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Biased signaling in naturally occurring mutations of G protein-coupled receptors associated with diverse human diseases

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Abstract

G protein-coupled receptors (GPCRs) play critical roles in transmitting a variety of extracellular signals into the cells and regulate diverse physiological functions. Naturally occurring mutations that result in dysfunctions of GPCRs have been known as the causes of numerous diseases. Significant progresses have been made in elucidating the pathophysiology of diseases caused by mutations. The multiple intracellular signaling pathways, such as G protein-dependent and β-arrestin-dependent signaling, in conjunction with recent advances on biased agonism, have broadened the view on the molecular mechanism of disease pathogenesis. This review aims to briefly discuss biased agonism of GPCRs (biased ligands and biased receptors), summarize the naturally occurring GPCR mutations that cause biased signaling, and propose the potential pathophysiological relevance of biased mutant GPCRs associated with various endocrine diseases.

Keywords

G protein-coupled receptor; naturally occurring mutation; biased signaling; pathophysiology

1. Introduction

G protein-coupled receptors (GPCRs), with about 800 members identified in human genome, constitute the largest superfamily of cell surface receptors [1, 2]. Their topology consists of an extracellular N-terminus, a bundle of seven transmembrane domains (TMDs, TMD1 to TMD7) within the plasma membrane, connected by three extracellular loops (ECLs, ECL1 to ECL3) and three intracellular loops (ICLs, ICL1 to ICL3), and an intracellular C-terminus. GPCRs are divided into six subfamilies, based on sequence homology and functional similarity, including rhodopsin-like (Family A), secretin-like

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Declaration of Interest

The authors declare that there is no conflict of interest with this manuscript.

(Family B), metabotropic glutamate (Family C), pheromone (Family D), cAMP (Family E), and frizzled (Family F) GPCRs (information on the GPCR database: https://gpcrdb.org). Only four families (A, B, C, and F) among them are found in humans. Family A rhodopsin-like GPCRs, consisting of more than half of all GPCRs, are the largest family in this superfamily.

GPCRs transmit a variety of extracellular signals into the cells. These signals comprise chemical and sensory stimuli, such as hormones, neurotransmitters, chemokines, ions, light, and odorants [1], highlighting the significant roles of GPCRs in mediating diverse physiological functions. Therefore, GPCRs have been exploited as therapeutic targets with particular longstanding interest over the past several decades. Another reason of GPCRs being good drug targets is that ligands bind to GPCRs (non-olfactory GPCRs) in the orthosteric binding site, a deep cleft on the extracellular side of the receptor, making it easier to develop drugs since they do not need to be structurally and chemically modified to cross the plasma membrane [3], compared to drugs targeting intracellular kinases and proteases. Up to 2017, 481 of all FDA-approved drugs target GPCRs, accounting for approximately 34% of all approved drugs. They mediate the effects via 107 GPCRs, which constitutes only 27% of the non-olfactory human GPCRs [4].

GPCR-mediated signal transduction starts with ligand binding to receptors, followed by receptor conformational change and activation of signal mediators (Fig. 1). The conventional intracellular signaling is mediated by heterotrimeric G protein consisting of α, β, and γ subunits. Once receptor is activated, the α subunit dissociates from βγ heterodimer and then activates enzymes generating the second messengers and downstream mediators to transduce the signal. Four ubiquitously expressed subtypes of α subunit of G protein, including $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$, have been discovered based on their different sequences and functions [5] (Fig. 1). Activated $G\alpha_s$ increases the activity of adenylyl cyclase (AC) (a membrane enzyme catalyzing the conversion of ATP to 3',5'-cyclic adenosine monophosphate (cAMP) and pyrophosphate) to induce intracellular cAMP generation and subsequently activate protein kinase A (PKA). Conversely, activated $G\alpha_{i/o}$ decreases adenylyl cyclase activity to inhibit intracellular cAMP generation and PKA activity. Activated $G\alpha_{q/11}$ triggers a distinct pathway by activating phospholipase C-β (PLCβ), which in turn induces diacylglycerol (DAG)-protein kinase C (PKC) pathway and inositol 1,4,5-trisphosphate (IP3)-Ca^{2+} mobilization. Activated $G\alpha_{12/13}$ primarily affect Rho-specific guanine nucleotide exchange factor (RhoGEF), further activating small GTPase RhoA and increasing Rho-kinase activity [6]. In addition to α subunit, βγ heterodimer mediates signaling processes and modulates activation of ion channels, such as G protein-regulated inwardly rectifying K^+ channels [7] and Ca^{2+} channels [8] (reviewed in [9]).

Naturally occurring mutations that affect GPCR signal transduction can cause either impaired or enhanced protein function, which are classified as loss-of-function or gain-of-function mutations, respectively [10–14]. Loss-of-function mutations prevent signaling through several ways, such as defective receptor biosynthesis or trafficking, impairing ligand binding, and/or impairing basal or agonist-induced signaling [12, 14, 15]. For instance, rhodopsin is a light-sensitive GPCR essential for visual phototransduction. The mutant rhodopsin P23H is the first identified mutant in GPCRs that results in human diseases [16].

Biochemical and biophysical studies reveled that P23H is trapped intracellularly by quality control system in endosomal reticulum due to misfolding, thus exhibiting signaling deficiency and causing retinitis pigmentosa, a degenerative eye disease associated with severe vision impairment [17, 18]. Since the discovery of rhodopsin P23H mutation, the list of diseases resulting from GPCR loss-of-function mutations keeps expanding [12], including idiopathic hypogonadotropic hypogonadism (IHH), Kallmann syndrome, oligospermia and subfertility, ovarian dysgenesis and other human diseases (Table 1).

Gain-of-function mutations can be due to decreased specificity, enhanced sensitivity in response to ligands, increased basal activity (constitutive activation), or increased receptor expression, thereby causing hyper-responsive phenotypes [11, 14]. The rhodopsin K296E is the first reported naturally occurring constitutively active mutation in GPCRs that is associated with human diseases [19]. Robinson et al. (1992) showed that the inverse agonist of rhodopsin, 11-*cis*-retinal, fails to suppress the constitutive activity of K296E since the retinal binding site is mutated, potentially explaining the mechanism that K296E causes retinitis pigmentosa with a persistent stimulation of phototransduction pathway [20]. Since then, numerous cases and studies have been reported, showing the gain-of-function mutations of GPCRs result in a wide range of human diseases [14, 21], including precocious puberty, spontaneous ovarian hyperstimulation, familial gestational hyperthyroidism and other diseases (Table 2).

Polymorphisms at GPCRs, generally defined as occurring in at least 1% of the population, can also be associated with various diseases [22]. Polymorphisms identified in coding regions usually result in dysfunctional receptors (e.g. rhodopsin, μ-opioid receptor (μ-OR), adrenergic receptors (ARs), follicle-stimulating hormone (FSH) receptor (FSHR), thyroid-stimulating hormone (TSH) receptor (TSHR), and vasopressin type 2 receptor (AVPR2)), while those identified in noncoding regions are believed to affect promoter activity or transcription (e.g. ARs, bradykinin B2 receptor, chemokine receptors, and dopamine receptors) (reviewed in [22]). Examination of these GPCR mutations and polymorphisms has a significant impact on our understanding of the relationship between genetic variants and various human diseases.

2. Multiple GPCR signaling pathways

For over two decades, biochemical and pharmacological studies demonstrated that a large number of GPCRs are pleiotropic receptors that can couple to multiple G proteins simultaneously [23]. For example, TSHR couples to all four G protein subtypes with similar potencies in thyroid gland [24, 25]. Other examples of GPCRs coupling to more than one G protein subtype include galanin receptor type 2 ($G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$) [26], parathyroid hormone (PTH) receptor (PTHR) ($G\alpha_s$, $G\alpha_i$, and $G\alpha_{q/11}$) [27], luteinizing hormone (LH) receptor (LHR) ($G\alpha_s$, $G\alpha_i$ and $G\alpha_q$) [28], melatonin receptor ($G\alpha_{i/o}$ and $G\alpha_q$) [29], and melanin-concentrating hormone receptor ($G\alpha_{i/o}$ and $G\alpha_q$) [30] (reviewed in [23]). Thus, the multiplicity of G protein coupling known for a variety of receptors provides one mechanism for the activation of multiple intracellular signaling pathways by a single receptor.

In the classical view, G protein signaling is typically terminated by desensitization, resulting from phosphorylation of the cytoplasmic tail and/or ICLs by G protein-coupled receptor kinases (GRKs) and recruitment of β-arrestins (β-arrestin-1 or β-arrestin-2) by phosphorylated receptor, with β-arrestins sterically competing with G-protein for binding to the GPCRs [31, 32]. Further, specific GRK-mediated phosphorylation pattern directs distinct β-arrestin conformations (phosphorylation "bar code" theory), thereby controlling the interaction partners and related functions of β-arrestins [33]. β-arrestins can act as the adaptors for clathrin, transporting phosphorylated receptors to clathrin-coated pits for receptor internalization. The internalized receptors can be further transported into endosomes where receptors can either recycle to the plasma membrane for resensitization or move into lysosomes for degradation. In addition, β-arrestins can serve as scaffolds to mediate signaling to mitogen-activated protein kinases (MAPKs) activation, such as extracellular signal-regulated kinases (ERKs), c-Jun-N-terminal kinases (JNKs), and p38 kinases, as well as other protein kinases [34–37] (reviewed in [32, 38, 39]) (Fig. 1). Although ERK1/2 activation can be caused by activated G protein, the ERK1/2 pathway mediated by β-arrestins has been shown to be segregated from G protein activation [40, 41]. In the case of type 1 angiotensin II receptor (AT1R), β-arrestins mediate slower and persistent ERK1/2 activation, whereas G protein mediates rapid and transient ERK1/2 activation [41].

In addition to G protein signaling from plasma membrane, the signaling originating from intracellular compartments, such as early endosomes and Golgi apparatus, also represents an important aspect of GPCR signaling and challenges the longstanding concept that GPCR only signals from plasma membrane and activation is silenced by receptor internalization [42, 43]. Up to now, several GPCRs have been reported to trigger sustained G protein signaling from intracellular compartments after internalization. Examples include PTHR, β_2-AR, TSHR and AVPR2 for $G\alpha_s$ signaling [44–47] and calcium-sensing receptor (CaSR) for $G\alpha_{q/11}$ signaling [48] from endosomes, and sphingolipid S1P receptor ($S1P_1R$) for $G\alpha_i$ signaling from Golgi [49]. Although binding of G proteins and β-arrestins to prototypical GPCRs was thought to be mutually exclusive, structural analyses have revealed that in some GPCRs, internalized receptor could form a super-complex with both β-arrestin and G protein, leading to this sustained endosomal G protein signaling [47, 50]. In this complex, the receptor couples to G protein in canonical fashion and also allows β-arrestin binding to function as a regulator for G protein signaling, along with β-arrestin-mediated signaling [42, 50].

