THE EUROPEAN SOCIETY OF MEDICINE
Medical Research Archives, Volume 12 Issue 9

REVIEW ARTICLE

Ligand-Free Signaling of G Protein Coupled Receptors: Addressing Unresolved questions with Antagonist Probes and Genomics

Wolfgang Sadee

[1] Professor Emeritus, Cancer Biology and Genetics, The Ohio State University
Professor Emeritus, Bioengineering and Therapeutic Sciences, Schools of Pharmacy and Medicine, University of California San Francisco
Chief Scientific Officer,
Aether Therapeutics Inc.

OPEN ACCESS

PUBLISHED
30 September 2024

CITATION
Sadee, W, 2024. Ligand-Free Signaling of G Protein Coupled Receptors: Addressing Unresolved questions with Antagonist Probes and Genomics. Medical Research Archives, [online] 12(9).
https://doi.org/10.18103/mra.v12i9.5666

DOI
https://doi.org/10.18103/mra.v12i9.5666

ISSN
2375-1924

ABSTRACT

G protein coupled receptors (GPCRs) exist each in multiple forms and aggregates distributed across various cell compartments, signaling along multiple pathways upon activation by agonists. In addition, pervasive ligand-free GPCR signaling also occurs with multiple functional states. I propose three distinct ligand-free receptor categories: first, low level slippage into active forms ('spontaneous basal signaling'); second, 'acutely activated ligand-free signaling' which sustains signaling after the agonist has dissociated from the receptor; and third, 'sustained ligand-free signaling' that is regulated to sustain cellular responses. Studies of the μ opioid receptor differentiate these three receptor forms and suggest that continued agonist stimulation can lead to sustained ligand-free signaling with a role in opioid dependence. The serotonin $5HT_{2A}$ receptor also appears to support ligand-free signaling of physiological and pharmacological relevance. Yet, systematic studies of distinct ligand-free receptor forms are scarce. Sustained ligand-free signaling can arise from various mechanisms, such as tethered extracellular peptide regions of GPCRs stabilizing an active receptor-G protein state, demonstrated with the glucagon-like peptide 1 receptor GLP1R. Accurate assays are needed to measure ligand-free signaling along specific pathways, localized to cellular sub-compartments. Neutral antagonists (no effect on ligand-free signaling) and inverse agonists (block ligand-free signaling) distinguish between the various forms of ligand-free signaling and can lead to distinct therapeutic applications, highlighted with opioid, serotonin, and peptide hormone receptors, targeting pain, depression and schizophrenia, and metabolic disorders. GPCR mutations that activate or suppress ligand-free signaling reveal (patho)physiological functions, but genetic effects on distinct ligand-free receptor signaling pathways are largely unresolved. Clarifying ligand-free signaling pathways of GPCRs has the potential to uncover hidden disease risk factors and novel targets for therapeutic interventions.

Introduction

This review focuses on the expanding field of ligand-free GPCR signaling, building on previous reviews [1,2]. Emerging advances reveal novel insights into the diversity of ligand-free signaling, its physiological relevance, and therapeutic opportunities.

Encoded by over 800 genes, GPCRs respond to diverse compounds, peptides, proteins, pH, mechanical stress, and photons, broadly regulating physiological functions and serving as the main drug target [3,4]. Each GPCR can exist in multiple conformations and aggregates with diverse signaling pathways [5]. As the activating stimulus conveys rather minute activation energy to the large receptor complex – for example the energy of a single photon – GPCRs are finely tuned to remain silent while responding to stimuli with high sensitivity. Yet, unstimulated GPCRs tend to slip into ligand-free active states or maintain sustained regulated ligand-free signaling, the extent of which varies for each receptor. Ligand-free GPCR signaling can have profound (patho)physiological functions [1,6,7], often revealed by gain-of function or loss-of function mutations [8]. Several GPCRs display high ligand-free signaling as part of their biological functions, for example the growth hormone secretagogue receptor 1 (GHSR1) with ~50% of maximal signaling occurring in ligand-free form [9]. For some GPCRs, such as the angiotensin II type 1 receptor (AT1), basal activity is difficult to detect but the membrane environment, interaction with autoantibodies, and mechanical stretch can increase ligand-free signaling [10].

Studies of ligand-activated GPCR signaling has revealed extensive complexity of the involved signaling pathways. Existing in various signaling complexes, each GPCR can be activated or blocked by biased agonists or antagonists, to engage diverse signaling pathways, involving distinct G proteins, b-arrestins, and more [11,13,14]. For drug development, biased agonists are employed to engage only a desirable signaling pathway with improved efficacy or reduced toxicity. Spatio-temporal bias introduces additional complexity to GPCR signaling demonstrated with the angiotensin receptor (AT_1R), V_2 vasopressin receptor (V_2R), and β_2-adrenergic receptor (β_2AR): preferential activation of G protein *versus* β-arrestin signaling changes over time upon receptor activation in step with receptor internalization into endosomal compartments [15]. Ligand-free signaling manifests itself also in different forms over time and cellular location for any given GPCR [16]. Varying properties of ligand-free GPCRs can be detected and exploited with use of biased antagonists [11]. While current genetics studies mostly address effects of mutations on the extent of overall ligand-free signaling, use of neutral antagonists and inverse agonists specific for a single pathway begins to unravel the biological relevance of each pathway separately.

Distinct categories of ligand-free receptor signaling

Most GPCRs display detectable ligand-free GPCR signaling, mostly of uncertain physiologic function, detectable with the opposing actions of neutral antagonists (do not block basal signaling) and inverse agonists (block basal signaling). Only a limited number of studies has addressed distinct ligand-free signaling receptor forms, supporting an equilibrium between three categories of active ligand-free receptor states. Each of these categories can exist in several sub-states determined by cellular location and composition of the receptor aggregate including accessory proteins, lipids, and various allosteric modulators (Fig. 1) [1,2]. The resting R^O form can spontaneously slip into a ligand-free active state termed R*, with poorly defined function. This type of signaling is often observed with GPCRs transfected into donor cells at high levels, overcoming the barrier imposed on spontaneous activation. Whether and how this slippage has physiological relevance remains unknown for most receptors.

