs. nuclear effects of MAPK activation by preventing ERK translocation to the nucleus [34, 36]. However, the β-arrestin dependent activation of MAPKs can also occur while the receptors are still located on the plasma membrane. Thus, it remains to be clarified what is the relative contribution of cell surface vs. endosomal MAPK signaling. Moreover, some GPCRs that are poorly internalized are nevertheless able to efficiently induce MAPK signaling. This can be at least partially explained by the existence of other mechanisms leading to MAPK activation. Yet another possible explanation for these findings comes from a recent study on the β_1-adrenergic receptor – which internalizes poorly upon agonist stimulation – indicating that receptor activation can lead to recruitment of β-arrestin to CCPs and MAPK signaling from CCPs in the absence of receptors [37].

New paradigm of GPCR signaling from intracellular compartments

Although classical, G protein-dependent signaling has long been believed to be restricted to the plasma membrane, studies performed in the last ten years have provided strong evidence that internalized GPCRs can continue signaling on intracellular membranes (Figure 1). A first indication came from experiments on the Ste2 receptor, which is implicated in pheromone signaling in yeast [38]. Subsequently, our group and that of Jean-Pierre Vilardaga independently showed that the TSH and PTH receptors induce a persistent phase of cAMP production after internalization, which could be prevented by interfering with CME [39, 40]. Signaling by internalized TSH receptors was shown to differ from the one occurring at the plasma membrane in that it was required for efficient phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) and actin depolymerization in response to TSH, which is involved in thyroglobulin reuptake and, thus, in thyroid hormone release [39]. In the case of the PTH receptor, signaling was shown to be turned off by retromer – which mediates retrograde trafficking from endosomes to the trans-Golgi network – and endosomal acidification [41, 42]. These findings challenged the classical model of GPCR signaling by indicating that G protein signaling can also occur on intracellular membranes. They also pointed to early endosomes, in the case of the PTH receptor, and the Golgi/trans-Golgi network, in the case of the TSH receptor, as likely sites of intracellular GPCR signaling (Figure 1).

Further important evidence for G protein signaling on early endosomes has been subsequently obtained for the β_2-adrenegic receptor using fluorescently-tagged conformation-sensitive nanobodies selectively recognizing the active receptor and G_s protein [43].

More recently, our group used a combination of sensors based fluorescence resonance energy transfer (FRET) and a nanobody recognizing the active G_s protein to localize the subcellular compartment where endogenous TSH receptors are signaling in primary thyroid cells [44]. We found that the TSH receptor co-internalizes with TSH and traffics retrogradely to the trans-Golgi network, where it activates an endogenous pool of G_s protein. This leads to a delayed phase of local cAMP production and PKA activation at a critical position near the nucleus, which appears required for efficient CREB phosphorylation and gene transcription in response to TSH [44]. In contrast to previous observations with the PTH receptor, however, retromer was found to promote persistent TSH receptor signaling [44]. A requirement of receptor internalization for gene transcription has also been demonstrated for the β_2 adrenergic receptor [45]. Moreover, signaling in the Golgi complex has also been demonstrated for the β_1-adrenergic receptor [46]. However, in the case of the β_1-adrenergic receptor, it has been suggested that adrenalin, which is hydrophilic, crosses cellular membranes via the organic cation transporter 3 (OCT3) and reaches a pool of β_1-adrenergic receptors that reside in the Golgi complex [46]. In the meantime, signaling at intracellular membranes has been reported for several GPCRs, including the dopamine D1 receptor [47], vasopressin V2 receptor [48], glucagon-like peptide 1 (GLP1) receptor [49], pituitary adenylate cyclase activating polypeptide 1 (PACAP1) receptor [50] and glucose-dependent insulinotropic peptide (GIP) receptor [51].

A question left open by these studies was related to the apparent contrasting role of β-arrestins, which have a well-established role in signal desensitization and, at the same time, have been suggested to promote endosomal signaling. Intriguingly, recent structural studies indicate that β-arrestins can engage with two different domains of GPCRs, i.e. with either the C-tail or the seven-transmembrane core [52]. Moreover, a complex consisting of a receptor with the G protein bound to its seven-transmembrane core and β-arrestin 1 simultaneously bound to its C-tail has been directly observed by cry-electron microscopy [53].