The diverse intracellular signaling pathways initiated by GPCRs have been shown to cause different cellular and physiological consequences and defects in these signaling pathways will lead to many diseases. Traditionally, functional characterizations of mutant receptors associated with diseases have primarily employed the canonical signaling pathway or preferential G protein, such as the $G\alpha_{s/i}$-cAMP or $G\alpha_q$-IP3/Ca^{2+} pathway. Thus, mutations are classified as loss-of-function or gain-of-function mutations according to the properties of this conventional pathway. However, with recent expansion of our understanding of the molecular pharmacology of GPCRs, this approach seems to be oversimplified, since the other signaling pathways may also be indispensable in mediating the physiological responses and contributing to pathophysiology when disrupted. Therefore, it is necessary to

determine whether the mutation affects multiple signaling pathways and whether the mutation differentially influences these signaling pathways, exhibiting biased signaling.

The traditional methods of measuring GPCR activation is to directly detect the GTP/GDP exchange ([^{35}S]-GTPγS binding assay widely used for $G\alpha_s$ and $G\alpha_q$ signaling) or second messenger levels (e.g. cAMP assay for $G\alpha_s$ and $G\alpha_i$ signaling, and inositol phosphate (IP) accumulation and intracellular calcium assays for $G\alpha_{q/11}$ signaling). Recent advances on biochemical and biophysical techniques added more dimensions to GPCR signaling assays and facilitated us to investigate multiple signaling pathways rather than only conventional one. Cell-based reporter gene assay provides a popular homogenous assay system for assessment of GPCR signaling owing to its high sensitivity and wide dynamic range. In the reporter gene assay, GPCR-mediated second messenger regulates specific transcription factor, thereby activating or inhibiting a responsive element located in the promoter of reporter gene, which in turn regulates the reporter protein expression and this process can be quantitatively measured [51]. The $G\alpha_s$-mediated cAMP production leads to activation of cAMP response element binding protein (CREB) [52]; $G\alpha_{q/11}$-mediated intracellular Ca^{2+} mobilization can activate nuclear factor of activated T cells (NFAT) [53]; $G\alpha_{12/13}$-mediated formation of filamentous actins activate serum response factor (SRF) [54]; ERK1/2 activation also activates SRF and downstream serum response element (SRE) [55]. The reporter can be β-galactosidase, luciferase, green fluorescent protein, or β-lactamase [51]. Of them, the luciferase reporter gene assay is the most commonly used for measurement of GPCR signaling.

Recently developed GPCR biosensors can monitor real-time GPCR activation in living cells under physiological conditions. For example, resonance energy transfer (RET) assay is based on the transfer of energy from an energy donor (fluorescent or bioluminescent molecule) attached to GPCR to an energy acceptor (another fluorescent molecule) attached to G protein or β-arrestin. The energy transfer can be changed and monitored when the proximity of GPCR to G protein or β-arrestin changes [56]. In the early studies of GPCR biased agonism, characterization of β-arrestin recruitment has significantly evolved with the development of FRET/BRET assays [56–58].

It is possible that differences may appear when different signaling assays are used to access GPCR activation [59]. For example, in one study of C-C chemokine receptor 7 (CCR7),both endogenous ligands (CCL19 and CCL21) activate G protein signaling (measured by [^{35}S]-GTPγS binding assay), but only CCL19 results in an increased β-arrestin recruitment (measured by translocation to cell membrane) and β-arrestin-dependent ERK1/2 activation, suggesting ligand bias of CCL21 at CCR7 [60]. However, in one later study, inconsistent result was observed when BRET biosensors were used for measurement of both G protein coupling and β-arrestin-recruitment. CCL21 is shown to be less potent in activating G proteins and recruiting β-arrestin, refuting the previous notion of ligand bias [61]. Therefore, the reliability of signaling properties of receptor is likely related to the signaling assays used. It is more reliable to directly detect G protein coupling or β-arrestin recruitment using FRET/BRET biosensors, compared to using different types of signaling assays. Future studies involving multiple GPCR signaling assays should take this into consideration.

3. Biased agonism of GPCRs

Biased signaling is the preferential activation of one signaling pathway over another or others, different from the classical view on GPCR signal transduction that multiple downstream signaling pathways are activated with similar efficacies [32, 62]. The phenomenon of biased agonism can be explained by the multi-state model of receptor activation. The theory states that receptor activation is a highly dynamic process in which multiple active conformations can be induced by different ligands to mediate distinct signaling pathways [32, 63, 64]. Biased agonism, in most cases, is a property of the ligand-receptor complex. Therefore, biased signaling initiated by the receptor may be due to either ligand effect (biased ligand) or receptor itself (biased receptor) (Fig. 2A). Additionally, for many GPCRs with constitutive activities, biased signaling can also be induced by receptor in the absence of ligand stimulation, known as biased constitutive signaling.

3.1. Biased ligands

Biased ligands selectively stabilize GPCRs to distinct conformations, stimulating or inhibiting one (or some) of the possible signaling pathways [65, 66]. Some biased ligands preferentially stimulate the signaling associated with beneficial responses, providing the potential to develop new therapeutics with fewer side effects, greater efficacy and higher safety (for excellent reviews, see [67–70] for examples). Here, we highlight several biased ligands, selectively activating either G protein or β-arrestin signaling, or signaling of different G protein subtypes.

G protein biased ligands exert therapeutic benefits via inducing G protein-dependent signaling with little or no β-arrestin signaling. Biased ligands targeting G protein-dependent signaling to opioid receptors (ORs) provide striking examples. μ-Opioid agonists like morphine, fentanyl, and oxycodone exhibit powerful analgesic effect by activating μ-OR. However, their clinical utility is greatly limited due to the adverse events including nausea and vomiting, respiratory depression, constipation, and sedation [69, 71, 72]. Studies with β-arrestin-2 knockout mice showed that the $G\alpha_i$ signaling is associated with powerful opioid analgesia, while β-arrestin-2 signaling is involved in adverse events in respiratory and gastrointestinal systems [73–75]. Therefore, identification of G protein-biased ligands devoid of β-arrestin activation may provide powerful analgesics with increased safety and tolerability [72]. TRV130, PZM21, and SR-17018, are three novel μ-OR ligands identified by high-throughput screening, structure-based drug design, and synthesis-driven structure-activity relationship, respectively [76–78]. These ligands stimulate $G\alpha_i$ signaling that is associated with analgesia without β-arrestin-induced nausea or vomiting [69, 72]. Compared to μ-OR agonists, κ-opioid receptor (KOR) agonists do not activate the reward pathway, thus exhibiting nonaddictive analgesic effects [79]. Likewise, KOR exerts analgesic properties by activating $G\alpha_i$ signaling, while β-arrestin signaling is associated with notable dysphoria [79]. 6'GNTI is a partial agonist of KOR that stimulates G protein signaling with potent analgesic effect but not β-arrestin signaling [79, 80].

In other GPCRs, β-arrestin-biased ligands are preferred therapeutic agents. Due to the important roles in regulating cardiovascular and pulmonary functions, the ARs are the most common therapeutic targets of therapeutic agents. However, activation of the ARs shows

biphasic effects in cardiovascular and pulmonary functions [81]. In β_1-AR, β-arrestin mediates the transactivation of epidermal growth factor receptor, exerting cardioprotective effect, whereas G protein signaling is thought to be cardiotoxic [82–84]. Therefore, identifying biased ligands to selectively target β-arrestin signaling in ARs shows therapeutic benefits [85]. Carvedilol is a β-arrestin-biased ligand of β_1-AR with improved cardioprotective effect [86, 87]. Rockman and colleagues revealed a previously unrecognized signaling mechanism of biased agonism resulting from carvedilol: 1). carvedilol results in the transition of the β_1-AR from $G\alpha_s$ coupling to $G\alpha_i$ coupling; 2). the recruitment of $G\alpha_i$ leads to a unique receptor conformation, initiating β-arrestin-biased signaling [85]. The β_2-AR biased ligands, propranolol, ICI 118,551, and BI-167107, selectively stimulate β-arrestin signaling, which is beneficial for hypertension and heart failure treatment [78, 88–91].

Some biased ligands activate receptors to couple with particular G protein subtype(s). For example, in α_2-AR, catecholamine analogues are more potent in activating $G\alpha_q$ signaling while imidazoline or ox-/thiazoloazepine analogues selectively activate $G\alpha_{i/o}$ signaling [92]. Ligand with different modification or concentration selectively activates different G protein signaling pathways. The acidic (sialylated) isoforms of gonadotropin activate coupling to $G\alpha_s$, whereas neutral/basic gonadotropin isoforms interact with $G\alpha_i$ signaling in gonadotropin receptors [93]. Different glycoforms of FSH also exert functional selectivity in Gs protein versus β-arrestin mediated signaling [94]. Thiazolidinone analogs activate $G\alpha_s$ signaling through FSHR at lower concentrations and $G\alpha_i$ signaling at higher concentrations, similar to the biased signaling induced by differentially glycosylated gonadotropins [93, 95, 96].

In addition to the G protein or β-arrestin biased ligands, ligands that activate the receptors with temporal or spatial preferences could be considered as a new type of biased ligands [42, 66]. The endosomal cAMP signaling potentially provides a mechanism to explain that the ligands exert temporal or spatial bias in signal generation at different subcellular localizations [97]. For example, analyses of FRET biosensors showed that PTHR, which plays an important role in regulating mineral ion homeostasis, exerts endosomal cAMP signaling [45]. PTH analogues and PTH-related peptide (PTHrP) bind to PTHR with different conformations, resulting in bias in signal generation [45, 97, 98]. The PTHrP preferentially triggers the transient cAMP signaling which are derived from ligand-receptor complexes at the plasma membrane, while the PTH analogues preferentially trigger the prolonged endosomal cAMP signaling [97, 99, 100]. The emerging endosomal PTHR cAMP signaling provides new insights into therapy for patients with hypoparathyroidism [42]. Due to the short half-life of the injected PTH (1–34), patients with hypoparathyroidism are currently treated by vitamin D and calcium supplements [101]. The LA-PTH is a PTH analog that preferentially trigger the prolonged endosomal cAMP signaling. Mouse studies showed that LA-PTH results in prolonged hypercalcemic and LA-PTH is now in preclinical development [42, 100].

