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Review

Biased Agonism or "*Biaism*" for Dummies: A Commentary

Jean A. Boutin [1,*] and Jérôme Leprince [1,2]

[1] Laboratory of Neuroendocrine Endocrine and Germinal Differentiation and Communication (NorDiC), University Rouen Normandie, Inserm, NorDiC UMR 1239, F-76000 Rouen, France; jerome.leprince@univ-rouen.fr
[2] High-Tech Research Infrastructures for Life Sciences (HeRacLeS), University Rouen Normandie, Inserm, US51, F-76000 Rouen, France
* Correspondence: jean.boutin@inserm.fr

Abstract: That signaling bias is a nth level of complexity in the understanding of G protein-coupled receptor (GPCR) activation is a first fact. That its exhaustive description, including the *mode d'emploi* of its quantitative measurement, remains a challenge is a second fact. That the use of this concept is promising for the design of drug candidates is a third fact. That the translation of signaling biases observed into in vivo specific effects is well documented is a fourth fact. However, the road to apply those aspects of receptology to a systematic description of a ligand and, a fortiori, of a drug candidate, still necessitates a huge body of studies. In the present commentary, the merits of the molecular description of receptor bias signaling are highlighted and the ligand induced-fit impact on GPCR structure, as well as on the functional repertoire of GPCRs, is discussed. An emphasis is given to the practical aspects during drug design, and, thus, the practical limitations of the current approaches, particularly in the context of as soon as the data are transferred to more integrated/living systems, might be a major limitation.

Keywords: G protein-coupled receptor; transduction pathway; signaling; bias; biaism; agonism; antagonism; ligands; potency; efficacy; conformational states

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1. Background

G protein-coupled receptor (GPCR) pharmacology gained an nth level of complexity [1] when it was realized that ligands are different from each other, not only in terms of structure, potency and efficacy, but also in terms of the transduction pathway(s) they trigger [2]. Indeed, different agonists at a given receptor initiate different intracellular events, such as Ca^{2+} release, IP_3 release, cAMP synthesis inhibition, intracellular protein phosphorylation and so on, which are mediated by G proteins, GPCR kinases, arrestins and probably less canonical proteins still unidentified. In brief, biased agonism is the capacity of two chemically different ligands of the same GPCR to transduce and/or modulate different signaling pathways (Figure 1A). By definition, this concerns mostly ligand–receptor pairs, except in the scarce cases in which a compound has a dual modality towards two pathways, elicited by its association with a unique receptor. Indeed, the same pharmacological modality is mandatorily required to quantitatively determine the bias (bias factor: $\Delta\Delta$Log(Emax/EC_{50}) or $\Delta\Delta$Log(t/Ka)) [3]. For instance, agouti-related peptide (AgRP) falls into this category of dual-modality ligands behaving as both an agonist and an antagonist at two different receptors, since this endogenous peptide does not act as a pure antagonist of α-MSH signaling, but rather like an inverse agonist reducing constitutive melanocortin receptor activity [4]. However, this peculiar modality is still debated and AgRP could be finally defined as a biased agonist of α-MSH-induced signaling, decreasing cAMP accumulation and stimulating ERK1/2 activation [5,6]. In addition to the ligand bias, there exists a system bias (Figure 1B), by which the involvement of all the non-ligand intracellular molecules of the signaling process might influence the functional response

of the receptor to the extracellular ligands [3]. We can also include, in this system bias, mutations in the sequence of the GPCR (see below). A thorough survey has been published on signaling bias that includes detailed analyses of the practical aspects of the concept, including experimental recommendations [3].