All these studies suggest the existence of multiple intracellular locations for GPCR signaling (Figure 1). Some receptors, like the PTH and the β_2-adrenergic receptor, seem to signal prevalently from early endosomes. In contrast, the TSH and the β_1-adrennergic receptor signal on membranes of the Golgi/trans-Golgi network. Furthermore, there is evidence for GPCR signaling at other intracellular compartments such as the nuclear envelope [54] and, more recently, mitochondria. Indeed, cannabinoid CB1 receptors have been shown to be located on brain mitochondrial membranes, where they have been suggested to play a role in the amnesic effects of cannabinoids [55]. Similarly, melatonin has been shown to be produced inside neuronal mitochondria, where it activates local MT1 receptors [56]. The resulting signaling prevents stress-mediated cytochrome *c* release and caspase activation, thus contributing to melatonin neuroprotective effects [56]. Although we are only beginning to understand the implications of such a high degree of spatial control and complexity in GPCR signaling, it is likely that these mechanisms play an important role in allowing to discriminate among the multitude of extracellular signals that converge on a single cell.

Role of receptor internalization and trafficking in physiology and disease

Consistent with their crucial role of in GPCR signaling, receptor internalization and trafficking are deeply implicated in human physiology and, most likely, also in disease. A first important aspect regards the correct subcellular localization of receptors. Indeed, genetic mutations affecting receptor trafficking and causing reduced cell surface localization of receptors are known to be implicated in various human diseases, such as TSH resistance, familial idiopathic hypogonadotropic hypogonadism, Leydig cell hypoplasia or familial glucocorticoid deficiency [57].

With the recent demonstration that GPCRs can continue signaling after internalization, GPCR signaling at intracellular sites is also emerging as an important aspect of GPCR biology with implications in physiology and disease.

For the TSH receptor, signaling at the Golgi/trans-Golgi network appears required for both rapid effects of TSH – such as actin depolymerization, which is implicated in thyroglobulin reuptake and, thus, thyroid hormone release – and late ones, such as those on gene transcription. Continued signaling by TSH receptors after internalization might contribute to hyperthyroidism in Grave´s disease, where autoantibodies chronically activate the TSH receptor. Moreover, it might play a role in the pathogenesis of toxic thyroid adenomas and congenital/familial non-autoimmune hyperthyroidism, which are caused by activating TSH receptor mutations that are often associated with intracellular receptor accumulation [58, 59].

For the PTH receptor, which plays a critical role in regulating Ca^{2+} homeostasis and bone turnover and is a major pharmacological target for the therapy of osteoporosis, it has been shown that PTH_{1-34} but not the PTH related peptide $PTHrP_{1-36}$ – which activates the PTH receptor in a paracrine fashion – is capable of inducing persistent cAMP signaling [40]. Moreover, a PTH analog (M-PTH_{1-34}) that produces a more sustained cAMP response than PTH_{1-34} has been shown to induce larger increases in trabecular bone volume and cortical bone turnover, although the responsible mechanisms have not been fully elucidated [60]. Similarly, vasopressin and oxytocin can both induce cAMP/PKA signaling upon binding to the V2 receptor but only vasopressin leads to a strong antinatriuretic and antidiuretic effect [61-63]. Feinstein et al showed that this difference in signaling strength possibly results from different spatial signaling patterns induced by these two ligands [48]. These examples also suggest the possibility of designing GPCR agonists capable of preferentially inducing cell-surface vs. intracellular signaling. This might allow developing a new generation of GPCR agonists with tailored biological effects, and thus, potentially improved efficacy and tolerability.

More recently, our group took advantage of mice expressing a FRET sensor for cAMP to investigate cAMP signaling in intact ovarian follicles [64]. We found that activation of LH receptors with LH induces two waves of cAMP production that propagate within the follicles. Importantly, blocking receptor internalization prevented the second phase and partially inhibited the LH-induce resumption of meiosis in the oocyte [64]. These data indicate that LH receptor internalization plays an important role in mediating the biological effects of LH. Future studies appear required to further investigate the role of LH receptor signaling at intracellular sites in both female and male reproduction and its alterations in gonadal disorders.

With the growing number of studies investigating GPCR signaling at intracellular sites, the physiological implications of this phenomenon are increasing. These include a role in insulin secretion for the GLP1 receptor [49, 65], in renal water and sodium reuptake for the vasopressin V2 receptor [48] and in the excitability of cardiac neurons for the PACAP1 receptor [50].