Five melanocortin receptors (MCRs), members of Family A GPCRs, are unique in that there are two endogenous antagonists, Agouti-signaling protein (ASIP) for MC1R and Agouti-related protein (AgRP) for MC3R and MC4R. In the grey squirrel, ASIP is an inverse

agonist at the wild-type (WT) MC1R but an agonist at a melanic variant [102]. We showed that although AgRP is an inverse agonist (negative antagonist) at the $G\alpha_s$-cAMP pathway [103], it is an agonist of ERK1/2 activation at both MC3R and MC4R [104, 105]. Several additional small molecule inverse agonists also serve as biased ligands at the MC4R [104] (reviewed in [106]). A small molecule, AP1189, has also been shown to be a biased agonist at the MC1R and MC3R, activating the ERK1/2 pathway but not the $G\alpha_s$-cAMP pathway [107].

3.2. Biased allosteric modulators

Allosterism has been shown to exist widely in various types of proteins since it was first described in the hemoglobin [108]. GPCRs are thought as natural allosteric machines because agonist binding on the extracellular portion of the receptor causes conformational change in the receptor, resulting in the binding of intracellular signal transducers such as G proteins or β-arrestins on the intracellular portion of the receptor [109]. Allosteric modulation of GPCRs, in recent years, has been emerging as a promising novel approach to develop therapeutic agents for treatment of various disorders, conferring advantages over orthosteric ligands such as potential enhanced selectivity for different subtypes of the same family of receptors and limited maximal signaling [67, 109, 110]. Allosteric modulators are a group of molecules that can change receptor response to its orthosteric ligand by allosterically binding to the receptor. They are generally classified as positive allosteric modulators (PAMs), which increase the receptor response to orthosteric ligand, negative allosteric modulators (NAMs), which decrease receptor responsiveness, and neutral allosteric ligands, which have no effects on the receptor responsiveness although allosterically bind to receptor [111].

Biased allosteric modulators are ligands that bind to allosteric sites of receptors and preferentially modulate particular signaling pathways triggered by the orthosteric ligands. This type of modulators is effective in modulation of GPCR signaling with therapeutic purposes. The allosteric modulators of the CaSR (cinacalcet, NPS-R568 and NPS-2143), promote biased signaling by exerting greater allosteric modulation of intracellular Ca^{2+} mobilization relative to ERK1/2 activation in response to extracellular Ca^{2+} [112]. The small molecule allosteric modulator, Org27569, displays biased allosteric effects on the CB1 cannabinoid receptor by blocking cAMP inhibition triggered by $G\alpha_{i/o}$ but having little or no effect on ERK1/2 activation with the presence of cannabinoid ligands [113].

One non-steroidal anti-inflammatory drug, fenoprofen, was shown to have positive allosteric modulator activity at some MCRs, with pro-resolving properties by promoting macrophage phagocytosis and efferocytosis [114]. Our recent study further showed that fenoprofen is a biased allosteric enhancer as it selectively induces ERK1/2 activation rather than cAMP production at both WT and naturally occurring mutant MC3Rs [115]. A variant of canine β-defensin 103 (a high-affinity agonist for MC1R [116]), cBD103ΔG23, is a weak allosteric agonist at the canine MC4R [117]. Although an orthosteric ligand for human MC4R [118], THIQ is an allosteric ligand at a fish MC4R [119].

3.3. Biased receptors

Biased receptor is a mutant receptor capable of selectively adopting particular active conformation upon ligand stimulation to induce specific signaling pathway, different from the WT receptor. Compared with extensive studies on identifying biased ligands, biased receptors are not widely investigated. However, the importance of characterization of biased receptors should also be highlighted since investigation of them provides an effective way to elucidate the relationship between receptor conformational change and downstream signaling. According to the multi-state model, it is possible that the signaling pathways can be selectively activated with specific amino acid modifications in GPCRs.

Indeed, mutagenesis studies have suggested that some residues are critical in maintaining the specific conformations of GPCRs, and the switches of different intracellular signaling pathways through mutant receptors are the consequences of mutations on the key residues. For instance, mutating the highly conserved DRY motif of AT1R to AAY leads to unbalanced activation of $G\alpha_{q/11}$ signaling and β-arrestin-dependent ERK1/2 signaling [120]. Novel mutations of β_2-AR designed based on evolutionary trace analysis, including T68F, Y132G, and Y219A, are shown to lead the receptor to adopt conformation incapable of activating $G\alpha_s$ but capable of recruiting β-arrestins to initiate receptor internalization and β-arrestin-dependent ERK1/2 signaling [121]. By predicting and mutating the key restudies potentially interacting with G proteins or β-arrestins, several engineered biased mutant D2 dopamine receptors, including β-arrestin-biased (A135R and M140D) and $G\alpha_{i/o}$ protein-biased (L125N and Y133L) mutants, have been characterized [122]. Biased signaling caused by mutating key residues were also reported in ghrelin receptor [123], apelin receptor [124, 125], neuropeptide Y receptors [126], and CB1 cannabinoid receptor [127] (reviewed in [128]).

We have used alanine scanning mutagenesis to systematically study the structure-function relationship of the neural melanocortin receptors, MC3R and MC4R. By comparing $G\alpha_s$-cAMP and ERK1/2 signaling, we have identified numerous biased mutant receptors [129–132]. For example, in our studies of L140 in human MC4R (numbered as 3.43 according to the Ballesteros and Weinstein numbering scheme [133]), a highly conserved amino acid in Family A GPCRs and shown to be important in constraining the receptor in inactive conformation (reviewed in Ref. [14]), in addition to confirming that this residue is also important in constraining the MC4R in inactive conformation (some mutants are constitutively active), we showed that some mutants have biased constitutive signaling and some mutants exhibit biased ligand-stimulated signaling [130], similar to a previous study [134].

Recently, biophysical and structural studies have started identifying the structural elements dictating biased signaling in different GPCRs. For example, using fluorine-19 nuclear magnetic resonance at the β_2-AR, it was shown that agonists and biased ligands induce different conformations, primarily at TMD6 and TMD7, respectively [135], with the biased ligands stabilizing the active conformation in TMD7 longer than balanced agonists [136]. Using double electron-electron resonance spectroscopy and molecular dynamic simulations at the AT1R, different conformational ensembles are observed with balanced and biased ligands [137, 138] (see these recent reviews [59, 139, 140] for additional examples in other

GPCRs). These studies will promote rational *in silico* design of biased ligands with better therapeutic potential (see [138] for example).

4. Biased signaling in naturally occurring GPCR mutations and endocrine diseases

Besides lab-generated biased mutant receptors, naturally occurring GPCR mutations that cause biased signaling have been identified and characterized based on different signaling assays. The majority of these mutations are associated with various disorders (Table 3). We will mainly focus on review of mutations in MC4R, CaSR, and PROKR2 since there are relatively more literature reporting biased mutations of these receptors. Mutations in other GPCRs will also be briefly discussed.

4.1. Neural MCR (*MC3R* and *MC4R*) mutations and biased signaling

Neural MCRs, including MC3R and MC4R, with high expression in the central nervous system, are two important regulators of energy homeostasis [141–143]. Targeted deletion of *Mc3r* or *Mc4r* in mice leads to obesity [144–146]. These two receptors have been extensively studied in obesity pathogenesis. Many naturally occurring mutations in *MC3R* and *MC4R* have been identified from obese patients, providing further evidence for their function in regulating energy homeostasis (for reviews, see Refs. [147–150]).

MC3R couples to $G\alpha_s$ protein stimulating cAMP-PKA pathway, and $G\alpha_{i/o}$ protein inducing ERK1/2 pathway through activation of phosphoinositide 3-kinase (PI3K) [151, 152]. β-arrestin-dependent internalization has also been suggested as an intracellular event after MC3R activation. Several MC3R mutants have been characterized in both cAMP and ERK1/2 pathway and shown to be biased receptors [153]. We showed that upon α-melanocyte-stimulating hormone (α-MSH) stimulation, I87T, N128S, M134I, and L297V mutants display biased activation of $G\alpha_s$-cAMP pathway, while F82S, I183N, and I335S mutants show biased activation of ERK1/2 pathway [153].

MC4R couples to multiple G proteins including $G\alpha_s$, $G\alpha_{i/o}$, and $G\alpha_{q/11}$, and G protein-independent pathways such as regulation of potassium channel (Kir 7.1) and β-arrestin-mediated internalization [151, 152, 154–156]. Until now, based on investigations of signaling profiles of naturally occurring *MC4R* mutations, a number of mutations resulting in biased signaling have been identified. These mutations are mostly distributed in the regions of TMDs, ICLs, and C-terminus, which potentially affect interaction of receptor and G protein or other signaling regulators/mediators (e.g. β-arrestins).

The first biased MC4R mutant associated with severe early-onset obesity is D90N [157, 158]. This mutant, without initiation of $G\alpha_s$-cAMP pathway despite normal α-MSH binding, is able to couple to $G\alpha_{i/o}$ protein thereby inhibiting cAMP production in pertussis toxin-sensitive manner [158]. D90 (2.50) is involved in interaction with NPxxY motif of TMD7 and plays a role in switch between inactive and active receptor conformation in other GPCRs, such as rhodopsin and TSHR [159, 160], possibly through changing the inter-helical space of receptors [135, 161]. This may suggest that D90 of MC4R is also important in inter-helical space change and thus critical for G protein coupling. We also investigated

biased signaling between $G\alpha_s$-cAMP and ERK1/2 pathways in 73 naturally occurring *MC4R* mutations, revealing 27 mutants with biased activation of either $G\alpha_s$-cAMP or ERK1/2 pathway [162] (Table 3). Some MC4R mutants with altered β-arrestin recruitment [155, 156] and biased $G\alpha_{q/11}$ activation [163] have also been identified, although another study just published reported no significant biased signaling in seven Class V mutants (mutants with no measurable change in receptor cell surface expression, ligand binding, and receptor-mediated intracellular signaling [15]) and two signaling defective mutants with the exception of one mutant, E308K [164].

