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Calebiro, Davide; Godbole, Amod

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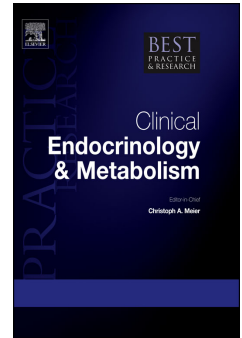
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Davide Calebiro, Amod Godbole



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Internalization of GPCRs: implication in receptor function, physiology and diseasesDavide Calebiro^{1,2,3*}, Amod Godbole^{1,4}¹Institute of Metabolism and Systems Research, University of Birmingham, Birmingham, UK²Centre of Membrane Proteins and Receptors (COMPARE), Universities of Birmingham and Nottingham, UK³Institute of Pharmacology and Toxicology and Bio-Imaging Center, University of Würzburg, Würzburg, Germany⁴Institute for Molecular Cell Biology, CMB-Center for Molecular Biomedicine, University Hospital Jena, Friedrich Schiller University Jena, Jena, Germany.

*Address correspondence to:

Davide Calebiro

Institute of Metabolism and Systems Research

College of Medical and Dental Sciences

Edgbaston

Birmingham

B15 2TT

Tel. +44 121 414 3928

Fax. +44 121 415 8712

1 Abstract

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

17

18 Keywords

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

20

21 Abbreviations

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

1 Introduction

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

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

13 A classic example of the role of these receptors in physiology is their involvement in the regulation of
14 heart contractility. β -adrenergic receptors located on the surface of cardiomyocytes mediate the
15 positive inotropic and chronotropic effects of adrenalin and noradrenalin, released upon sympathetic
16 activation. Binding of adrenalin or noradrenalin to these receptors, which are coupled to the G_s protein,
17 activate adenylyl cyclases to produce cAMP, which stimulates protein kinase A (PKA). PKA, in turn,
18 phosphorylates different molecules involved in cardiac contractility, including L-type Ca²⁺ channels,
19 phospholamban and troponin I, ultimately leading to enhanced cardiomyocyte contractility [5]. In
20 addition, cAMP directly promotes the opening of pacemaker (HCN) channels in the conductive tissue,
21 thus increasing heart rate [6, 7]. Parasympathetic activation counteracts these effects via release of
22 acetylcholine, which binds to muscarinic (M2) receptors coupled to G_{i/o}, thus inhibiting adenylyl cyclase
23 activation. In addition, the $\beta\gamma$ subunits released upon G_{i/o} activation stabilize the membrane potential via
24 activating potassium (GIRK) channels in the conductive tissue [8-12]. In the endocrine system, GPCRs
25 play an essential role as receptors for several hormones, hypothalamic releasing factors and local
26 modulators. All major known hypothalamic releasing (TRH, GnRH, CRH, GHRH) and inhibiting
27 (somatostatin, dopamine) hormones act via specific GPCRs [13-17]. With the exception of GH and PRL,
28 anterior (TSH, LH, FSH, ACTH, MSH) and posterior (vasopressin, oxytocin) pituitary hormones also signal
29 through activation of GPCRs [18]. For an extensive discussion of the specific roles of GPCRs and G
30 proteins in human physiology we refer the reader to the comprehensive review by Wettschureck and
31 Offermanns [19].

32

1

2 Mechanisms of GPCR internalization

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

20

21 Role of receptor internalization in MAPK signaling

22 While rapid desensitization was shown to occur before receptor internalization and be mediated by
23 receptor phosphorylation and β -arrestin recruitment, it also began to emerge that β -arrestin
24 recruitment and receptor internalization might also exert other functions. In experiments using a
25 dominant-negative dynamin mutant, Daaka et al. showed that receptor internalization is required for
26 efficient ERK activation in response to β_2 -adrenergic receptor stimulation [32]. Subsequently, it was
27 shown that β -arrestins can bind several components of mitogen-activated protein kinase (MAPK)
28 pathways [33, 34], thus promoting G protein-independent MAPK signaling. Since some GPCR are found
29 on early endosomes in complex with β -arrestins, it has been suggested that these events result in
30 endosomal MAPK signaling (Figure 1) [35]. Intriguingly, the activation of arrestin-bound ERK has been
31 shown to favor cytoplasmic vs. nuclear effects of MAPK activation by preventing ERK translocation to
32 the nucleus [34, 36]. However, the β -arrestin dependent activation of MAPKs can also occur while the

1 receptors are still located on the plasma membrane. Thus, it remains to be clarified what is the relative
2 contribution of cell surface vs. endosomal MAPK signaling. Moreover, some GPCRs that are poorly
3 internalized are nevertheless able to efficiently induce MAPK signaling. This can be at least partially
4 explained by the existence of other mechanisms leading to MAPK activation. Yet another possible
5 explanation for these findings comes from a recent study on the β_1 -adrenergic receptor – which
6 internalizes poorly upon agonist stimulation – indicating that receptor activation can lead to recruitment
7 of β -arrestin to CCPs and MAPK signaling from CCPs in the absence of receptors [37].