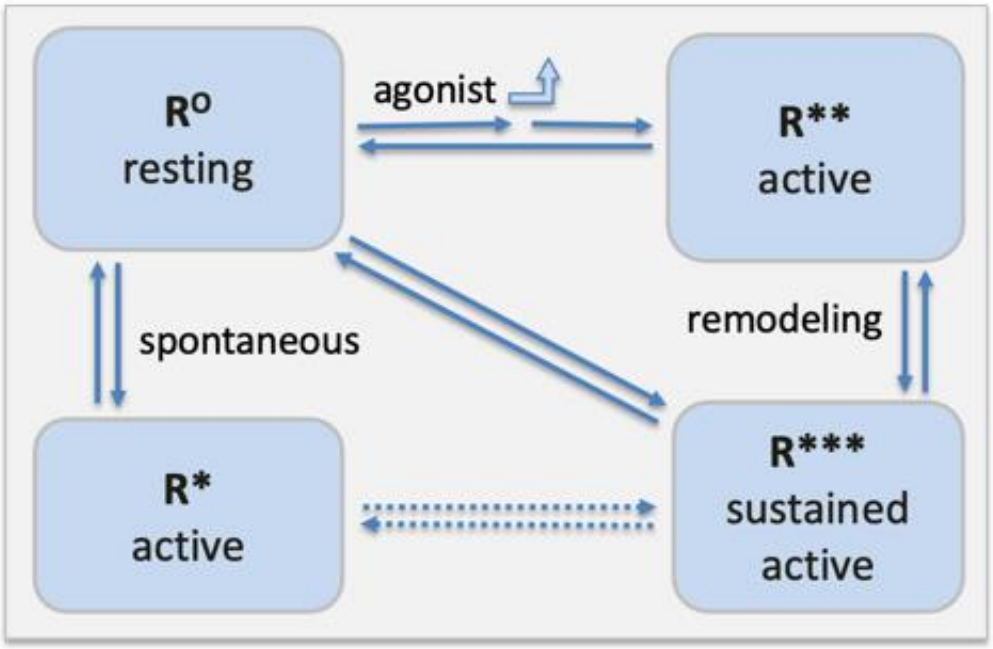

Figure 1. Model of distinct ligand-free states of a GPCR. All states are assumed to interconvert among each other at rates that fluctuate with the cellular environment and external stimuli. Receptor activation by an agonist can result in acutely activated R** if the ligand-free receptor continues signaling after agonist dissociation.

Agonist binding leads to a ternary agonist-receptor-G protein or b-arrestin complex – typically considered the active GPCR state [17]. However, activation of receptor signaling triggers profound changes in the composition and cellular location of the receptor complex, which can result in loss of high agonist affinity, agonist dissociation, and continued ligand-free receptor signaling [18] - defined here as the acutely activated R** state (Fig. 1). Duration of R** signaling can be a main factor in determining the time course of drug response [1]. Spontaneously activated R* and agonist-activated R** can be differentiated with use of biased antagonists [11]. For example, naloxone and naltrexone act as neutral antagonist at the spontaneously signaling ligand-free μ opioid receptor (MOR*) but as inverse agonists at the acutely agonist-generated MOR** and the sustained active MOR*** forms which continue signaling after agonist dissociation [2,19]. Many GPCRs display substantial sustained ligand-free receptor signaling by R***, a process under cellular regulation endowed with physiological functions, and a novel target for therapeutic interventions. Continued agonist stimulation can be one factor regulating the level of R*** signaling, for example, elevating sustained μ opioid receptor by MOR*** as a factor in opioid dependence (reviewed in [1]). Agonist pretreatments followed by washout generating R*** forms also changes antagonist efficacy (inverse agonism, neutral antagonism, and weak agonism) at the ligand-free forms of the Δ and κ opioid receptors [19,20]. Continued receptor signaling after agonist activation and dissociation may be a general phenomenon of GPCRs with profound physiological and pharmacological implications, illustrated further below with opioid and serotonin receptors.

Just as orthosteric agonists have the capacity to convert silent R^O into ligand-free signaling R**, allosteric ligands can stabilize distinct receptor forms and affect their equilibrium. A positive allosteric modulator can maintain MOR in a fully activated conformation, determined by cryogenic electron microscopy [21]. In contrast, a negative allosteric modulator is shown to stabilize a silent MOR conformation, and by binding adjacent to naloxone, enhances naloxone's potency while reducing withdrawal behavior in morphine dependent mice [22]. Both orthosteric and allosteric ligands need to be studied for their ability to shift the equilibrium between ligand-free receptor forms (Fig. 1), with potential for enhanced therapeutic opioid management. The proposed accelerated conversion of sustained ligand-free μ opioid receptor (MOR***) signaling back to the resting state MOR^O by 6β-naltrexol, proposed to reverse opioid dependence [2], serves as an example for novel therapies through modulation of receptor state equilibria. Select GPCRs will serve here to illustrate the relevance of agonist-activated and sustained ligand-free GPCR signaling (R** and R***, respectively).

Agonist-activated ligand-free receptor signaling (R**): implications for agonist potency

Experimental documentation of continued R** signaling after agonist dissociation came unexpectedly from interactions involving retinal covalently bound to rhodopsin. When retinal dissociates from rhodopsin, the ligand-free rhodopsin-G protein complex continues to signal, allowing covalent rebinding of retinal over several cycles [18]. This cycle can be interrupted by inverse agonists, which could thus serve to treat retinitis pigmentosa caused by activating mutations of the rhodopsin gene [18].

Beyond the rhodopsin example, little direct evidence documents continued ligand-free R** signaling after the agonist dissociates. Nevertheless, pharmacological effects unaccounted for by common receptor models make a compelling case for a key role of R** in the immediate response to agonists, potentially applicable to many GPCRs. If the rate of agonist dissociation is faster than the decay rate of active R**, ligand-free R**can become the main receptor form mediating the response to an agonist, while also accounting for extreme potency [1,2]. The ultrapotent μ opioid receptor (MOR) agonist etorphine displays an EC50 of ~0.1 mg/kg for antinociception in rats; this dose delivers enough molecules to the brain to occupy only 2% of available MOR sites [23]. In addition, the *in vivo* dissociation from MOR is fast ($t_{1/2}$ 50 sec *versus* >30 min *in vitro*), while circulating drug levels rapidly decline [23]. These results support the hypothesis that etorphine serves as a trigger generating MOR**, while rapidly dissociating and rebinding to activate more receptors, thereby, account for the exceptional potency of etorphine. When measured in MOR-transfected cells, opioid agonist-MOR dissociation rates are rapid for morphine, DAMGO, and fentanyl [24]. Yet, the *in vivo* potencies of opioid agonists such as morphine and fentanyl are much higher than expected from *in vitro* studies [24], likely a results of the same trigger mechanism described for etorphine, requiring only low receptor occupancy. The inability of morphine even at lethal doses to fully displace opioid antagonist tracers from MOR *in vivo* further supports a key role of ligand-free MOR** signaling, having low affinity for agonists but high affinity for antagonists [25]. It remains to be determined what role R** plays with GPCRs in general.

The same mechanism implicated for etorphine at MOR could also account for the extreme potency of lysergic acid diethylamide (LSD), which causes hallucinations at or above 30 mg per dose in humans [26], capable of reaching only low occupancy at its target receptor $5HT_{2A}$. LSD displays high affinity binding to a $5HT_{2A}$-G protein complex, with long dissociation half-lives [27,28]. As suggested for etorphine, its extreme potency *in vivo* could result from rapid dissociation while signaling continues, with rapid rebinding to inactive receptors, triggering a robust overall response even at low receptor occupancy.