The physiological relevance of intracellular signaling initiated by neural MCRs have been shown by several studies (reviewed in [165]). The conventional $G\alpha_s$-cAMP signaling pathway through MC4R mediates energy expenditure since $G\alpha_s$ deficiency in central nervous system leads to defect in energy expenditure without changing food intake [166, 167]. Food intake is mediated by several other intracellular mediators, such as $G\alpha_{q/11}$-IP3/Ca^{2+}, AMPK, ERK1/2, and potassium channel (Kir 7.1) [154, 165–171]. For example, $G\alpha_q$ deletion in the paraventricular nucleus causes hyperphagic obesity without affecting energy expenditure [171]; MC4R activation decreases AMPK phosphorylation thereby inhibiting food intake [168] and inhibitory effects of α-MSH on AMPK phosphorylation can be blocked by ERK1/2 inhibitors (U0126 and PD184352) [170]; MC4R coupling to closure of potassium channel (Kir 7.1) results in depolarization of neurons within paraventricular nucleus of hypothalamus, generating an anorexigenic effect [154]. ERK1/2 activation through MC3R is also involved in regulation of feeding behavior [172]. In addition to regulation of energy homeostasis, MC4R-medtaied $G\alpha_s$ signaling results in increased blood pressure and heart rate through sympathetic activation, causing the cardiovascular side effects and potentially limiting the therapeutics targeting MC4R [171, 173–176].

The biased agonism at neural MCR mutants provides new understanding on obesity pathogenesis and its therapeutics. Mutant D90N MC4R with both inactivated $G\alpha_s$ and activated $G\alpha_{i/o}$ signaling, resulting in severe defect of cAMP production, potentially account for the obesity phenotype of patients carrying this mutation [177]. Biased defect of either $G\alpha_s$-cAMP or ERK1/2 pathway of MC3R and MC4R mutants might also be one cause of disrupted energy homeostasis and obesity phenotype of patients with corresponding mutations. Indeed, among MC4R mutants, five Class V mutants including C40R, V50M, T112M, A154D, and S295P are defective in ERK1/2 activation despite normal cell surface expression, ligand binding, and ligand-stimulated $G\alpha_s$-cAMP signaling, suggesting potentially important role of ERK1/2 activation in regulation of energy homeostasis other than $G\alpha_s$-cAMP pathway [162]. This may suggest that different therapeutic strategies should be adopted for obese patients carrying either Gαs signaling defective or ERK1/2 defective mutations.

Two recent studies identified β-arrestin-biased MC4R mutations and proposed that β-arrestin-mediated signaling plays a significant role in MC4R-mediated body weight regulation [155, 156]. Gillyard *et al.* suggest that β-arrestin recruitment is a marker of normal MC4R function [156]. In their study, five of seven Class V MC4R mutants show altered activities in β-arrestin recruitment upon α-MSH and/or β-MSH stimulation. Another study on 61 naturally occurring gain-of-function (in terms of canonical $G\alpha_s$-cAMP

pathway) MC4R variants clarified that β-arrestin-mediated MC4R signaling is critical in human weight regulation and not associated with cardiovascular side effects. In their study, five MC4R gain-of-function variants, including T11S, T101N, F201L, G231S, and R236C, are shown to result in biased signaling of cAMP production, whereas four variants, including V103I, I251L, I289L, and I317V, exhibits signaling bias toward β-arrestin recruitment and enhanced ERK1/2 activation [155]. By performing a comprehensive genetic association analyses, they found that people carrying homozygous β-arrestin-biased *MC4R* alleles, compared to noncarriers, have approximately 50% lower risks of obesity, type 2 diabetes, and coronary artery disease. The risks of these diseases are intermediate even in carriers with one β-arrestin-biased *MC4R* allele. Carriers with β-arrestin biased variants also have normal blood pressure and heart rate. However, population carrying gain-of-function variants with biased cAMP production do not exhibit the lower risks of such diseases but are associated with increased blood pressure [155]. This supports that β-arrestin biased signaling is clearly associated with protection from obesity and its related cardio-metabolic diseases.

Characterization of MC4R biased mutations further dissects the molecular mechanisms underlying diseases caused by genetic variants, and thereby identify other clinically relevant pathways which could be preferentially modulated in obesity treatment to achieve more effective and safer therapeutic effects. Considering the protective effects of biased β-arrestin signaling, some biased ligands or biased allosteric modulators could be used to modulate this pathway with minimal alteration on conventional $G\alpha_s$ pathway to avoid cardiovascular complications.

4.2. *CASR* mutations and biased signaling

The CaSR is a Family C GPCR, expressed in parathyroid gland, kidney, bone, intestine, and brain [178]. By sensing the extracellular Ca^{2+}, activated CaSR suppresses PTH secretion from parathyroid cells and Ca^{2+} reabsorption in the kidney tubules [178, 179], and stimulates calcitonin secretion from thyroid gland [180]. Activation of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ proteins through CaSR has been suggested as signaling pathways related to the inhibition of PTH release in response to extracellular Ca^{2+} stimulation [181, 182]. In addition, CaSR-mediated ERK1/2 activation, a convergent point of several signaling pathways mediated by $G\alpha_{q/11}$ and $G\alpha_{i/o}$, as well as other signal mediators, such as β-arrestins [183, 184], is also believed to be involved in inhibition of PTH secretion [185, 186]. Until now, more than 230 mutations have been identified in human *CaSR* [187]. Most of these mutations are clinically relevant and associated with human disorders related to dysregulation of extracellular Ca^{2+} level, such as familial hypocalciuric hypercalcemia (FHH) and autosomal dominant hypocalcemia (ADH). However, in many earlier studies, mutations were classified as loss-of-function or gain-of-function mutations simply based on $G\alpha_{q/11}$ signaling pathway by measuring IP accumulation or intracellular Ca^{2+} mobilization, which may lead to neglect of other clinically important signaling pathway(s) and the therapeutic potential.

Recently, several groups have focused on biased agonism of CaSR and identified many CaSR mutations which can induce signaling bias between $G\alpha_{q/11}$-Ca^{2+} mobilization and ERK1/2 activation [188–190]. Most of these biased mutations are located in the TMDs and extracellular domain or loops. In particular, some biased mutations appear at disease-switch

residues (different mutations on the same residue cause opposite phenotypes, leading to either FHH or ADH) [190], suggesting that biased agonism of CaSR may play important role in disease pathogenesis. Investigation of biased signaling triggered by mutant CaSRs will provide us insights into developing new targets for therapies of disorders related to extracellular calcium regulation.

Leach and colleagues investigated the signaling properties of 21 mutant CaSRs on both $G\alpha_{q/11}$-Ca^{2+} mobilization and ERK1/2 activation [188]. These naturally occurring mutations included several loss-of-function mutations associated with FHH and gain-of-function mutations associated with ADH. Functional studies demonstrated that some of these mutations lead to pathway-selective signaling [188]. Some FHH-causing mutations switch the CaSR from preferentially coupling to $G\alpha_{q/11}$-Ca^{2+} mobilization to ERK1/2 activation. In contrast, some of the ADH-causing mutations (Q681H, E767K, L773R, S820F, and F821L) induce a signaling bias toward $G\alpha_{q/11}$-Ca^{2+} mobilization with increased potencies but do not significantly change either potency or maximal response in ERK1/2 activation. Of particular interest is the fact that three mutations (L727Q, G778D, and V836L) show biased impact on maximal responses in these two pathways [188] (Table 3).

Gorvin *et al.* showed that an ADH-causing mutation, R680G, located at the extracellular end of TMD3, differentially impacts the intracellular signaling pathways of CaSR. Different from cysteine and histidine substitution on R680 (R680C and R680H) (which impair receptor cell surface expression, $G\alpha_{q/11}$-Ca^{2+} mobilization, and ERK1/2 activation [188]), glycine substitution on arginine 680 (R680G) does not affect intracellular $G\alpha_{q/11}$-Ca^{2+} signaling but enhances G protein-independent and β-arrestin-dependent ERK1/2 activation [190], leading to gain-of-function in MAPK signaling. The signaling bias towards β-arrestin-dependent ERK1/2 activation has been considered as a consequence of disruption of R680-E767 salt bridge, an important interaction between TMD3 and ECL2 of CaSR [189]. Based on structure model of metabotropic glutamate receptor 1, which also belongs to Family C GPCRs and may have similar structural topology to the CaSR TMDs, this disruption might lead to disruption of TMD3 and ECL2 interaction and outward shift of the TMD3, TMD4 and/or TMD5 of CaSR, thereby facilitating β-arrestin binding to the cytoplasmic side of receptor [189, 191]. The outward shift of TMDs and insertion of the β-arrestin finger loop was also observed in β_2-AR [192]. The signaling bias of R680G provides further support for the role of CaSR-mediated MAPK signaling in inhibition of PTH synthesis and release, and also clear insights into structure-function relationship of CaSR.

Gorvin *et al.* further identified some disease-switch residues that may determine the CaSR signaling bias [190]. By using bias factor calculation formula [193, 194], in which both EC_{50}s and maximal responses are calculated, several FHH- or ADH-causing mutations were shown to result in signaling bias towards either intracellular Ca^{2+} signaling or ERK1/2 signaling. It is possible that mutations at these disease-switch residues may switch receptor conformation between G protein coupling and β-arrestin coupling since many of these mutations are located in the critical regions (extracellular domain or TMDs) for ligand binding and receptor activation. For example, a disease-switch residue N178, located at hinge region of the extracellular domain, has been shown to appear at the interface of CaSR

dimer upon receptor activation [195]. Different mutations on N178 showed distinct signaling bias between intracellular Ca^{2+} signaling and ERK1/2 signaling. FHH-causing mutation N178D has impaired Ca^{2+} signaling (increased EC_{50} and reduced maximal response) but normal ERK activation, thus typical biased Ca^{2+} signaling. However, ADH-causing mutation N178Y reinforces both Ca^{2+} and ERK signaling pathways. Two other disease-switch residues, S657 and S820, are located at the TMD2 and TMD6, respectively. ADH-causing mutation S657C causes biased intracellular Ca^{2+} signaling with decreased ERK1/2 activation, but FHH-causing mutation S820A impairs intracellular Ca^{2+} signaling without altering ERK1/2 activation [190].

CaSR can undergo clathrin-mediated endocytosis [196], indicating that signal transduction could be regulated by this process. Indeed, some mutations on σ subunit of adaptor protein-2 (AP2σ) (a critical protein recognizing clathrin and connecting it with receptor in coated vesicles) have impact on receptor activity and are linked with FHH [197]. These hypercalcemia-associated AP2σ mutations disrupt CaSR endocytosis, therefore increasing cell surface expression of CaSR. However, paradoxically, they impair CaSR-mediated intracellular signaling rather than generating signal amplification [198]. Hence, Gorvin *et al.* hypothesized that in addition to the signaling from plasma membrane, CaSR might also signal from endosomal compartment like some other GPCRs (such as AVPR2 and PTH1R), and disruption of receptor endocytosis might attenuate overall signal transduction [46, 48, 199]. They further found that endosomal CaSR could predominantly couple to $G\alpha_{q/11}$ to generate a sustained intracellular signaling distinct from rapid signaling originated from plasma membrane and this pathway is independent of β-arrestins [48]. The discovery of endosomal CaSR signaling has enriched our knowledge on relationship between CaSR-related diseases and CaSR-mediated intracellular signaling pathways, providing a new scope for further investigation. Although there is no CaSR mutation so far reported to cause biased signaling through endosomal pathway, it is worthy to evaluate signaling properties of CaSR mutations in light of this endosomal pathway considering its novel roles in regulating receptor activity and therapeutic potential for CaSR-related diseases.