8

9 **New paradigm of GPCR signaling from intracellular compartments**

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

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

29 More recently, our group used a combination of sensors based fluorescence resonance energy transfer
30 (FRET) and a nanobody recognizing the active G_s protein to localize the subcellular compartment where
31 endogenous TSH receptors are signaling in primary thyroid cells [44]. We found that the TSH receptor
32 co-internalizes with TSH and traffics retrogradely to the trans-Golgi network, where it activates an

1 endogenous pool of G_s protein. This leads to a delayed phase of local cAMP production and PKA
2 activation at a critical position near the nucleus, which appears required for efficient CREB
3 phosphorylation and gene transcription in response to TSH [44]. In contrast to previous observations
4 with the PTH receptor, however, retromer was found to promote persistent TSH receptor signaling [44].
5 A requirement of receptor internalization for gene transcription has also been demonstrated for the β_2
6 adrenergic receptor [45]. Moreover, signaling in the Golgi complex has also been demonstrated for
7 the β_1 -adrenergic receptor [46]. However, in the case of the β_1 -adrenergic receptor, it has been
8 suggested that adrenalin, which is hydrophilic, crosses cellular membranes via the organic cation
9 transporter 3 (OCT3) and reaches a pool of β_1 -adrenergic receptors that reside in the Golgi complex [46].
10 In the meantime, signaling at intracellular membranes has been reported for several GPCRs, including
11 the dopamine D1 receptor [47], vasopressin V2 receptor [48], glucagon-like peptide 1 (GLP1) receptor
12 [49], pituitary adenylate cyclase activating polypeptide 1 (PACAP1) receptor [50] and glucose-dependent
13 insulinotropic peptide (GIP) receptor [51].

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

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

1 these mechanisms play an important role in allowing to discriminate among the multitude of
2 extracellular signals that converge on a single cell.

3

4 **Role of receptor internalization and trafficking in physiology and disease**

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

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

14 For the TSH receptor, signaling at the Golgi/trans-Golgi network appears required for both rapid effects
15 of TSH – such as actin depolymerization, which is implicated in thyroglobulin reuptake and, thus, thyroid
16 hormone release – and late ones, such as those on gene transcription. Continued signaling by TSH
17 receptors after internalization might contribute to hyperthyroidism in Grave's disease, where
18 autoantibodies chronically activate the TSH receptor. Moreover, it might play a role in the pathogenesis
19 of toxic thyroid adenomas and congenital/familial non-autoimmune hyperthyroidism, which are caused
20 by activating TSH receptor mutations that are often associated with intracellular receptor accumulation
21 [58, 59].

22 For the PTH receptor, which plays a critical role in regulating Ca^{2+} homeostasis and bone turnover and is
23 a major pharmacological target for the therapy of osteoporosis, it has been shown that PTH_{1-34} but not
24 the PTH related peptide PTHrP_{1-36} – which activates the PTH receptor in a paracrine fashion – is capable
25 of inducing persistent cAMP signaling [40]. Moreover, a PTH analog (M- PTH_{1-34}) that produces a more
26 sustained cAMP response than PTH_{1-34} has been shown to induce larger increases in trabecular bone
27 volume and cortical bone turnover, although the responsible mechanisms have not been fully elucidated
28 [60]. Similarly, vasopressin and oxytocin can both induce cAMP/PKA signaling upon binding to the V2
29 receptor but only vasopressin leads to a strong antinatriuretic and antidiuretic effect [61-63]. Feinstein
30 et al showed that this difference in signaling strength possibly results from different spatial signaling
31 patterns induced by these two ligands [48]. These examples also suggest the possibility of designing
32 GPCR agonists capable of preferentially inducing cell-surface vs. intracellular signaling. This might allow

1 developing a new generation of GPCR agonists with tailored biological effects, and thus, potentially
2 improved efficacy and tolerability.

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

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

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

20 So far, endosomal GPCR signaling has been mostly investigated in cellular models or using *ex vivo*
21 preparations. Whereas these studies indicate that receptor internalization is required to mediate the
22 biological effects of several hormones and neurotransmitters, further studies are required to investigate
23 these processes *in vivo*. Interestingly, two recent studies have provided first *in vivo* evidence for a
24 relevant physiological role of endosomal GPCR signaling. A first study investigated the role of
25 internalization of the neurokinin 1 (NK1) receptor, which mediates the effects of substance P, on pain
26 sensing [66]. As a result of pain stimuli, substance P is released from the terminals of primary sensory
27 neurons in the dorsal horn of the spinal cord, where it induces activation and internalization of NK1
28 receptors expressed in second-order neurons [67, 68]. The results of the study indicate that inhibiting
29 NK1 internalization and the resulting endosomal signaling attenuate nociception *in vivo*. This study also
30 reports an innovative pharmacological strategy to selectively inhibit receptor endosomal signaling. For
31 this purpose, the authors developed a cholesterol-conjugated antagonist, which accumulates in
32 endosomes and is capable of inhibiting endosomal NK1 receptor signaling – which is required for

1 nociception – without affecting NK1 receptors at the cell surface. Similar results were obtained by the
2 same group for the calcitonin receptor-like receptor, which binds the calcitonin-gene related peptide
3 (CGRP), and is also implicated in pain transmission [69].

4 Altogether, these new findings reinforce the view that receptor internalization and signaling are
5 inextricably linked and cooperate to mediate the effects of several hormones and neurotransmitters.

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

16 17 **Author contributions**

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

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24 Graduate School of Life Sciences, University of Würzburg.

25 26 **Conflicts of interests**

27 The authors declare no competing financial interests.

28 29 **Practice points**

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

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

11

12 **Research agenda**

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

18

19 **Figure legend**

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

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