Several GPCRs display high ligand-free signaling, for example growth hormone stimulation receptor (GHSR1) (~50% of maximum signaling capacity) [29] or are maintained in the active ligand-free states by portions of their extracellular regions reaching towards the binding pocket. The protease activated GPCR family acquires an intrinsic tethered agonist ligand by proteolysis of the N-terminus [30], while several peptide hormone receptors are sustained in an active ligand-free conformation coupled to G_s by allosteric actions of their extracellular loops without an orthostatic ligand [31], presumably in a sustained R*** state further discussed below.

Upon activation at the cell membrane, GPCRs can internalize into cellular compartments, mostly after agonist dissociation. As a result, polar GPCR ligands such as biogenic amines, glutamate, and peptides do not

have access to the cell's interior unless membrane transporters facilitate entry [32]. The role of intracellular ligand-free GPCR signaling captures increasing attention, for example regarding $5HT_{2A}$ [33].

Ligand-free receptor signaling (R**): neutral antagonist and inverse agonist effects

Antagonists of varying efficacy reveal the presence of ligand-free GPCR signaling (Fig. 2). Endogenous GPCR antagonist such as Agouti polypeptide at melanocortin receptors [34] and Leap2 at growth hormone secretagogue receptor (GHSR1) [35] act as inverse agonists, implicating a physiological role of ligand-free signaling that is under regulatory control.

Upon activation at the cell membrane, GPCRs can internalize into cellular compartments, mostly after agonist dissociation. As a result, polar GPCR ligands such as biogenic amines, glutamate, and peptides do not have access to the cell's interior unless membrane transporters facilitate entry [32]. The role of intracellular ligand-free GPCR signaling captures increasing attention, for example regarding $5HT_{2A}$ [33].

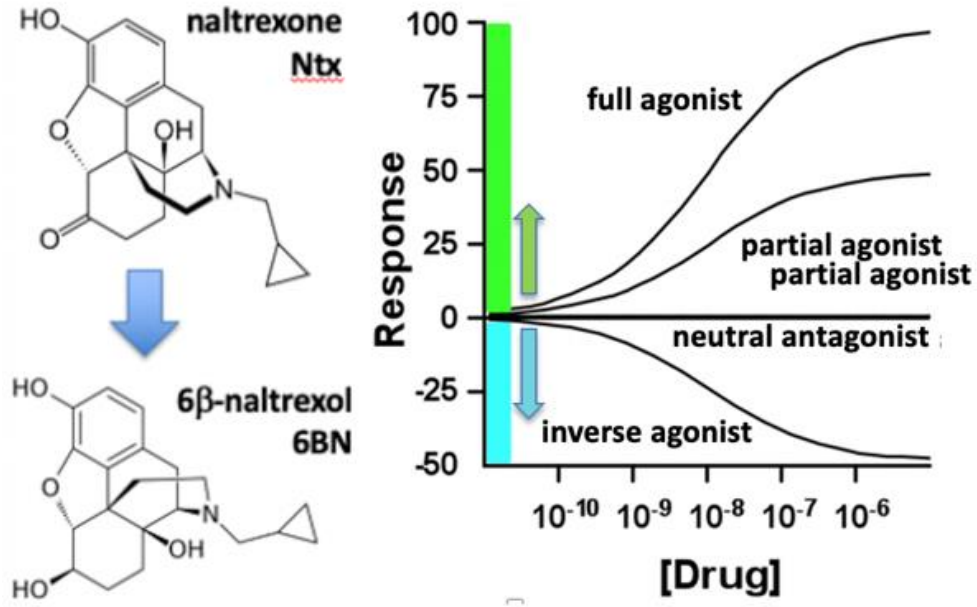

Figure 2. GPCR signaling response. A ligand's efficacy can range from full agonist to inverse agonist. Ligand-free signaling (at 0) can vary with cellular conditions. In the case of MOR, Ntx is a neutral antagonist at MOR* but an inverse agonist at MOR** and MOR***. Its major metabolite 6BN is neutral at all three ligand-free signaling states of MOR.

As each receptor state shown in Figure 1 can exist in distinct configurations, one can expect that any given antagonist can have different efficacy at each form. Whereas naloxone and naltrexone are neutral antagonists at spontaneous basal μ opioid receptor (MOR*) signaling but inverse agonists at MOR** and MOR***, 6β-naltrexol is a neutral antagonist at all three MOR forms [19]. Similar results have been documented with various antagonist at the δ and κ opioid receptors stimulated by their respective agonists before testing ligand-free receptor signaling [19,36]. For example, naloxone turns into an inverse agonist at the δ opioid receptor [19,36], and 6β-naltrexol is neutral before agonist treatment of the κ opioid receptor but turns inverse after κ agonist stimulation, whereas naltrexone remains neutral [19], with as yet undetermined pharmacological consequences.

The distinct receptor interaction of naltrexone compared to 6β-naltrexol can account for dramatic differences in antagonist potency at the μ opioid receptor (MOR), documented in animal studies. Even though 6β-naltrexol has near equal affinity to MOR compared to naltrexone, it is ~100-fold less potent in both blocking morphine antinociception and causing withdrawal in opioid-dependent rhesus monkeys [37], even though 6β-naltrexol readily penetrates the blood-brain-barrier in this species (J. Oberdick, unpublished data). This discrepancy could have resulted from the inverse activity of naltrexone at acutely activated ligand-free MOR** without any competition with the agonist, whereas 6β-naltrexol does not block MOR** signaling but can block receptor activation only in competition with the agonist at resting MOR^O. This large potency difference implicates MOR** as the main receptor form carrying out the response to the agonist trigger. Potency differences between neutral antagonists and inverse agonists should be assessed for each GPCR separately.

Endowed with substantial ligand-free signaling, the cannabinoid-1 receptor (CB1R) is targeted with antagonists to reduce drug taking behaviors, but clinical trials with the inverse agonist rimonabant were abandoned because of excessive toxicity [37]. Testing the neutral CB1R antagonist AM6527 in an animal model of cocaine self-administration proved effective while lacking adverse effects [38], exemplifying the need to consider antagonist efficacies.