4.3. *PROKR2* mutations and biased signaling

Prokineticin (PROK) receptor 2 (PROKR2), a Family A GPCR, is known as an important GPCR in olfactory bulb morphogenesis and sexual maturation. Dysfunctional PROKR2 signaling may cause Kallmann syndrome, a disorder associated with idiopathic hypogonadotropic hypogonadism (IHH) and olfactory abnormalities (anosmia or hyposmia) [200, 201]. The migration of embryonic GnRH neurons from nasal epithelium into forebrain is disrupted due to early impaired development of the peripheral olfactory system [202]. Naturally occurring mutations in the genes encoding PROKR2 and cognate ligand PROK2 have been identified in patients with this syndrome, implicating defective PROKR2 signaling in disease pathogenesis.

PROKR2 can trigger several different intracellular signaling pathways, including $G\alpha_{q/11}$, $G\alpha_{i/o}$, and $G\alpha_s$-dependent pathways [203]. In addition, β-arrestin recruitment through PROKR2 was also observed upon PROK2 stimulation [203], although the interaction

between PROKR2 and β-arrestins is not considered as an event for receptor internalization and ERK1/2 activation [204].

To date, several mutations in human *PROKR2* associated with Kallmann syndrome have been characterized in multiple signaling pathways in order to evaluate whether other pathways, in addition to the conventional $G\alpha_{q/11}$, are related to disease pathogenesis. Two groups reported that 10 missense mutations result in biased signaling through PROKR2 with respect to different G protein subtypes and β-arrestins (see details in Table 3) [203, 205]. Of these mutations, some impair at least one G protein-dependent signaling but not β-arrestin recruitment (A51T, R85C, R85H, R164Q, R268C, M323I, and V331M). One mutation (R80C) results in defect in β-arrestin recruitment and $G\alpha_s$-dependent signaling without impacting $G\alpha_{q/11}$ and $G\alpha_{i/o}$ pathways [203]. Two other mutations (L173R and V334M) have been shown to preferentially impair $G\alpha_s$ but not $G\alpha_{q/11}$-dependent signaling [205]. A very recent functional study revealed that two IHH-associated mutations, L218P and R270H, impair $G\alpha_{q/11}$-dependent signaling but maintain normal $G\alpha_s$ and ERK1/2 signaling [206].

Investigation of biased mutations of PROKR2 provides a distinctive view to better understand the molecular mechanisms of PROKR2 signaling and structure-function relationship of this receptor. For instance, defective β-arrestin recruitment of R80C could be explained by the unique location of this mutation. R80, highly conserved among PROKR2s of different species, is located at ICL1, the region that does not directly interact with G proteins but is close to TMD7 and the eighth α helix known for β-arrestin recruitment [135, 203, 207–209]. The ICL3 may be a critical region controlling biased coupling of G proteins (impairment of $G\alpha_q$ coupling). This intracellular loop is thought to interact with various G proteins [210, 211]. The structural model of PROKR2 shows that the residue R268 of ICL3 is located in the region that interacts with G proteins [203, 205]. Mutation R268C selectively impairs $G\alpha_q$ signaling (measured by IP accumulation), although no significant change when intracellular Ca^{2+} release was monitored [203]. Another biased mutation, R270H, is also presented in ICL3. R270 is only two residues distal to R268. Thus, it is possible that R270H selectively disrupt the interaction of ICL3 to $G\alpha_q$ protein, leading to a biased $G\alpha_s$ coupling and ERK1/2 signaling [206]. To demonstrate this, the glutathione S-transferase (GST)-tagged PROKR2-ICL3s (WT and R270H) were pulled down to detect the binding of $G\alpha_s$ and $G\alpha_q$ proteins [206]. Indeed, the R270H mutation only impaired $G\alpha_q$ binding to ICL3, with normal $G\alpha_s$ interaction. This biased signaling occurs only when substitution is from arginine to other basic amino acids, such as histidine and lysine, but not other non-basic amino acids [206].

The signaling properties of these biased PROKR2 mutants indicate that the conventional $G\alpha_q$ signaling is not the only pathway relevant for disease pathogenesis. Many biased mutations described above cause defects in other G protein signaling and/or β-arrestin recruitment, suggesting pathogenic involvement of these pathways. Further studies are needed to explore the relationship of these pathways to Kallmann syndrome. It is interesting to note that one of these mutations, R268C, resulting in severe impairment of $G\alpha_{i/o}$ pathway, is now considered to be nonpathogenic since this mutation is frequently present in African American population (even in homozygous state), indicating that $G\alpha_{i/o}$-dependent pathway plays a minor role in Kallmann syndrome pathogenesis. R268C is speculated to be a

positively selected mutation which is protective in infection process in which infectious agents, such as whooping cough bacterial agent and the human malaria parasite [212, 213], use $G\alpha_{i/o}$ pathway to exert detrimental effects [203].

4.4. Other GPCR mutations and biased signaling

4.4.1. *KISS1R* mutations—G protein-coupled receptor 54, GPR54 (encoded by *KISS1R*, also called kisspeptin receptor), and the cognate ligand, kisspeptin, are known as important regulators for gonadotropin-releasing hormone (GnRH) secretion [214, 215]. GnRH is a critical hormone in hypothalamic-pituitary-gonadal axis and regulator of reproduction. Kisspeptin activates various intracellular signaling pathways via GPR54, including $G\alpha_{q/11}$-mediated IP3 accumulation, Ca^{2+} mobilization, PKC activation, and MAPK (ERK1/2 and p38) activation, important events in kisspeptin-dependent GnRH secretion [216, 217]. Recruitment of β-arrestin is involved in desensitization, internalization, and signal transduction of GPR54 [218]. Recently, Ahow et al. reported that kisspeptin-dependent luteinizing hormone secretion (downstream action of GnRH secretion) is impaired in mice lacking either β-arrestin-1 or β-arrestin-2 [219], further supporting the idea that β-arrestin-dependent signaling plays a significant role in reproductive function through GPR54.

Loss-of-function mutations in human *KISS1R* have been shown to be a cause of IHH [220], a disorder characterized by the absence of spontaneous sexual maturation due to deficiency in GnRH secretion. One *KISS1R* mutation associated with IHH and infertility, L148S, has been shown to impact on the receptor with strong uncoupling from $G\alpha_{q/11}$-dependent IP accumulation (downstream metabolite of IP3) although it does not affect receptor cell surface expression and ligand binding [215, 221]. Further, Ahow et al. reported that L148S mutant retains β-arrestin-dependent ERK1/2 activation despite defective $G\alpha_{q/11}$ signaling, suggesting that L148S is a biased receptor, potentially explaining why some patients with loss-of-function *KISS1R* mutations in terms of IP accumulation display variable gonadotropic deficiency [219]. $G\alpha_{q/11}$-independent signaling through biased GPR54 mutants might partially account for GnRH secretion and prevent complete gonadotropin deficiency in those patients. For example, the loss-of-function mutation L102P delays pubertal maturation but does not impede it, which might be due to other $G\alpha_{q/11}$-independent signaling through the mutant receptor, such as β-arrestin-biased ERK1/2 activation [219, 222].

The only gain-of-function *KISS1R* mutation is R386P identified from a girl with idiopathic central precocious puberty [223 932]. Functional studies showed that R386P mutant has comparable EC_{50} and maximal response in IP accumulation as WT receptor, but displays a prolonged activation of intracellular signaling pathways, which indicates that this mutation might preferentially disrupt β-arrestin-dependent receptor desensitization and increase the stimulatory effects of kisspeptin on GnRH synthesis and secretion, eventually leading to precocious puberty [223].

4.4.2. *FSHR* mutations—The FSHR is mainly expressed by the granulosa cells of the ovarian follicle and the Sertoli cells of the seminiferous tubules, playing critical roles in

reproduction. The gonadotropin from pituitary gland, FSH, is the endogenous ligand for FSHR in the gonads. FSH action through FSHR is fundamental for ovarian follicle maturation, whereas it is not essential for males to develop fertility although necessary to achieve normal testis and sperm production [224, 225].

Several loss-of-function mutations in human *FSHR* associated with IHH have been described (reviewed in [226]). A189V was the first reported *FSHR* loss-of-function mutation [227]. Five Finnish men (the population where A189V is frequently present) carrying A189V mutation exhibit variable degrees of oligospermia and subfertility [225]. The mutation impairs FSH-stimulated cAMP production [227, 228]. One recent study demonstrated that although A189V mutant is defective in $G\alpha_s$-cAMP pathway with undetectable cAMP production, PKA activation, and cAMP/PKA transcriptional-associated response, this mutant receptor can still induce normal β-arrestin-dependent ERK1/2 signaling at low cell surface expression level, suggesting that A189V FSHR is a biased receptor [229]. The biased signaling properties provide the plausible explanation for the mild abnormalities (oligospermia and subfertility) detected in men harboring A189V mutation. Compared with the complete inhibition of all potential FSH-dependent signaling pathways due to failure of mutant FSH binding and activating FSHR, the biased A189V FSHR mutant impairs $G\alpha_s$-cAMP pathway but not β-arrestin-dependent ERK1/2 signaling, which is suspected to be also involved in regulating reproductive function in males [229].

N431I is a gain-of-function *FSHR* mutation detected in an asymptomatic man with normal spermatogenesis, suppressed serum FSH, and normal or increased levels of biomedical markers of FSH action [230]. The impaired agonist-stimulated desensitization and internalization caused by this mutation are thought to be one of the reasons for the apparent constitutive activity and increased agonist-induced cAMP production when receptor cell surface expression was normalized [230].