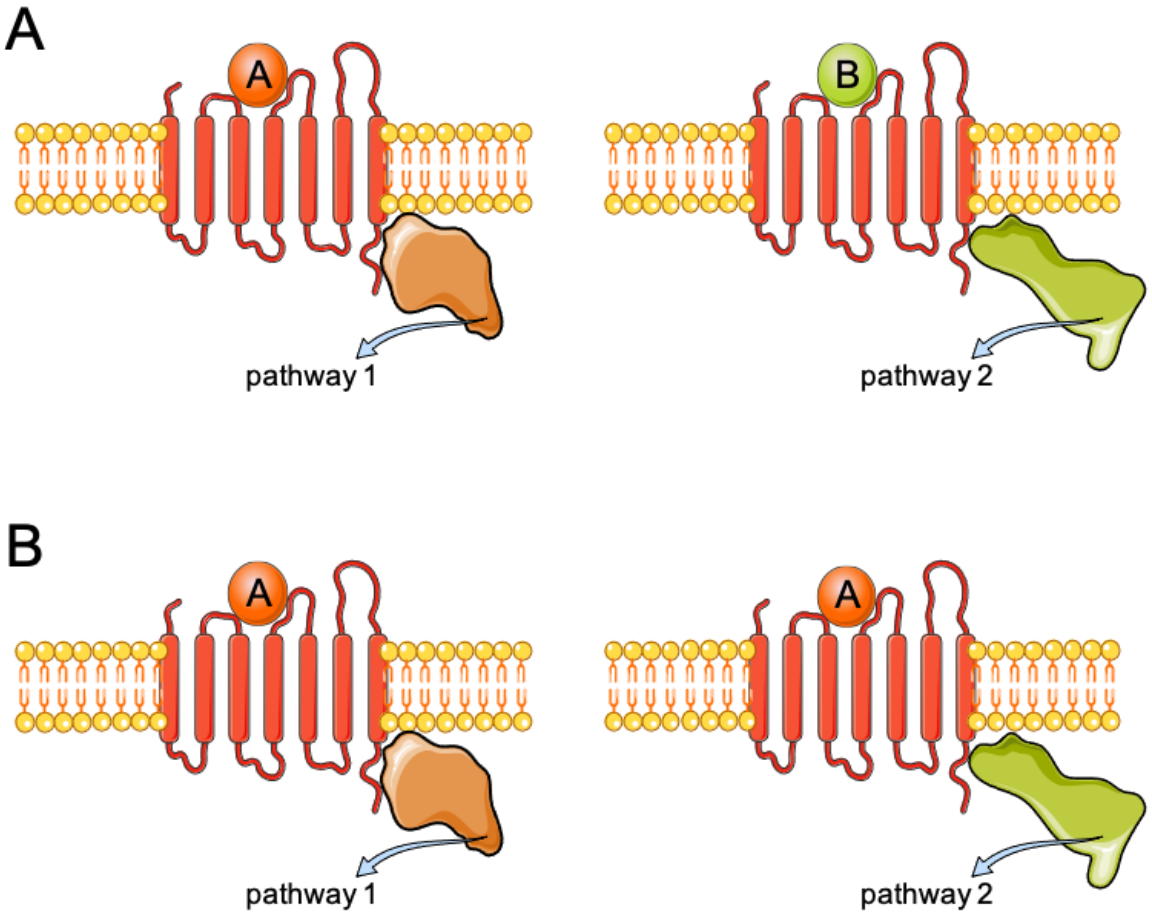

Figure 1. Schematic representations of ligand and system bias. (**A**) Ligand biased signaling (ligand biaism) between two different agonists of the same receptors in a same or a different cell. (**B**) System biased signaling (system biaism) of an agonist at a same receptor in two different cells.

The molecular foundation of this concept is that by interacting with a different set of amino acids in the binding pocket of the GPCR, an agonist promotes, by drastic conformational changes in the receptor structure, the intracellular recruitment of different G proteins or other transducer proteins, eliciting a different route than the next agonist of kin, even if the structures of both agonists are minimally different. More subtle modifications in the structure of the receptor can be caused by fine and local variations in the energy of interaction with the ligand, which result in modulations of the efficiency and/or potency of the ligands on the same pathway. This aspect must also be considered as a signaling bias. These features started to be systematically explored from a structural point of view by fluorescence approaches [7–9], crystallography and docking approaches [10,11] and by mass spectrometry, through hydrogen/deuterium exchange experiments, for instance in [12,13]. If we consider that a residue of the receptor can no longer be in direct contact with part of the agonist when the structure of the latter varies, it is easy to conceive conversely that, for the same agonist, the change in the initial residue of the receptor by another moiety modifies its conformation and, thus, its activation capacities in the same way, in terms of intensity or even transduction pathway. This is strongly suggested by the fact that mutations in the arginine vasopressin type 2 receptor lead to two different coupling

pathways [14,15], revealing that, also, the interaction of the agonist with a given amino acid in the vicinity of the receptor binding site drives, at least in part, the nature of the activated functional pathway.