As ligand-free signaling by each GPCR can involve multiple receptor conformations, antagonists could display distinct properties at each sub-from (biased antagonism) [11]. This question was tested on signaling pathways of the serotonin receptor $5HT_{2A}$. Enhanced ligand-free signaling is implicated in schizophrenia, since the antipsychotic ketanserin has been identified as an inverse agonist at $5HT_{2A}$, a property thought to contribute to its superior efficacy compared to neutral $5HT_{2A}$ antagonists [39]. Muneta Arrate *et al.* [40] determined efficacy of several $5HT_{2A}$ antagonists in activating or blocking G protein signaling (G_{i1-3}, G_o, $G_{q/1}$) by ligand-

free $5HT_{2A}$ in *post mortem* human brain cortex, showing that each of the tested antipsychotic drugs displays a distinct efficacy profile at each signaling pathway. For example, altanserin and pimavanserin displayed inverse agonism at G_{i1} but neutral antagonism at $G_{q/11}$, the former implicated in agonist-mediated hallucinogenic effects and the latter in memory effects [41]. However, Wallach *et al*. [42] contend that a threshold level of G_q activation is required to induce psychedelic-like effects. Use of BRET analyses of interactions between $5HT_{2A}$ with various G protein and β-arrestin enabled fingerprint classification of antipsychotic drugs [43], supporting the notion that G_{i1} signaling is a target of further antipsychotic drug development. However, these studies were done in transfected cells without agonist pretreatment that could have altered antagonist efficacy as shown for opioid receptors. Also, it remains uncertain whether these studies target ligand-free $5HT_{2A}$ states at the plasma membrane or endosomal compartments, reflecting possible spatio-temporal antagonist bias. Nevertheless, these results begin to address a critical open issue, namely, the properties of various ligand-free receptor states. Similar studies are needed at other GPCRs with relevant ligand-free signaling to guide drug development.

Sustained ligand-free receptor signaling (R***): regulation and physiological implications

Multiple GPCRs display substantial R*** signaling, often involving intracellular sites after agonist activation of surface receptors followed by receptor internalization [1]. Sustained high ligand-free signaling is physiologically relevant, demonstrated with opioid receptors, growth hormone secretagogue receptor 1 (GHSR1), melanocortin receptors, and more [44]. For example, continuous signaling of GSHR1 is required to sustain growth in early childhood [45]. Yet, little is known about the equilibration process between R^o and R*** receptor states (Fig. 3). As GPCRs tend to heterodimerize with multiple other GPCRs, receptors with sustained high R*** signaling can convey their active status to heterodimeric GPCR partners, for example GHSR1 to dopamine receptors D1 and D2, melanocortin receptor 3, cannabinoid receptor B1, and serotonin receptor 5-HT2C [46]. This process spreads ligand-free R*** signaling across many GPCRs, generating a complex network of physiological relevance.

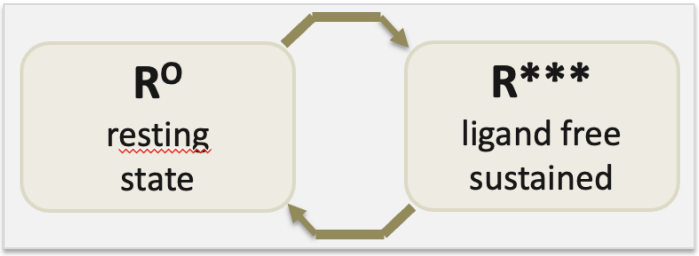

Figure 3. GPCRs in resting state (R^O) in equilibrium with a sustained regulated ligand-free signaling state (R***). R*** can have profound physiological effects, but regulation and interconversion with RO is poorly understood.

The sustained ligand-free signaling state of the μ opioid receptor (MOR***) is thougth to be a driving force for opioid dependence (reviewed in [2]). Whereas the neutral antagonist 6β-naltrexol binds to but does not block R*** signaling, it gradually and potently appears to accelerate reversal to the resting MOR^O state ([2]). Thereby, 6β-naltrexol suppresses opioid dependence, possibly accounting for 6β-naltrexol's exceptional potency of reversing the opioid dependent state in rodents without blocking analgesia nor causing withdrawal [2]. This reversal of receptor states was directly demonstrated in rat peripheral nociceptors expressing silent μ opioid receptors ($MOR^{O)}$ which is activated to ligand-free sustained signaling (MOR***) by inflammatory stimuli [47]. Unexpectedly, the inverse agonist naltrexone alone caused silent MOR^o conversion to active MOR*** in peripheral nociceptors [47], even though inverse agonists are thought to lock the receptor into the silent R^o state - this assumption may need to be revisited. In contrast, the neutral antagonist 6β-naltrexol reversed ligand-free MOR signaling back to the silent MOR^O [46]. Similarly, pretreatment of δ opioid receptor-transfected HEK293 cells with its inverse agonist ICI17486 increased receptor/G protein coupling, characteristic of an enhanced activity state, turning the inverse antagonist naloxone into a partial agonist [20], opposite to the effect of agonist pretreatment [19,36]. Beyond these example, little is known about what cellular factors and ligands regulate the equilibrium between R^o and R*** of GPCRs, as quantitative studies are still lacking. These results indicate that agonist receptor stimulation leads to incremental increases of sustained ligand-free receptor signaling - requiring further studies for other GPCRs as well.

Many GPCR ligands cannot readily penetrate cell membranes so that intracellular ligand levels are low. Yet, many GPCRs are present in intracellular locations and most internalize after agonist-activation, often in ligand-free form after agonist dissociation. For example, the type I metabotropic glutamate receptor mGLUR5, a target for developing autism spectrum disorder therapies [48], resides largely intracellularly in neurons [49], likely endowed with ligand-free signaling since glutamate does not readily enter the cells. To study glutamate activation of internalized metabotropic receptors in cell culture, a glutamate transporter had to be co-transfected [32]. The adrenergic receptor β1 also displays ligand-free signaling with intracellular locations where epinephrine has access only with co-transfection of a membrane transporter [50]. Irannejad *et al*. [51] argue that intracellular GPCRs have distinct signaling properties and propose the term location bias, further showing that that currently used β-blockers differ markedly in access to and ability to antagonize Golgi signaling. Clinical relevance of these findings remains to be clarified.

For some GPCRs, the extracellular loops and C-terminus function as internal, masked or open, covalent ligands or as allosteric factors stabilizing an active G

protein coupling state, creating sustained signaling of ligand-free receptors (reviewed in [1]). Protease activated receptors PAR1-4 are permanently activated by proteolysis of the extracellular N terminus [31]. Even if the local concentration of a tethered ligand is high, the active signaling process could reduce binding affinity of the tethered agonist and permit inverse agonist or allosteric modulators to shut down signaling or alter signaling pathways [29].

Three peptide hormones receptors offer further insight into protracted ligand-free signaling. Involved in glucose homeostasis, glucagon-like peptide-1 (GLP-1), glucagon (GCG), and glucose-dependent insulinotropic polypeptide (GIP) activate signaling cascades of their cognate receptors GLP1R, GCGR, and GIPR, respectively [52], therapeutic targets for type 2 diabetes and obesity. Ligand-free signaling of all three receptors, including signaling from intracellular sites, has been shown to enhance glucose-induced insulin secretion [53], demonstrating its physiological relevance. A β-arrestin 1 complex with the glucagon receptor GCGR has been shown to form a complex with G protein promoting sustained ligand-free signaling in endosomes [54]. *Cong et al*. [52] used cryo-electron microscopy to demonstrate for all three receptors the formation of a receptor state induced by G_s binding and stabilized with binding of their extracellular loop regions acting as allosteric ligands, resembling that of the ternary peptide agonist-receptor-G protein complex presumed to be the active receptor form. Since these peptide hormones are highly potent agonists and are mobilized when metabolic conditions change, it is possible that sustained ligand-free signaling of receptor forms is a key part of their duration of action, with implications for optimal dosing regimen.