4.4.3. *TSHR* mutation—TSHR, expressed in thyroid gland, is essential for thyroid hormone (TH, including thyroxine and triiodothyronine) synthesis and secretion. Upon endogenous agonist TSH binding, TSHR primarily couples to $G\alpha_s$-cAMP pathway. In addition, the receptor also couples to $G\alpha_{q/11}$-IP3/Ca^{2+} pathway when higher TSH concentration is present [231]. These two intracellular G protein-dependent signaling pathways have been suggested to mediate distinct physiological processes. The $G\alpha_s$-cAMP pathway is thought to mediate TSH-stimulated TH secretion, growth and differentiation of follicular cells, and iodide uptake through sodium iodide symporter, while the $G\alpha_{q/11}$-IP3/Ca^{2+} pathway regulates TH synthesis by inducing production of H_2O_2 which is critical for activity of thyroid peroxidase, a key enzyme in iodination and production of TH [232, 233].

Loss-of-function mutations has been known to cause hypothyroidism and resistance to TSH [231]. However, one novel familial missense mutation, L653V, has been reported in individuals with euthyroid hyperthyrotropinemia [234]. Homozygous individuals carrying L653V *TSHR* mutation exhibit normal free and total TH levels but markedly increased serum TSH levels. In addition, these individuals have higher radio-iodide uptake in thyroid gland compared with heterozygous and normal family members, suggesting imbalanced

iodide trapping and iodination [234]. Functional study revealed that in COS-7 cells, L653V mutant has comparable cell surface expression and TSH binding as WT receptor. The potencies of TSH for cAMP and IP production are affected equivalently by this mutation with same fold-change on EC_{50}s for both pathways. However, reduced efficacy is observed in IP production with severely impaired maximal response in contrast to normal efficacy of TSH for cAMP production, indicating that L653V TSHR exhibits biased signaling [234].

Because $G\alpha_s$-cAMP and $G\alpha_{q/11}$-IP/Ca^{2+} mediate distinct physiological responses [232, 233], severely defective $G\alpha_{q/11}$-IP3/Ca^{2+} pathway may be the reason for the development of clinical phenotype of individuals carrying this mutation as discussed above [234]. In those patients, serum concentration of TSH are increased to overstimulate the mutant TSHR and compensate for defective TH synthesis due to impaired $G\alpha_{q/11}$-IP3/Ca^{2+} signaling (negative feedback regulation of hypothalamic-pituitary-thyroid axis). Elevated TSH, on the other hand, also overstimulates the $G\alpha_s$-cAMP pathway to induce further increased iodide trapping [234]. Hence, it is assumed that patients with L653V mutation exhibiting normal TH levels but increased iodine uptake is due to biased signaling of the two G protein pathways.

4.4.4. *AVPR2* mutations—The system of arginine vasopressin (AVP) and AVPR2 are important in the regulation of water homeostasis. After AVP binding, AVPR2 predominantly signals through coupling to $G\alpha_s$ and stimulating cAMP-PKA pathway to phosphorylate aquaporin 2 water channel (AQP2), thus promoting water reabsorption in the kidney distal collecting tube [235–237]. Moreover, ERK1/2 and $G\alpha_{q/11}$ signaling through AVPR2 can also be activated after AVP binding [238, 239]. Mutations in *AVPR2* gene may lead to disorders of water homeostasis, with either nephrogenic diabetes insipidus (NDI) (failure in water reabsorption) or nephrogenic syndrome of inappropriate antidiuresis (NSIAD) (excessive water reabsorption, hyponatremia, and seizures) [235, 236, 240].

L83Q, Y128S, R137H, and R180C, are four NDI-associated mutations. L83Q and Y128S mutants are retained in the endoplasmic reticulum or endoplasmic reticulum-Golgi intermediate compartment [241, 242]. Rescue of these two mutants with pharmacoperones causes AVP-induced biased $G\alpha_s$ signaling in L83Q and biased constitutive $G\alpha_s$ signaling in Y128S [239]. R137H cannot activates $G\alpha_s$ but can recruit β-arrestin and induce endocytosis [243]. Constitutive β-arrestin recruitment and receptor endocytosis cause loss of function by decreasing the number of receptors at the cell surface [243–245]. R181C mutant exhibits $G\alpha_s$ biased signaling incapable of signaling to $G\alpha_{q/11}$-IP3 accumulation or recruiting β-arrestins [246].

Opposite to the clinical outcomes of R137H, two other mutations (R137C and R137L) on the same residue lead to NSIAD. R137C and R137L display higher basal cAMP accumulation compared to R137H, although they are also associated with defective AVP-stimulated cAMP production and constitutive β-arrestin recruitment as R137H. The balance between constitutive $G\alpha_s$-cAMP signaling and β-arrestin-dependent endocytosis seems to determine the extent of basal cAMP levels, providing an explanation for the different disease phenotypes between NDI- and NSIAD-causing mutations [245].

Three other NSIAD-associated mutations, I130N, F229V, and L312S, exhibit similar biased signaling properties with constitutive cAMP production and defective constitutive β-arrestin recruitment [247–249]. This further confirms that constitutive $G\alpha_s$ signaling in mutant AVPR2 is the major determinant of NSIAD [249]. Tolvaptan, an aquaretic drug, is proposed to be used for treatment of hyponatremia in patients with NSIAD, due to its role of inverse agonist at constitutive cAMP production of I130N, F229V, and L312S mutants [247–249].

4.4.5. *MTNR1A* and *MTNR1B* mutations—Melatonin, the principal hormone of the pineal gland, is crucial in sleep induction, circadian rhythm regulation, and immune response [250]. Melatonin signaling is mediated by melatonin receptor 1 and 2 (MT1R and MT2R). RT-PCR or *in situ* hybridization showed that the transcripts of *MTNR1A* (encoding MT1R) are localized in the suprachiasmatic nuclei, cortex, hippocampus, thalamus and cerebellum, and transcripts of *MTNR1B* (encoding MT2R) are distributed in the retina, whole brain, and hippocampus (reviewed in [251]). MT1R and MT2R activation by melatonin modulates several signal transduction pathways, typically inducing $G\alpha_i$ signaling to inhibit cAMP-PKA activation, and stimulating ERK1/2 and/or JNK activation (reviewed in [252]). In some cells, these receptors also change intracellular diacylglycerol, IP3, and Ca^{2+} concentration as well as cGMP levels [253].

Alteration in melatonin signaling has been reported in autism spectrum disorders (ASD), circadian disorders, and diabetes. ASD is a developmental disability that affects communication, behavior, and sleep [254]. Chaste et al. [255] reported that in 295 ASD patients, six nonsynonymous mutations in *MTNR1A* and ten in *MTNR1B* are identified with altered receptor function. Particularly interesting mutants are G166E MT1R (no significant difference of sleep between patients and controls), I212T MT1R (identified from patients with severe mental retardation and autism), and V124I MT2R (identified from patient with seasonal affective disorder). The two MT1R mutants show reduced cell surface expression. They also display biased signaling by completely inhibiting $G\alpha_i$ signaling and retaining partial activity in ERK1/2 activation. V124I MT2R preferentially impairs ERK1/2 signaling and shows normal $G\alpha_i$ signaling in comparison to the WT receptor [255]. The biased signaling of these three mutants potentially accounts for the phenotypes of some patients.

Moreover, some *MTNR1B* mutations have been most extensively associated with increased fasting plasma glucose and increased type 2 diabetes risk. Functional analysis of 40 MT2R mutants revealed that nine mutants (W22L, A52T, A74T, R138H, R138L, L166I, R222H, R330W, and I353T) display ERK1/2 biased signaling without $G\alpha_i$ activation [256]. Identification of these mutants and validation of the biased signaling may provide further information for the development of precision therapies for ASD and type 2 diabetes patients.

4.4.6. *MC1R* mutations—Melanocortin-1 receptor (MC1R) is a $G\alpha_s$-coupled receptor expressed in epidermal melanocytes. MC1R regulates production of melanin pigment, serving as a major determinant of skin pigmentation, skin phototype, ultraviolet radiation sensitivity and non-melanoma skin cancer risk [257, 258]. After endogenous ligand (α-MSH or other proopiomelanocortin-derived peptides) binding, MC1R activates cAMP-PKA and ERK1/2 signaling [259].

The *MC1R* is highly polymorphic with more than 100 nonsynonymous mutations reported so far [259, 260]. R151C, R160W, and D294H, are the commonest three loss-of-function *MC1R* mutations associated with the red hair color (RHC) phenotype (such as red hair, fair skin, freckling, poor tanning, and increased risk for melanoma and nonmelanoma skin cancers) [261, 262]. These three mutants display reduced cAMP signaling but activate ERK1/2 signaling as efficiently as WT MC1R, suggesting that they are biased receptors [263]. Impaired cAMP signaling in R151C, R160W and D294H may account for the loss-of-function in photoprotection [264, 265], and ERK1/2 activation plays a minor effect on MC1R-dependent pigmentation in human skin compared with cAMP pathway [259]. Biased constitutive signaling has also been reported for a naturally occurring mutation, E92K [266].

4.4.7. *MC2R* mutation—Adrenocorticotropic hormone (ACTH) and its cognate receptor, MC2R, play a pivotal role in adrenal development, carbohydrate metabolism, and stress response by regulating synthesis and secretion of aldosterone and glucocorticoid [267]}. ACTH induces $G\alpha_s$-cAMP-PKA pathway through activating MC2R [267]. Dysfunctions of ACTH-MC2R system such as abnormal secretion of ACTH or *MC2R* mutations result in severe adrenal diseases. For example, pathological ACTH over-secretion results in Cushing's syndrome (glucocorticoid over-secretion) and adrenocortical hyperplasia whereas loss-of-function mutations in *MC2R* cause familial glucocorticoid deficiency syndrome [267, 268].

A naturally occurring mutation in human *MC2R* (F278C) has been identified with ACTH-independent Cushing's syndrome. F278C mutant displays similar ACTH-induced cAMP production but increased basal cAMP accumulation with impaired desensitization and internalization compared to WT receptor [269]. Desensitization of MC2R results from PKA-mediated phosphorylation [270]. Mutation of phosphorylation site S208 of MC2R also causes failure in desensitization. One possibility of the F278C-induced dysfunction in receptor desensitization is that F278C affects the phosphorylation sites, serine 280 and/or 294. These findings suggest that failure of desensitization in MC2R appears to be associated with enhanced basal receptor activity, causing Cushing's syndrome [269].

5. Pathophysiological implications of biased mutant GPCRs in endocrine diseases

Over the past decade, biased ligands at GPCRs, with more beneficial effects and less detrimental side effects, have been suggested as novel therapeutic agents for disease treatments. Biased mutant GPCRs identified so far also provide us implications on pathogenesis and treatment of many diseases. Herein, we proposed four types of biased mutant receptor models relevant to disease pathogenesis (Fig. 2B).