The ligand bias has been summarized by Kolb et al. [3], as sketched in Figure 1A. Many examples of ligand bias have been reported in the literature, including our own contribution on melatonin receptors [16]. The pre-existence of multiple conformations of a receptor, evolving in equilibria between a single inactive and a single or multiple signaling-competent active conformation prototypes [17], stabilizes preferentially one of these structures, leading to a particular G protein recruitment for instance, while another agonist would stabilize a different one. These pre-existing populations comprise all the possible GPCR–transducer protein complexes capable of recognizing a limited number of possible agonists, including the synthetic and newly discovered ones [18]. Alternatively, another hypothesis could be that the binding of a given agonist to a receptor alters its intracellular moiety, leading to the recruitment of a specific G protein among the population of available G proteins, without the obligatory pre-formation of GPCR–transducer protein complexes.

An exhaustive summary of the state of the art in the structural field of GPCR applied to signaling bias was published by Sexton and Christopoulos group in 2018 [19] and remains a source of inspiration. Nevertheless, progression is mandatory in the structural definition of GPCR and GPCR complexes—using Artificial Intelligence (AI), for instance [20,21]—in order to be able to achieve ab initio designs of new ligands targeting predetermined signaling pathway(s). Some very recent works point already in this direction, in particular by systematically using cryo-electronic microscopy approaches rather than crystallographic ones, as reported for GPR101 [22], GPR34 [23], GPR84 [24] or, to a lesser extent, 5-HT_6 [25], following the pioneering work of Liang et al. on GLP1R [26]. This issue was also largely discussed by Seufert et al. [27].

Interestingly, as a first step in this direction, GPCR isoform minute changes in amino acid sequences lead to predictions of the changes in the actual structure, particularly of the C-terminal (in cellular) portion, causing possible variations in the coupling of the receptor isoforms to various downstream signaling pathways. Those observations pave the way towards an understanding of the variation in binding effectors, due to minute variations in the structure of the GPCR; as predicted, variation occurs in various ligand bindings [28]. Furthermore, it is clear that such variations in the GPCR–effector interface will also have an influence on the allostery and the dynamics of the associations and, thus, on the end result of the functionality of the receptor. Another aspect of the signaling bias that, to our understanding, might be under-regarded, is indeed the dynamics with which one given receptor is bound to its downstream effector proteins (G proteins and others). Indeed, a competition might be established between those effectors, depending on the variations in the contact surface to which they bind. And those surfaces might change upon the influence of ligand bindings, and, of course, this would depend on the structural modification of those surfaces initiated by structurally different ligands. For example, if ligand A leads to a stronger change in the surface than ligand B, it might have a direct impact on the speed at which an A-dependent pathway effector protein binds to the GPCR–ligand A complex, as opposed to a slower one for a ligand B-dependent one [29].

The field of signaling bias would like to answer several questions regarding the textbook case that the drug X, active on disease Y through the GPCR Z, has several effects on the various associated downstream functional pathways, represented with a radar plot. Question 1: can this profile be found with a completely different molecule, leading to another drug on the same diseases? Question 2: is the radar plot obtained for a given recombinant system really relevant/identical/similar to the one that could be obtained for GPCR Z-expressing cells derived from a patient with the disease Y? Question 3: can the profile explicitly explain the activity of the compound? Question 4: would it be true to say that the disease Y is due to a malfunction of receptor Z? Question 5: what are the roles of the other functional pathways of GPCR Z—unexplored at this stage—and the

allosteric molecules—whether positive (PAM) or negative (NAM)—on the biased functional determinations, and is this important for the profile of the molecule?

2. Basic Rules, Theory and Practicability Warnings of Signaling Bias

Basically, the signaling bias of a receptor can be measured, provided that, firstly, several agonists (including or not the endogenous one) are known for this receptor—even if their structures are closely related—and, secondly, that experiments are conducted with the largest panel of signaling pathway measurements for a full characterization—as exemplified for the melatonin receptors [16]. T.P. Kenakin established the systematization of these features, reporting at several occasions the use, the aim, the practicability and the recipes (including the mathematical ones) for exploring such a molecular scale phenomenon [30–33]. The many contexts of the theoretical evaluation of bias onto receptor pharmacology have been established, including at the highest level of receptology [3]. Thus, the practical use of these approaches at the experimental level is solidly established. The non-specialist reader would nevertheless poorly perceive the practicability of the approach in non-native systems; a complete description of the biased signaling would necessitate measurements in the presence of different stoichiometries of receptors and downstream molecules, reflecting natural intercellular variations and causing imperfect transpositions to in vivo systems [31]. Indeed, the notion of partial agonists depends on the density of the receptor, opening the first door towards the differences found between tissues, in which receptors might be expressed at different levels [31].