A role for extracellular loop regions stabilizing active ligand-free receptor forms was also demonstrated for orphan receptor GPCR52 [55] and adhesion receptor ADGRG5 [31], suggesting a rather common mechanism maintaining R*** activity. The regulation of sustained ligand-free signaling involves multiple mechanisms, including internal allosteric and orthosteric ligands, diverse cellular components, and homo- and hetero-oligomerization between GPCRs [5,12,14,56], all applicable to agonist activated receptor signaling while explored in much less detail for ligand-free receptor forms.

Endogenous antagonists of ligand-free signaling GPCRs

Endogenous antagonists play a role in controlling ligand-free signaling of several GPCRs, including melanocortin receptors MCR1,3,4 [34,57], growth hormone secretagogue receptor GSHR1a [35], and interleukin 1 receptor IL1R1 [58]. The anti-inflammatory IL1R1 antagonist peptide of IL1RN is physiologically and therapeutically relevant to the pathogenesis of both acute and chronic inflammatory diseases [58]. The Agouti signaling peptide ASIP targets the melanocortin-1 receptor MCR1, interfering with pigmentation [34], while Agouti related neuropeptide AGRP block of MCR4 leads to obesity and late onset hyperglycemia [59]. Both Agouti peptides are inverse agonists, highlighting the relevance of ligand-free receptor signaling.

The hormone ghrelin activates the growth hormone secretagogue receptor 1a (GHSR1a), whereas the protein LEAP-2 serves as an inverse agonist of GHSR1a, blocking ghrelin's effects on food intake and hormonal secretion [35]. The LEAP-2/ghrelin molar ratio in blood increases with food intake and obesity affecting energy intake. Both proteins are circulating in minute quantities, highlighting their role in potently regulating sustained ligand-free GHSR1a. A systematic genome-wide search for endogenous GPCR antagonist will likely reveal more such examples with therapeutic implications.

Sustained ligand-free receptor signaling of the serotonin 2A receptor ($5HT_{2A}$)

Agonist activation leading to a transient active ligand-free receptor state (Fig. 1) is one likely regulatory factor that subsequently elevates sustained ligand-free signaling, as proposed for the μ opioid receptor driving dependence [2,60]. One would expect long-lasting effects of low agonist doses that go beyond the initial receptor trigger. The serotonin $5HT_{2A}$ receptor could transition from an acutely agonist-upregulated R** state to a long-lasting ligand-free R*** signaling state, largely with intracellular location, leading to enhanced sustained ligand-free R*** signaling. Sustained effects of one or a few doses of hallucinogens such as lysergic acid diethylamide (LSD) or psilocybin, presumably acting intracellularly *via* $5HT_{2A}$-G protein signaling [33], can result in persistent growth of dendritic spines, enhanced neuroplasticity [33,61], and neurological effects lasting for several days long after the drugs are largely eliminated. A low dose of LSD (26 μg) given to individuals with depressive symptoms results in positive mood and stimulant-like effects and reduces depression scores 48h after dosing [26]. A single dose of psilocybin (25mg/70kg) alters emotions and brain function for up to one month [62].

These hallucinogenic compounds are also under study for treating depression, which could involve a pathway from intracellular $5HT_{2A}$ signaling to tyrosine kinase B (TRKB) as target [63], while direct binding to TRKB has also been invoked [64]. Endogenous serotonin metabolites such dimethyltryptamine exist intracellularly at low concentrations, with low potency as hallucinogens. Nevertheless, they have been proposed to represent intracellular endogenous regulators of $5HT_{2A}$ [65], possibly by upregulating ligand-free receptor signaling. Microdosing of psychedelics at doses below those causing hallucinations has been applied to treat various mood disorders including depression [66]. Possibly, sub-acute levels of hallucinogens sufficiently enhance sustained ligand-free serotonin receptor $5HT_{2A}$ signaling to yield lasting therapeutic benefits. Experimental studied are needed to test these hypothesis. Inverse $5HT_{2A}$ agonists targeting the involved signaling pathway would be expected to block these effects. It remains unknown whether neutral $5HT_{2A}$ antagonists can accelerate reversion of the sustained ligand-free signaling state to the silent receptor state as proposed for the neutral MOR antagonist 6β-naltrexol.

Genetics of ligand-free GPCR signaling

The purpose of this review is not to cover this broad topic comprehensively, but rather to address the question what can be learned about the functions of innate ligand-free GPCR signaling. Both gain- and loss-of-function mutations affecting ligand-free GPCR signaling can reveal physiological or pathophysiological relevance. Yet, it remains mostly unknown what specific signaling

pathways are affected, or whether a mutation affects only ligand-mediated or ligand-free signaling, or both.

Multiple reviews and large databases document a wealth of information on GPCR mutations [3,7,8,66-68]. Main topics address cancers, often associated with gain-of-function driver mutations [69-71]. Among the ~800 GPCRs, mutations in at least 55 GPCR genes cause 66 inherited monogenic diseases in humans, involving classic gain- or loss-of-function variants [8]. In addition, multiple mechanisms can result in pathophysiological conditions, including biased signaling, ectopic expression, gene fusion, and gene dosing [8]. GPCR variants further can disrupt ligand binding, G protein coupling, receptor desensitization and receptor recycling, while gain-of-function variants can result in biased signaling or constitutive ligand-free activity [7, 72]. For example, activating and inactivating variants of the calcium sensing receptor gene *CaSR* cause familial hypocalciuric hypercalcemia and autosomal dominant hypocalcemia, respectively, treatable with calcimimetics and calcilytics [7,73]. *In* silico alanine-scanning mutations of GPCRs demonstrates the fluid nature of intramolecular thermodynamic interactions between the receptor's structural elements, revealing underlying mechanisms of disease causing GPCR mutations [16].