Whereas receptor internalization has been mostly associated with prolonged cAMP signaling from intracellular sites, and thus mostly with slow biological effects, in the case of dopamine D1 receptors, it has been shown that these receptors are internalized very rapidly after agonist stimulation (within one minute) and that the resulting cAMP signaling from endosomal membranes increases neuronal excitability in striatal neurons [47].

So far, endosomal GPCR signaling has been mostly investigated in cellular models or using *ex vivo* preparations. Whereas these studies indicate that receptor internalization is required to mediate the biological effects of several hormones and neurotransmitters, further studies are required to investigate these processes *in vivo*. Interestingly, two recent studies have provided first *in vivo* evidence for a relevant physiological role of endosomal GPCR signaling. A first study investigated the role of internalization of the neurokinin 1 (NK1) receptor, which mediates the effects of substance P, on pain sensing [66]. As a result of pain stimuli, substance P is released from the terminals of primary sensory neurons in the dorsal horn of the spinal cord, where it induces activation and internalization of NK1 receptors expressed in second-order neurons [67, 68]. The results of the study indicate that inhibiting NK1 internalization and the resulting endosomal signaling attenuate nociception *in vivo*. This study also reports an innovative pharmacological strategy to selectively inhibit receptor endosomal signaling. For this purpose, the authors developed a cholestenol-conjugated antagonist, which accumulates in endosomes and is capable of inhibiting endosomal NK1 receptor signaling – which is required for nociception – without affecting NK1 receptors at the cell surface. Similar results were obtained by the same group for the calcitonin receptor-like receptor, which binds the calcitonin-gene related peptide (CGRP), and is also implicated in pain transmission [69].

Altogether, these new findings reinforce the view that receptor internalization and signaling are inextricably linked and cooperate to mediate the effects of several hormones and neurotransmitters. While genetic defects in receptor trafficking have been associated with selected human diseases and we are beginning to explore the physiological implications of new exciting discoveries in this field, further studies are needed to investigate the involvement of receptor internalization and signaling at intracellular sites in a large repertoire of diseases. Furthermore, there is an urgent need in drug development to move away from oversimplified models of GPCR signaling to take into account the complex interplay between signaling and internalization. This might allow going far beyond the concept of either activating or inhibiting a receptor – on which current drugs are based – and design more selective drugs capable of modulating receptor signaling at the desired time and subcellular location. The clinician should keep an eye on these exciting developments, which might revolutionize the way of treating common diseases in the near future.

Author contributions

A.G. and D.C. wrote the manuscript.

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Conflicts of interests

The authors declare no competing financial interests.

Practice points

- GPCRs are the largest family of receptors and mediate the effects of several hormones and neurotransmitters

- GPCRs are major pharmacological targets (at least 30% of all drugs on the market target these receptors)
- Prolonged stimulation with hormones or drugs leads to GPCR internalization
- Receptor internalization serves different functions and has been unexpectedly shown to be required for the biological effects of hormones and neurotransmitters
- Defects in receptor trafficking are involved in some genetic disorders and their involvement in common diseases needs to be further explored.
- The new finding that GPCRs signal not only at the plasma membrane but also on membranes of endosomes and the Golgi/trans-Golgi network might allow to develop a new generation of drugs with improved efficacy and less side effects.

Research agenda

- Further explore the role of GPCR internalization in human physiology.
- Investigate the involvement of receptor internalization and GPCR signaling on intracellular membranes in the pathogenesis of human diseases where GPCRs play an important role.
- Develop new drugs capable of selectively activating or inhibiting GPCR at the cell surface vs. at intracellular sites or to modify GPCR internalization and/or intracellular trafficking.

Figure legend

Figure 1: The complex interply between GPCR signaling and innternalization. Binding of a ligand to a receptor (**1**) induces a first phase of G protein-dependent signaling at the plasma membrane (**2**). This is followed by GRK-mediated phoshorylation of the receptor and β-arrestin binding, which results in rapid desensitization. At the same time, β-arrestin promotes MAPK signaling (**3**). β-arrestin also induces receptor internalization via clathrin-mediated endoctosis (CME). The internalized receptor can induce a second phase of G protein-dependent signaling from either early endosomes or the Golgi/trans-Golgi network (**4**). This second signaling phase has been shown to be biologically relevant for a growing number of GPCRs. Afterwards, the receptor is either degraded in lysosomes or recycled back to the plasma membrane (**5**) to undergo another round of signaling.