It seems to us, nevertheless, that the theoretical approach has led to a somewhat obscure picture of the situation, leading less-fundamental scientists (the "*dummies*" of the title) away from the reality of the description of this new concept. This has important consequences for compounds used as benchmarked agonists, because they lead to a functional response (often the single one measured in a given set of experiments), which might be something indeed very different in "physiological reality"—that is, in an in vivo context—where some parameters are less controlled than in an artificial system, as very well explained elsewhere [33,34].

It is certainly scary to understand the in-depth meaning of these observations. Indeed, molecules classified as agonists at a given receptor could activate very different pathways, leading, on a theoretical basis, to various cellular events that can be profoundly different from one another. By activating cAMP, one can generate the activation of the protein kinase A and further phosphorylations, leading to a cascade of events, while activating the calcium mobilization can lead to different cellular responses and a cascade of completely different events. That, of course, applies to most standard pathways described to date. But the fact that receptors, following the binding of small molecules—whether natural or synthetic agonists [18]—can recruit protein(s) to initiate alternative unsuspected pathways, even though the affinity of these ligands at these receptors are weak, would lead to questions about our understanding of the way molecules actually work in in vivo systems. Indeed, there might be a huge difference between the affinity of a reference agonist at its receptor and the circulating quantity of this particular compound, suggesting that, despite clear descriptions of its affinity in an artificial context (cloning and expression in host cells), this compound will not activate this receptor in a tissue. For example, some dopamine receptors have a millimolar affinity for dopamine, which is in line with the dopamine concentrations in synaptic clefts in brain, while, in other locations of the body, the same receptor with the same poor affinity for dopamine would be barely activated.

Overall, though, a recent publication [35] warns about the variation in the bias due to the overexpression of a receptor (the angiotensin AT2 receptor) in a host cell, convincingly demonstrating the caution necessary when applying the basic rules of signaling bias to artificial contexts.

3. Practicability

We have used below the made-up term "*biaism*", a contraction of bias and agonism, to distinguish the pharmacological signaling bias from the widespread term "bias", which refers often to design flaws in experimental conditions (there are more than 311,266 entries currently on PubMed for "bias" as a query-23 January 2024). This term easily accommodates ligand bias and system bias through ligand biaism and system biaism.

As pointed out, an important parameter of the determination of the biaism of a given agonist is to describe it in several conditions in which the stoichiometry of the receptor and the pools of various signaling molecules differ. Even if the inducible vectors [31] are the key to such appreciation, it remains an under-regarded task. Furthermore, the whole functional machinery of the receptor should be present, to permit a fair measurement of the activated system in those conditions. Indeed, one should have not only a cellular system expressing a wide variety of G proteins and other transducers, their respective downstream machineries and the experimental tools to independently measure them, but also various cellular systems differentially expressing the targeted receptor to be studied.

Several ways to fulfill this task should be considered. For the expression of a transgene, the use of the integrase system leads to a perfectly mastered system in which the transgene is expressed at a control level, similarly to different genes, once inserted in a given region of the cell genome that was described for its neutrality [36]. Furthermore, from one clone to the next, the stable expression of the transgene is of a comparative level, leading to populations of cells expressing similar amounts of the transgene, a very useful tool whenever one wants to compare a mutant to a wild type, or mutant receptors to each other [37]. Finally, as pointed out above, further cautions should be applied in interpreting data on biaism obtained from overexpressing systems [35].