Among pertinent examples, loss of ligand-free signaling of the growth hormone secretagogue receptor GSHR1a has been associated with familial short stature [45]. For example, the A203E mutation of *GHSR1a* ablates ligand-free signaling leading to short stature in human carriers [74]. Nevertheless, the mutant receptor still responds to ghrelin activation, but ghrelin fails to affect food intake in mice [74]. One might conclude that either the acute ghrelin signaling response differs from that of ligand-free GHSR1a signaling, or the latter is an integral part of the combined innate and agonist-dependent response. In another example of a basally active GPCR, the thyroid-stimulating hormone receptor (TSHR), 'inverse agonist' *TSHR* mutations not only lower basal activity but also attenuate agonist-induced signaling, with pathogenic consequences [75]. An immune checkpoint in the tumor micro-environment and an emerging target for cancer treatment, the adenosine A_{2A} receptor (A_{2A}AR) features multiple cancer-associated gain-of-function mutations that are immunosuppressive in the tumor micro-environment [76]. Scanning the Genomic Data Commons (GDC) yielded 15 tumor associated *A_{2A}AR* mutants that were expressed in HEK293T cells for testing the potency of antagonists [76], supporting drug development. Detailed analyses of the ligand-free signaling pathway(s) and their cellular location are needed to reveal the impact of GPCR mutations.

Genetic factors can alter ligand-free GPCR signaling by multiple mechanism other than directly through GPCR variants themselves. Allosteric factors, lipids, proteins associated with the multicomponent receptor complex, membrane potential, and endogenous antagonist peptides all impinge on the formation and stability of ligand-free GPCR signaling. These indirect factors modulating ligand-free GPCR signaling can be addressed by measuring ligand-free signaling in a sufficiently large number of subjects with available genomic information. Thereby, genome-wide association studies could serve to discover hidden genetic variants indirectly affecting GPCRs that impinge on ligand-free signaling.

Conclusions

This review highlights a pervasive role of ligand-free signaling of GPCRs, providing a roadmap to further studies, and revealing novel therapeutic opportunities beyond those afforded by typical GPCR ligands. Numerous publications already address diverse aspects of ligand-free signaling; yet, critical gaps remain to be resolved. Whereas the multifaceted structure and function of each GPCR with bound agonist or antagonist have been thoroughly investigated, much less is known about the diversity of ligand-free signaling states for each GPCR individually. Key factors determining these states include spatial and temporal events that lead to new functions (spatio-temporal bias) dependent on membrane-bound and intracellular locations.

To address these gaps, new methods are needed to measure quantitatively ligand-free signaling over time, including cellular location and receptor complex composition including signaling proteins (such as G proteins and arrestins). The analysis of serotonin receptor $5HT_{2A}$ signaling with neutral antagonists and inverse agonists provides a roadmap for developing quantitative assays for individual signaling pathways [40,43]. Phenotypic *in vivo* effects of inverse agonists could also serve as downstream measures of ligand-free receptor signaling, for example, naloxone withdrawal symptoms as a surrogate measure of ligand-free μ opioid receptor signaling.

Once ligand-free signaling can be measured accurately over time, open questions about treatment outcomes can be resolved, for example, the long-lasting effects of psychedelic drugs [26,33,65] that may result from sustained serotonin receptor $5HT_{2A}$ signaling. For receptors with known functional ligand-free signaling, such as growth hormone secretagogue receptor GHSR, one should ask whether continuous or sporadic agonist treatments are more effective, with agonists serving as trigger regulating acute and long-term ligand-free receptor activation. As to genetics, GPCR mutations affecting ligand-free signaling should be assessed for differential effects on the spectrum of receptor states, revealing novel phenotypic associations. Genome-wide association studies (GWAS) can further reveal hidden variants that affect ligand-free GPCR signaling. Overall, resolving diverse aspects of ligand-free GPCR signaling has the potential to reveal novel physiological functions and therapies.

Conflicts of Interest:

WS is Chief Scientific Officer of Aether Therapeutics Inc.

Acknowledgement:

Research related to opioid receptros was funded by NICHD R21HD085496, NIDA R44DA045414, Aether CRADA NCATS agreement Ref. No. 2020-04.