The first model proposes that two intracellular signaling pathways are both required for mediating a certain physiological response. Impaired or enhanced activation on either one will induce lower or higher degree of such response, eventually leading to the abnormal phenotypes. This type of biased mutant receptors includes L148S GPR54, A189V FSHR, and some mutant PROKR2s, CaSRs, and neural MCRs [153, 158, 162, 188, 189, 203, 205, 219, 229]. In the second model, two intracellular signaling pathways are thought to be

responsible for two distinct physiological responses, and defect in either one will lead to its distinct phenotype. Biased L653V TSHR belongs to this type [234].

In the third model, β-arrestin mediated receptor desensitization and internalization are considered as a counteractive pathway for G protein-dependent signaling cascades. Inactive or overactive β-arrestin coupling will lead to either hyperactivated or suppressed G protein-dependent signaling and then consequent abnormal phenotype. This type of biased receptor is represented by some mutant GPCRs, such as R386P GPR54, N431I FSHR, F278C MC2R, and some mutant AVPR2s [223, 230, 245–247, 269]. Considering the increasing evidence of endosomal signaling triggered by internalized GPCRs, we also present the model of biased endosomal signaling (model 4), in which mutant receptor may preferentially strengthen G protein activation through endosomal pathway, leading to a more sustained G protein signaling and relevant physiological response. Although there is no GPCR mutant characterized so far in this way, future research along this line will likely provide better understanding of the molecular and cellular basis of disease pathogenesis.

Comprehensive understanding of signaling properties of biased mutant GPCRs in pathophysiology facilitates us to select appropriate ligands that could specifically and precisely target the abnormal pathways without affecting the normal ones. Biased ligands and biased allosteric modulators described above might be better drug candidates considering their desirable effects on modulation of clinically relevant signaling pathways of normal receptors. However, to our knowledge, there is no report of such effective ligands for selective modification of the impaired pathways of biased mutant GPCRs. Hence, future studies are still required to identify or develop these ligands and to test their pharmacological abilities in terms of biased mutant GPCRs. The investigation of signaling properties of biased mutant GPCRs also provides a better perspective on structure-function relationship of these receptors and will be of benefit to develop drugs targeting some residues critical for receptor signaling and meet therapeutic effects in patients with loss-of-function or gain-of-function mutations.

6. Conclusions

Naturally occurring mutations in GPCRs have been known to cause various endocrine diseases. GPCR mutations that result in biased signaling have been identified and characterized in recent years, making significant progresses in elucidating the pathogenesis of diseases. The investigation of biased agonism of these GPCR mutations will strengthen our understanding of correlation between signaling properties and clinical phenotypes, thereby providing pathophysiological implications and novel therapies for patients with such mutations.

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Abbreviations:

AC	adenylyl cyclase
ACTH	adrenocorticotropic hormone
ADH	autosomal dominant hypocalcemia
AQP2	aquaporin 2
AR	adrenergic receptor
ASD	autism spectrum disorders
AT1R	angiotensin II receptor type 1
AVP	arginine vasopressin
AVPR2	type 2 arginine vasopressin receptor
cAMP	cyclic adenosine monophosphate
CaSR	calcium-sensing receptor
CCR	C-C chemokine receptor
DAG	diacylglycerol
ECL	extracellular loop
ERK	extracellular signal-regulated kinase
FHH	familial hypocalciuric hypercalcemia
FSH (FSHR)	follicle-stimulating hormone (receptor)
GnRH	gonadotropin-releasing hormone
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
hCG	human chorionic gonadotropin
ICL	intracellular loop
IHH	idiopathic hypogonadotropic hypogonadism
IP	inositol phosphate
IP3	inositol 1,4,5-trisphosphate
JNK	c-Jun-N-terminal kinase
LH (LHR)	luteinizing hormone (receptor)
MAPK	mitogen-activated protein kinase

MCR	melanocortin receptor
MSH	melanocyte-stimulating hormone
MT1/2R	melatonin receptor 1 and 2
NDI	nephrogenic diabetes insipidus
NSIAD	nephrogenic syndrome of inappropriate antidiuresis
OR	opioid receptor
PI3K	phosphoinositide 3-kinase
PKA/C	protein kinase A/C
PLC	phospholipase C
PROK2 and PROKR2	prokineticin 2 and prokineticin receptor 2
PTH (PTHR)	parathyroid hormone (receptor)
RHC	red hair color
RhoGEF	Rho-specific guanine nucleotide exchange factor
TH	thyroid hormone
TMD	transmembrane domain
TSH (TSHR)	thyroid-stimulating hormone (receptor)

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Highlights:

- Naturally occurring mutations in GPCRs are the causes of numerous diseases.
- Biased ligands and mutations can be found in GPCRs.
- Some disease-causing GPCR mutations cause biased signaling.
- Characterization of biased mutant GPCRs elucidates the pathophysiology of these diseases.

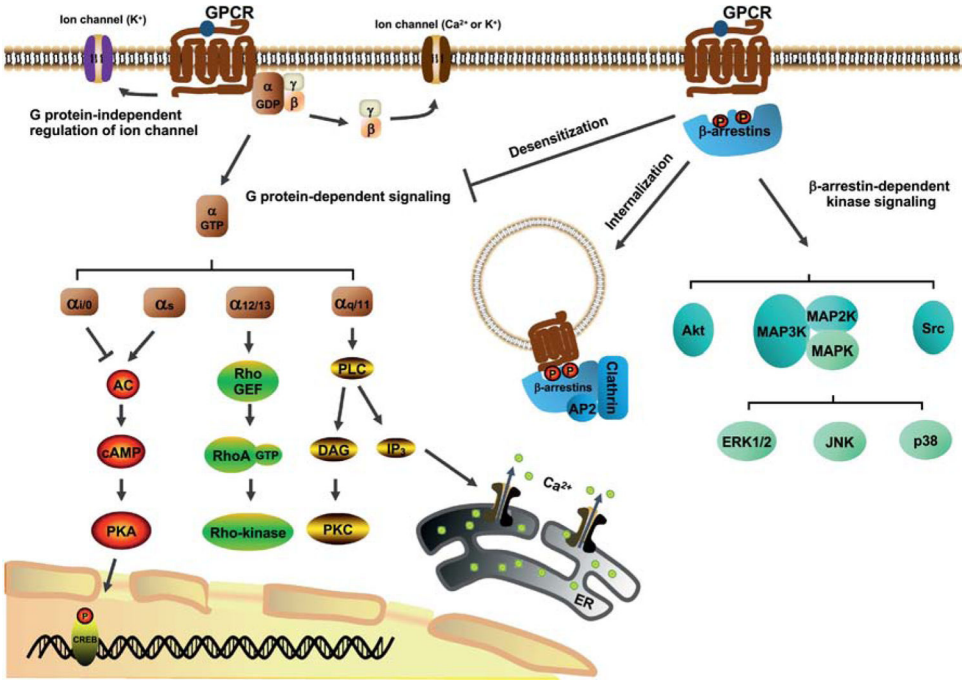

Figure 1.

Intracellular signaling pathways triggered by GPCRs. Agonist binding to receptors triggers a variety of distinct downstream signaling pathways. Signal propagation can be mediated through G proteins ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, and $G\beta\gamma$), thereby regulating activities of several kinases (PKA, PKC, and Rho-kinase) and Ca^{2+} mobilization. Recruitment of β-arrestins resulted from phosphorylation of GPCRs desensitizes G protein coupling, mediates receptor internalization, and initiates β-arrestin-dependent signaling and kinase activation.

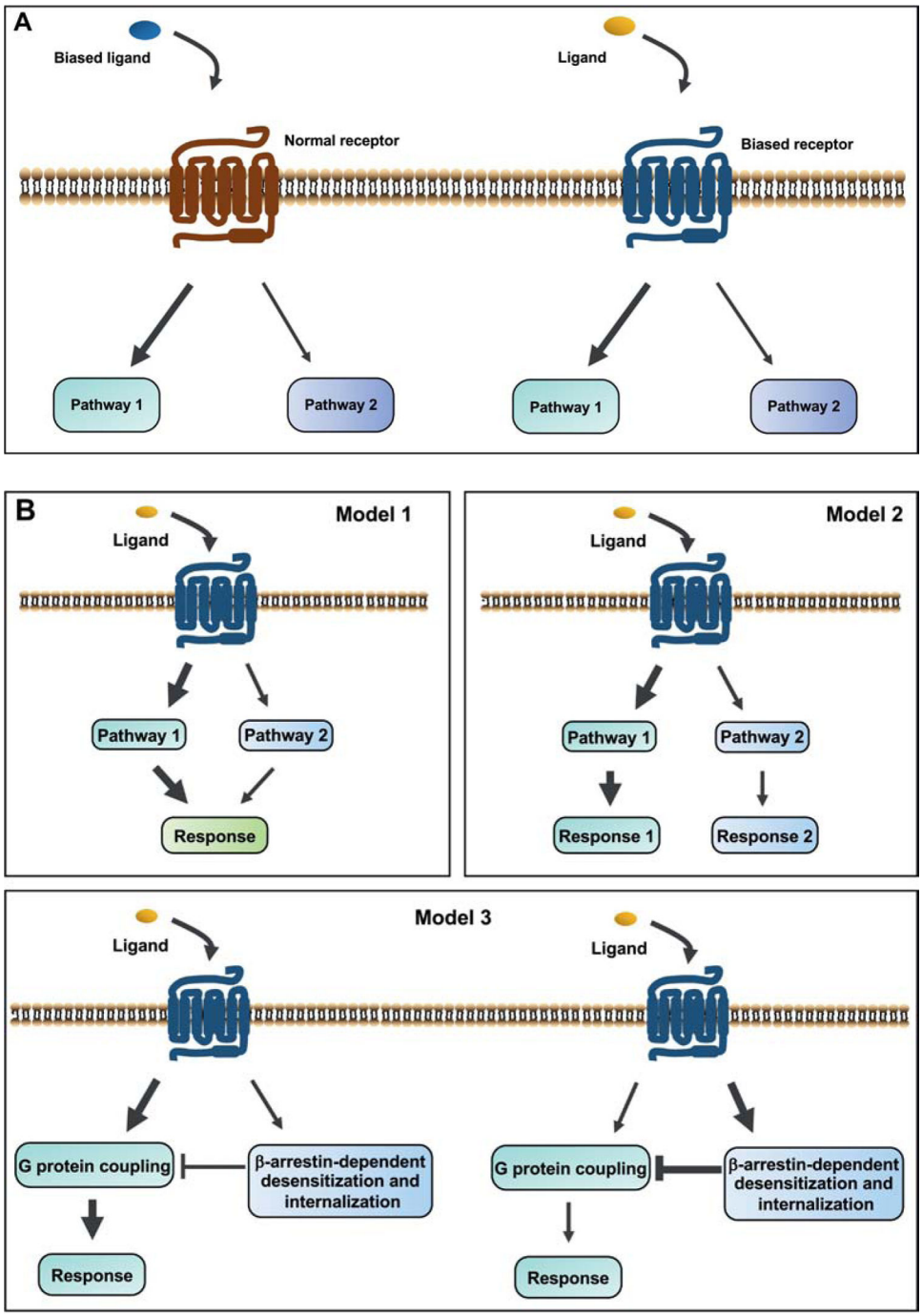

Figure 2.