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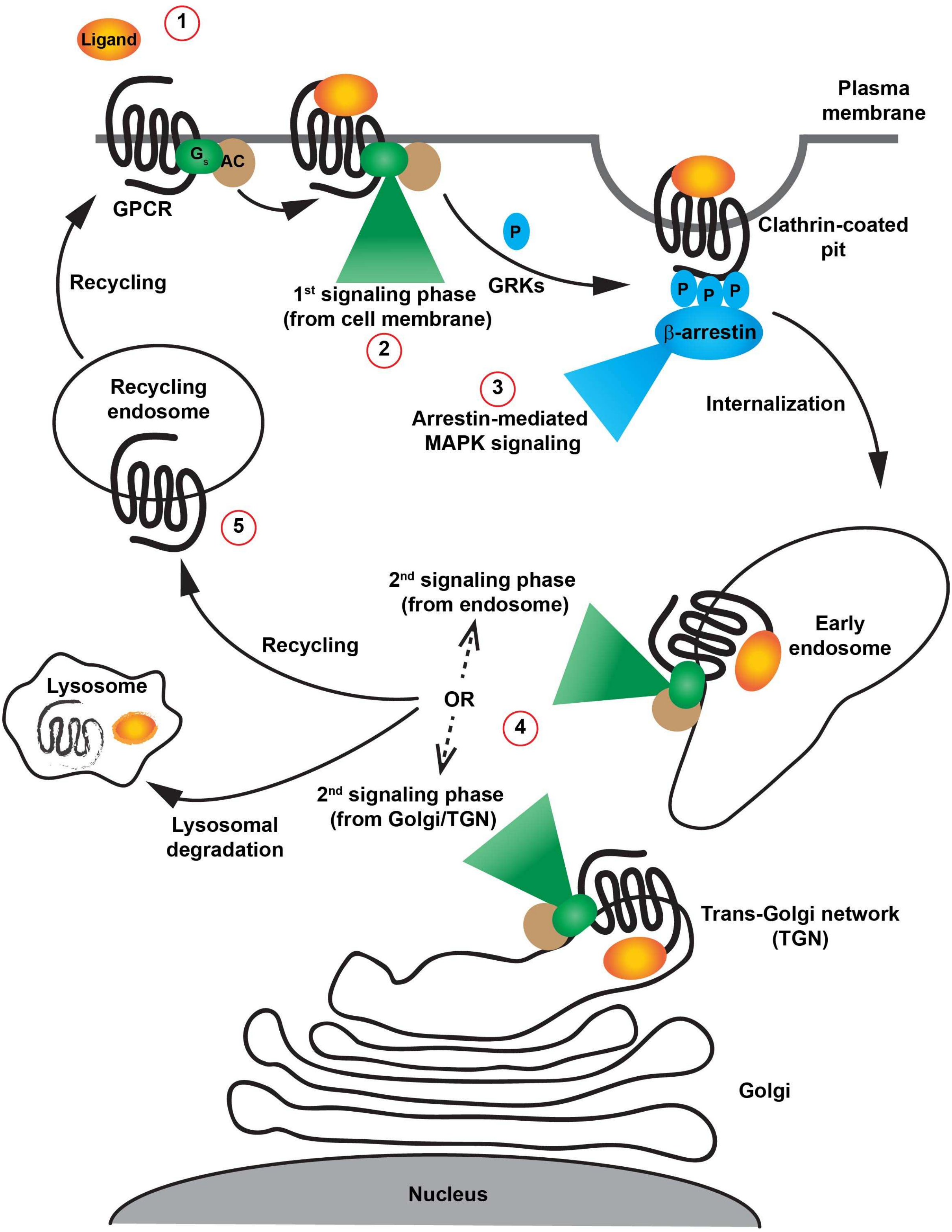

Ligand
1
Plasma membrane
GPCR
Gs
AC
P
Clathrin-coated pit
Recycling
1st signaling phase (from cell membrane)
GRKs
P P P
β-arrestin
2
Recycling endosome
3
Arrestin-mediated MAPK signaling
Internalization
5
2nd signaling phase (from endosome)
Early endosome
Recycling
Lysosome
OR
4
2nd signaling phase (from Golgi/TGN)
Lysosomal degradation
Trans-Golgi network (TGN)
Golgi
Nucleus