References

1. Sadee W. Ligand-free signaling of G-protein-coupled receptors: physiology, pharmacology, and genetics. *Molecules*. 2023;28:6375. https://doi.org/10.3390/molecules28176375
2. Sadee W, McKew JC. Ligand-free signaling of G-protein-coupled receptors: Relevance to μ opioid receptors in analgesia and addiction. *Molecules.* 2022;27:5826. https://doi.org/10.3390/molecules27185826
3. Pándy-Szekeres W, Caroli J, Marmybekov A, et al. GPCRdb in 2023: State-specific structure models using AlphaFold2 and new ligand resources. *Nucleic Acids Res.* 2023, *51*, D395–D402.
4. Sriram K, Insel PA. G protein-coupled receptors as targets for approved drugs: How many targets and how many drugs? *Mol Pharmacol.* 2018;93:251–258.
5. Perez DM, Karnik SS. Multiple signaling states of G-protein-coupled receptors. *Pharmacol Rev.* 2005;57:147–161.
6. Meye FJ, Ramakers GM, Adan RA. The vital role of constitutive GPCR activity in the mesolimbic dopamine system. *Transl Psychiatry.* 2014;4:e361. doi:10.1038/tp.2013.130.
7. Thompson MD, Percy ME, Cole DEC, Bichet DG, Hauser AS, Gorvin CM. G protein-coupled receptor (GPCR) gene variants and human genetic disease. *Critic Rev Clinical Labor Sci*. 2024; 1–30. https://doi.org/10.1080/10408363.2023.2286606.
8. Schöneberg T, Liebscher I. Inherited GPCR-related diseases. *Pharmacol Rev*. 2021;73(1):89-119.
9. Kern A, Grande C, Smith RG. apo-Ghrelin receptor (apo-GHSR1a) regulates dopamine signaling in the brain. *Front Endocrinol.* 2014;5:129. doi: 10.3389/fendo.2014.00129
10. Unal H, Karnik SS. Constitutive activity in the angiotensin II type 1 receptor: discovery and applications. *Adv Pharmacol*. 2014;70:155-174.
11. Sadee W, Oberdick J, Wang Z. Biased opioid antagonists as modulators of opioid dependence: Opportunities to improve pain therapy and opioid use management. *Molecules.* 2020;25:4163.
12. Wingler LM, Lefkowitz RJ. Conformational basis of G protein-coupled receptor signaling versatility. *Trends Cell Biol.* 2020;30(9):736–747.
13. Kurose H, Kim SG. Pharmacology of antagonism of GPCR. *Biol Pharm Bul.* 2022;45:669–674.
14. Zhou B, Giraldo J. An operational model for GPCR homodimers and its application in the analysis of biased signaling. *Drug Discov Today.* 2018;23:1591–1595.
15. Tóth AD, Szalai B, Kovács OT, et al. G protein-coupled receptor endocytosis generates spatiotemporal bias in β-arrestin signaling. *Sci Signal*. 2024;17(842):eadi0934. doi: 10.1126/scisignal.adi0934.
16. Anantakrishnan S, Naganathan AN. Thermodynamic architecture and conformational plasticity of GPCRs. *Nat Commun*. 2023;14(1):128. doi: 10.1038/s41467-023-35790-z.
17. Weis, W.I.; Kobilka, B.K. The molecular basis of G protein-coupled receptor activation. *Annu. Rev. Biochem.* 2018, *87*, 897–919.
18. Schafer CT, Fay JF, Janz JM, Farrens DL. Decay of an active GPCR: Conformational dynamics govern agonist rebinding and persistence of an active, yet empty, receptor state. *Proc Natl Acad Sci USA.* 2016;113:11961–11966.
19. Wang D, Sun X, Sadee W. Wang D, Sun X, Sadee W. Different effects of opioid antagonists on mu-, delta-, and kappa-opioid receptors with and without agonist pretreatment. *J Pharmacol Exp Ther*. 2007;321(2):544-552.
20. Piñeyro G, Azzi M, deLean A, Schiller PW, Bouvier M. Reciprocal regulation of agonist and inverse agonist signaling efficacy upon short-term treatment of the human delta-opioid receptor with an inverse agonist. *Mol. Pharmacol.* 2005;67:336–348.
21. Kaneko S, Imai S, Uchikubo-Kamo T, Hisano T, Asao N, Shirouzu M, Shimada I. Structural and dynamic insights into the activation of the μ-opioid receptor by an allosteric modulator. *Nat Commun*. 2024;15(1):3544. doi: 10.1038/s41467-024-47792-6.
22. O'Brien, E.S., Rangari, V.A., El Daibani, A. *et al.* A μ-opioid receptor modulator that works cooperatively with naloxone. *Nature.* 2024;631:686-693.
23. Perry DC, Rosenbaum JS, Kurowski M, Sadée W. ^{3}H-Etorphine receptor binding *in vivo*: small fractional occupancy elicits analgesia. *Mo. Pharmacol.* 1982:21,272–279.
24. Alhosan N, Cavallo D, Santiago M, Kelly E, Henderson G. Potency, dissociation kinetics and reversibility of fentanyls and nitazenes by naloxone at the μ opioid receptor. *BioR$_X$*. 2024; doi: https://doi.org/10.1101/2024.06.04.597322 June 5, 2024.
25. Rosenbaum JS, Holford NHG, Richard ML, Aman RA, Sadee W. Discrimination of three types of opioid binding sites in rat brain in vivo. *Mol Pharmacol.* 1984;25:242–248.
26. Molla H, Lee R, Tare I, deWit H. Greater subjective effects of a low dose of LSD in participants with depressed mood. *Neuropsychopharmacol.* 2024;49:774–781 (2024).
27. Kim K, Che T, Panova O, DiBerto JF, et al. Structure of a hallucinogen-activated Gq-coupled 5-HT$_{2A}$ serotonin receptor. *Cell*. 2020;182(6):1574-1588.
28. Wacker D, Wang S, McCorvy, JD, et al. Crystal structure of an LSD-bound human serotonin receptor. *Cell*. 2017;168:377-389.
29. Cornejo MP, Mustafá ER, BarrileF, Cassano,D, et al.The intriguing ligand-dependent and -independent actions of the growth hormone secretagogue receptor on reward related behaviors. *Neurosci Biobehav Rev.* 2021;120: 401–416.
30. Canto I, Soh UJ, Trejo J. Allosteric modulation of protease-activated receptor signaling. *Mini Rev. Med. Chem.* 2012;*12*:804–811.
31. Wilde C, Fischer L, Lede V, et al. The constitutive activity of the adhesion GPCR GPR114/ADGRG5 is mediated by its tethered agonist. *FASEB J.* 2016;30:666–673.
32. Purgert CA, Izumi Y, Jong YJ, Kumar V, Zorumski CF, O'Malley KL. Intracellular mGluR5 can mediate synaptic plasticity in the hippocampus. *J Neurosci.* 2014;34:4589–4598.
33. Vargas MV, Dunlap LE, Dong C, et al. Psychedelics promote neuroplasticity through the activation of intracellular 5-HT2A receptors. *Science.* 2023;379:700–706.
34. Kanetsky PA, Swoyer J, Panossian S, Holmes R, Guerry D, Rebbeck TR. A polymorphism in the agouti signaling protein gene is associated with human pigmentation. *Am J Hum Genet*. 2002;70:770-775.