Models for biased signaling and pathophysiological relevance of biased mutants in GPCRs. (A) Two cases of biased agonism: the biased response is initiated by binding of biased ligand to the GPCR or a ligand to biased receptor. (B) Four types of biased mutant models that are proposed to be pathophysiologically relevant. Biased signaling can be initiated by mutant receptor between G protein and β-arrestins, or different G protein subtypes (models 1 and 2). The β-arrestin mediated receptor desensitization and internalization are considered

as a counteractive pathway for G protein-dependent signaling (model 3). Mutant receptors can also trigger biased constitutive signaling in the absence of ligand. Sustained endosomal G protein signaling also represents an important pathway mediating physiological function of receptor (model 4).

Table 1.

Loss-of-function GPCR mutations associated with diverse human diseases.

Receptor	Disease	Clinical features	References
GPR54	Idiopathic hypogonadotropic hypogonadism	Attenuated secretion of GnRH, sexual development disruption	[215]
PROKR2	Kallmann syndrome	Impaired development of the olfactory system and reproductive axis	[271]
FSHR	Oligospermia and subfertility Ovarian dysgenesis	Small testes with impaired spermatogenesis in males Absence of follicular maturation in females	[272]
TSHR	Euthyroid hyperthyrotropinemia Congenital hypothyroidism	TSH resistance with normal biological effects of TH TSH resistance with reduced biological effects of TH	[234, 273]
CaSR	Familial hypocalciuric hypercalcemia	Lifelong moderate elevation of serum calcium concentration and inappropriately normal PTH levels	[274, 275]
	Neonatal severe hyperparathyroidism	Neonatal marked elevation of serum calcium and PTH levels	
AVPR2	Nephrogenic diabetes insipidus	Polyuria, polydipsia, hypernatremia, low urine osmolality, and AVP resistance	[236]
MC1R	Pigmentation defect	Red hair color phenotype: red hair, fair skin, poor ability to tan, and increased risk of melanoma	[276]
MC2R	Familial glucocorticoid deficiency	Low or undetectable cortisol levels and elevated ACTH levels	[277]
MC4R	Obesity	Hyperphagia, increased fat and lean mass, and increased bone mass	[278]
PTHR1	Blomstrand chondrodysplasia	Advanced endochondral bone maturation associated with breast development and tooth impaction	[279, 280]
Rhodopsin	Retinitis pigmentosa	Loss of vision, insensitivity in dim light	[16]

Table 2.

Gain-of-function GPCR mutations associated with diverse human diseases.

Receptor	Disease	Clinical features	References
GPR54	Central precocious puberty	Premature secretion of GnRH, early pubertal development	[223]
FSHR	Spontaneous ovarian hyperstimulation	Hydrothorax, ascites, and ovarian enlargement	[281, 282]
TSHR	Familial gestational hyperthyroidism	Severe nausea and vomiting, weight loss, persistent tachycardia, and sweating during gestation	[283]
CaSR	Autosomal dominant hypocalcemia	Decreased serum calcium concentrations and inappropriately low PTH levels, hyperphosphatemia, hypercalciuria	[284, 285]
	Bartter syndrome type V	Defective renal reabsorption of sodium and chloride, hypokalemia, and increased blood pH	
AVPR2	Nephrogenic syndrome of inappropriate antidiuresis	Hyponatremia, concentrated urine in adults and seizures in infants	[235]
MC2R	Cushing's syndrome	Hypercortisolism	[269, 286]
PTHR1	Jansen metaphyseal chondrodysplasia	Short-limbed dwarfism, disorganized metaphyseal regions	[287]
Rhodopsin	Congenital night blindness	Insensitivity in dim light without progressive loss of day vision	[288]

Table 3.

Summary of naturally occurring GPCR mutations resulting in biased signaling.

Receptor	Mutation	Signaling properties	ligand	Cell system	References
GPR54	L148S	Strongly impairs $G\alpha_q$-IP accumulation but retains β-arrestin-dependent ERK1/2 activation	Kisspeptin-54	HEK293	[219]
	R386P	Normal IP accumulation but defective receptor internalization	Kisspeptin-10	COS-7	[223]
PROKR2	A51T, R85C, R85H, R164Q, R268C, M323I, and V331M	Impair at least one G protein-dependent signaling ($G\alpha_q$-Ca^{2+}, $G\alpha_s$-cAMP, and $G\alpha_{i/o}$-ERK1/2) but not β-arrestin recruitment (A51T: defective in $G\alpha_s$ and $G\alpha_{i/o}$; R85C: defective in $G\alpha_q$; R85H, R164Q, and V331M: defective in $G\alpha_q$ and $G\alpha_{i/o}$; R268C and M323I: defective in $G\alpha_{i/o}$)	PROK2	HEK293	[203, 205, 289]
	R80C	Impairs $G\alpha_s$-cAMP signaling and β-arrestin recruitment but not $G\alpha_q$-Ca^{2+} or $G\alpha_{i/o}$-ERK1/2 signaling			
	L173R and V334M	Preferentially impair $G\alpha_s$-cAMP but not $G\alpha_q$-IP signaling			
	L218S, R270H, R268C and V331M	Preferentially impair $G\alpha_q$ (IP or Ca^{2+}) but not $G\alpha_s$-cAMP signaling			
FSHR	A189V	Undetectable cAMP production, PKA activation, and cAMP/PKA transcriptional-associated response but inducible β-arrestin-dependent ERK1/2 activation	FSH	HEK293N	[229]
	N431I	Increases basal and ligand-induced cAMP production but impairs β-arrestin-dependent desensitization and internalization (normalized to cell surface expression)	FSH	HEK293	[230]
TSHR	L653V	Normal maximal cAMP production but impaired IP accumulation	TSH	COS-7	[234]
CaSR	L727Q and V836L	Reduced efficacy on intracellular Ca^{2+} mobilization but enhanced potency or efficacy on ERK1/2 activation	Ca^2	HEK293	[188, 189]
	R680G	Normal intracellular Ca^{2+} mobilization but enhanced β-arrestin-dependent ERK1/2 activation			
	S657C	Increased intracellular Ca^{2+} mobilization but decreased ERK1/2 activation			
	G778D, N178D, and S820A	Not detectable intracellular Ca^{2+} mobilization but inducible ERK1/2 activation (G778D); Impaired intracellular Ca^{2+} mobilization without altering ERK1/2 activation (N178D and S820A)			
	G778D	Not detectable Ca^{2+} mobilization but inducible ERK1/2 activation			
AVPR2	L83Q	$G\alpha_s$-biased signaling after rescue by pharmacoperone	AVP	HeLa	[239]
	Y128S	Constitutive $G\alpha_s$-biased signaling after rescue by pharmacoperone			
	R137H	Inducible β-arrestin recruitment and endocytosis but resistance to AVP-stimulated cAMP production and ERK1/2 activation (constitutive β-arrestin recruitment and endocytosis)	AVP	HEK293	[245]
	R181C	$G\alpha_s$-biased receptor incapable of signaling to $G\alpha_{q/11}$-IP accumulation or recruiting β-arrestin	AVP	PC12	[246]

Receptor	Mutation	Signaling properties	ligand	Cell system	References
	R137C/L	Inducible β-arrestin recruitment and endocytosis but resistance to AVP-stimulated cAMP production and ERK1/2 activation (high basal cAMP levels and constitutive β-arrestin recruitment and endocytosis)	AVP	HEK293	[245]
	I130N, F229V, and L312S	Higher basal cAMP production and reduced constitutive β-arrestin recruitment and/or $G\alpha_{q/11}$-IP accumulation		HEK293T HEK293FT	[247–249]
MT1R MT2R	G166E and I212T (MT1R)	Bias towards ERK1/2 activation	Melatonin	COSM1 HEK293	[255, 256]
	V124I (MT2R)	Bias towards $G\alpha_i$ activation			
	W22L, A52T, A74T, R138H, R138L, L166I, R222H, R330W, and I353T (MT2R)	Bias towards ERK1/2 activation without $G\alpha_i$ activation			
MC1R	R151C, R160W, and D294H	Defective cAMP production but normal ERK1/2 activation	[Nle4,D-phe7]-α-MSH	PC12	[263]
MC2R	F278C	Normal cAMP production but defective receptor internalization	ACTH	Mouse Y6	[269]
MC3R	I87T, N128S, M134I, and L297V	Biased cAMP production without significant activation of ERK1/2	α-MSH	HEK293T	[153]
	F82S, I183N, and I335S	Biased activation of ERK1/2 without increasing cAMP production			
MC4R	D90N	Biased coupling to $G\alpha_{i/o}$ protein rather than $G\alpha_s$ protein	α-MSH	HEK293 GT1–7	[158]
	V50M, H76R, and I170V	Biased cAMP production and defective β-arrestin recruitment	α- and/or β-MSH	CHO- K1	[156]
	V103I and H158R	Biased $G_{q/11}$ activation	α- and β-MSHs	HEK293	[163]
	G55V, I69T, M79I, N97D, G98R, I125K, W174C, A175T, I194T, A219V, P260Q, F261S, C271Y, Q307X, and C326R	Biased activation of ERK1/2 without increasing cAMP production			[155, 162]
	R165G, R165W, and C172R	Biased ERK1/2 constitutive signaling			
CCR6	A89T, R155W, and A369V	Decreased basal and ligand-induced $G\alpha_i$ signaling but unaffected β-arrestin recruitment	Chemokine (C-C motif) ligand 20	HEK293	[290]
β_3-AR	S165P and S257P	Impaired $G\alpha_s$-cAMP production but normal $G\alpha_i$-ERK1/2 activation	CL316,243	HEK293 CHO-K1	[291]
GPR56	R565W and L640R	Altered $G\alpha_{12/13}$ signaling but no effects on $G\alpha_q$-independent Ca^{2+} channel		HEK293T/17	[292]