35. Lu X, Huang L, Huang Z, Feng D, Clark RJ, Chen C. LEAP-2: an emerging Endogenous Ghrelin Receptor Antagonist in the Pathophysiology of Obesity. *Front Endocrinol.* 2021;12:717544. doi: 10.3389/fendo.2021.717544.
36. Liu JG, Prather PL. Chronic agonist treatment converts antagonists into inverse agonists at delta-opioid receptors. *J Pharmacol Exp Ther.* 2002;302:1070–1079.
37. Ko MC, Divin, MF, Lee H, Woods JH, Traynor JR. Differential in Vivo Potencies of Naltrexone and 6β-Naltrexol in the Monkey. *J Pharmacol Exp Ther.* 2006;316:772–779.
38. Soler-Cedeño O, Alton H, Bi GH, et al. AM6527, a neutral CB1 receptor antagonist, suppresses opioid taking and seeking, as well as cocaine seeking in rodents without aversive effects. *Neuropsychopharmacol.* 2024; https://doi.org/10.1038/s41386-024-01861-y
39. Kantrowitz JT. Targeting serotonin 5-HT_{2A} receptors to better treat schizophrenia: Rationale and current approaches. *CNS Drugs.* 2020;34:947–959.
40. Muneta-Arrate I, Miranda-Azpiazu P, Horrillo I, Diez-Alarcia R, Meana JJ. Ligand bias and inverse agonism on 5-HT_{2A} receptor-mediated modulation of G protein activity in post-mortem human brain. *Br J Pharmacol*. 2024; Apr 21. doi: 10.1111/bph.16368. Epub ahead of print.
41. Kossatz, E, Diez-Alarcia R, Gaitonde SA, et al. G protein-specific mechanisms in the serotonin 5-HT_{2A} receptor regulate psychosis-related effects and memory deficits. *Nat Commun.* 15:4307 (2024). https://doi.org/10.1038/s41467-024-48196-2
42. Wallach J, Cao AB, Calkins MM, *et al.* Identification of 5-HT_{2A} receptor signaling pathways associated with psychedelic potential. *Nat Commun.* 14, 8221 (2023). https://doi.org/10.1038/s41467-023-44016-1
43. Gaitonde, SA, Avet C, de la Fuente Revenga M, *et al.* Pharmacological fingerprint of antipsychotic drugs at the serotonin 5-HT_{2A} receptor. *Mol Psychiatry.* 2024; doi: 10.1038/s41380-024-02531-7. Online ahead of print.
44. Müller A, Berkmann JC, Scheerer P, Biebermann H, Kleinau G. Insights into basal signaling regulation, oligomerization, and structural organization of the human G-protein coupled receptor 83. *PLoS ONE.* 2016;*11*, 68260.
45. Pantel J, Legendre M,Cabrol S, et al. Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *J. Clin. Investig.* 2006;116:760–768.
46. Wellman M, Abizaid A. Growth hormone secretagogue receptor dimers: A new pharmacological target. *eNeuro.* 2015:, ENEURO.0053-14.2015.
47. Sullivan LC, Chavera TS, Jamshidi RJ, Berg KA, Clarke WP. Constitutive desensitization of opioid receptors in peripheral sensory neurons. *J Pharmacol Exp Ther.* 2016;359:411–419.
48. Matrisciano F, Locci V, Dong E, Nicoletti F, Guidotti A, Grayson DR. Altered Expression and *In Vivo* Activity of mGlu5 Variant a Receptors in the Striatum of BTBR Mice: Novel Insights Into the Pathophysiology of Adult Idiopathic Forms of Autism Spectrum Disorders. *Curr Neuropharmacol*. 2022;20(12):2354-2368.
49. Hubert GW, Paquet M, Smith Y. Differential subcellular localization of mGluR1a and mGluR5 in the rat and monkey substantia nigra. *J Neurosci.* 2001;21(6):1838–1847.
50. Nash CA, Wei W, Irannejad R, Smrcka AV. Golgi localized β1-adrenergic receptors stimulate Golgi PI4P hydrolysis by PLCε to regulate cardiac hypertrophy. *Elife.* 2019,*8*:e48167.
51. Irannejad R, Pessino V, Mika D, Huang B, Wedegaertner PB Conti M, von Zastrow M. Functional selectivity of GPCR- directed drug action through location bias. *Nat Chem Biol.* 2017;13,799–806.
52. Cong Z, Zhao F, Li Y, et al. Molecular features of the ligand-free GLP-1R, GCGR and GIPR in complex with G_s proteins. *Cell Discov*. 2024;10(1):18. doi: 10.1038/s41421-024-00649-0.
53. Shuai H, Xu Y, Ahooghalandari P, Tengholm A. Glucose-induced cAMP elevation in beta-cells involves amplification of constitutive and glucagon-activated GLP-1 receptor signalling. *Acta Physiol.* 2021;231:e13611.
54.. Chen K, Zhang C, Lin S, et al. Tail engagement of arrestin at the glucagon receptor. *Nature*. 2023;620(7975): 904-910.
55. Lin X, Li M, Wang N, et al. Structural basis of ligand recognition and self-activation of orphan GPR52. *Nature.* 2020;579:152–157.
56. Ferré S, Casadó V, Devi LA, et al. G protein-coupled receptor oligomerization revisited: Functional and pharmacological perspectives. *Pharmacol. Rev.* 2014;*66*:413–434.
57. Milligan G. Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol*. 2003;64(6):1271-1276.
58. Lennard AC. Interleukin-1 Receptor Antagonist. *Crit Rev Immunol*. 2017;37(i2-6):531-559.
59. Kleinau G, Heyder, NA, Tao YX, Scheerer, P. Structural complexity and plasticity of signaling regulation at the melanocortin-4 receptor. *Int. J. Mol. Sci.* 2020;21(16):5728. doi: 10.3390/ijms21165728
60. Wang Z, Bilsky, EJ, Porreca F, Sadée W. Constitutive receptor activation as a regulatory mechanism underlying narcotic tolerance and dependence. *Life Sci.* 1994;54:339–350.
61. Shao LX, Liao C, Gregg I, Davoudian PA, Savalia NK, Delagarza K, Kwan A.C. Psilocybin induces rapid and persistent growth of dendritic spines in frontal cortex in vivo. *Neuron.* 2021;109:2535–2544.e4.
62. Barrett FS, Doss MK, Sepeda ND, Pekar JJ, Griffith RR. Emotions and brain function are altered up to one month after a single high dose of psilocybin. *Sci Rep.* 2020;10:2214. https://doi.org/10.1038/s41598-020-59282-y
63. Slocum ST, DiBerto JF, Roth BL. Molecular insights into psychedelic drug action. *J Neurochem.* 2022;162:24–38.
64. Moliner R, Girych M, Brunello CA, et al. Psychedelics promote plasticity by directly binding to BDNF receptor TrkB. *Nat Neurosci.* 2023;26:1032–1041.
65. Barker SA, McIlhenny EH, Strassman R. A critical review of reports of endogenous psychedelic N,N-dimethyltryptamines in humans. 1955–2010. *Drug Test Anal.* 2012;4:617–635.
66. Rootman JM, Kryskow P, Harvey K. et al. Adults who microdose psychedelics report health related motivations and lower levels of anxiety and depression compared to non-microdosers. Sci Rep. 2021;11:22479 (2021). https://doi.org/10.1038/s41598-021-01811-4

67. Kazius J, Wurdinger K, VanIterson M, Kok J, Bäck T, Ijzerman AP. GPCR NaVa database: Natural variants in human G protein-coupled receptors. *Hum Mutat.* 2008;29:39–44.
68. Vassart G, Costagliola S. G protein coupled receptors: mutations and endocrine diseases. *Nat Rev Endocrinol.* 2011;7:362-372.
69. Chaudhary PK, Kim S. An insight into GPCR and. G proteins as cancer drivers. *Cells.* 2021;10:3288. doi: 10.3390/cells10123288
70. Bongers BJ, Gorostiola González M, Wang X, et al. Pan-cancer functional analysis of somatic mutations in G protein-coupled receptors. *Sci. Rep.* 2022;12: 21534. https://doi.org/10.1038/s41598-020-59282-y
71. Raymond, J.H.; Aktary, Z.; Larue, L.; Delmas, V. Targeting GPCRs and their signaling as a therapeutic option in melanoma. *Cancers* 2022, *14*, 706.
72. Parnot C, Miserey-Lenkei S, Bardin S, Corvol P, Clauser E. Lessons from constitutively active mutants of G protein-coupled receptors. *Trends Endocrinol Metab*. 2002;13(8):336-343
73. Ma S, Yin X, Pin JP, Rondard P, Yi P, Liu J. Absence of calcium-sensing receptor basal activity due to inter-subunit disulfide bridges. *Commun Biol*. 2024; 7(1):501. doi: 10.1038/s42003-024-06189-3.
74. Torz LJ, Osborne-Lawrence S, Rodriguez J, et al. Metabolic insights from a GHSR-A203E mutant mouse model. *Mol Metab.* 2020;39,101004.
75. Kleinau G, Jaeschke H, Mueller S, Worth CL, Paschke R, Krause G. Molecular and structural effects of inverse agonistic mutations on signaling of the thyrotropin receptor--a basally active GPCR. *Cell Mol Life Sci*. 2008;65(22):3664-3676.
76. Feng C, Wang X, Jespers W, et al. Cancer-Associated Mutations of the Adenosine A_{2A} Receptor Have Diverse Influences on Ligand Binding and Receptor Functions. Molecules. 2022;27(15):4676. doi: 10.3390/molecules27154676.