Another aspect is the artificial expression of functional protein machinery in the experimental cells used in the biaism measurements of an agonist drug candidate. Indeed, cells used in cell biology experiments, when they are not cancer cells, are laboratory tools, such as HEK 293 cells, a human kidney-derived cell (more probably adrenal gland-derived cells) with 64 chromosomes (instead of the usual 46 in humans), including three chromosomes "X" and no "Y" [38]. Thus, this lineage has a widely different protein baggage when comparing not only clones from different laboratories but even to those clones from the same laboratory, and obviously from the diploid human cells. Moreover, at a upper level, protein expression patterns in cancer cells, such as PC12 cells, are widely different from chromaffin cells, both for G proteins and other proteins [39]. Furthermore, these machineries are not the 'physiological' ones that normal cells would express and use in the organ of origin. To be complete, one should mention other non-cancer cells used in cell biology studies, particularly CHO cells, an aneuploid hamster-derived epithelial cell line. Even though this cell line has been very carefully analyzed [40], it remains that the multitude of clones derived are slightly different from each other [41,42], leading to a similar view to PC12 cancer cells, for which clones have widely different expression profiles, leading to completely different conclusions in GPCR pathways [43]. Therefore, the suitability of these host cell lines for the study of "physiological-like" conditions should be questioned. Finally, protein expression in cultured mammalian cells is regulated at different levels from transcription, post-transcription, translation and post-transcription, to protein processing by culture conditions [44] mainly comprising the origin of the serum used. Thus, the systematization of the experimental conditions for these biaism measurements is also a source of variations between laboratories and should be addressed.

One way to render the model somewhat comparable is to use stem cell-derived specialized cells. This is a feature that is widely described in the literature [45] but is still in its infancy in the context of receptology research [46]. Furthermore, these stem cells can be stably transfected and can express "at will" transgenes. On the other hand, they also are supposed to differentiate into organ-like cells, similar to those in in vivo situations, leading also, intuitively, to a baggage of proteins for the functional GPCR pathways that should resemble those of these organs. Even if the target GPCR is expressed at a high level, the

surrounding machinery would include the "physiological" one. In a reference review on biaism [31], the systems considered are basically recombinant systems, and are therefore based on experiments in cells stably expressing the receptor of interest. We have also used the same approach with HEK 293 cells overexpressing melatonin receptors [16], as have others with taste receptors [34], GPR68 [47,48] or CB_1 receptors [49], to name but a few. A counter example is the remarkable work of Lind et al., in which the biological assay is based on genuine human neutrophiles [50].

In other words, the context of the description of those undeniable phenomena is the cells in which the target is expressed. Basic knowledge has been acquired over the years that, despite their high and frequent use, these laboratory cell tools are poor models for physiology, although they were the only ones until recently.

Obviously, new attempts of GPCR studies using organoids, although still artificial, might also bring more correct information for the ultimate in vivo translation, when compared to in situ living systems. These attempts are still rather scarce in the literature [51–53].

4. Background to a *Modus Operandi*

One might regard the present commentary as a critic of how biaism is investigated. On the contrary, we feel that there are a series of predeterminants to assess and to validate. For example, in the drug discovery context that we deal with herein, the biaism profile of a given compound has an interest if, and only if, it corresponds to the desired physiotherapeutic effect. If this is the case, this particular profile, obtained in a validated model system (which is artificial by nature), can be the *fil rouge* of the medicinal project.

One might also want to progress step-by-step with the description of the starting compound in systems closer and closer to the pathological system. For example, if the GPCR Z is the target, it will be stably expressed in a recombinant cellular system and all the signaling pathways iteratively assayed in that system. The ultimate step would be to use either natural material—for example natural organ-derived membranes—or induced pluripotent stem cells (iPS) derived in the targeted cell type. None of these approaches are simple and *biasless*, but they could help us to understand how the endogenous system will work with the drug candidate.

Practically, a profile predictive of the desired therapeutical effect, such as for compound A in Figure 2, should then be obtained. This profile has been obtained for an arbitrarily fixed number of 12 pathways of the GPCR of interest. A bet is made at this stage that the therapeutical effect is due to this receptor and the functional pathways thereof. The screening of other compounds is performed on the same 12 pathways, and radar plots are constructed, as in Figure 2 for compounds B, C and D. Regardless of their chemical structures, one can identify the similar profile of compound D and select it as a hit for the process of drug development. This pattern will be limited by the following: (i) the capacity of the initial compound A to interact or not with other targets (GPCR or others) that might be implicated in the therapeutical effect; (ii) the possibility that compound A is a precursor of the active drug, and thus, not completely representative; and (iii) that the compound D, if from a different chemical family, might act on targets untouched by the initial compound A. This is the pitfall of industrial programs of medicinal chemistry based generally on the use of an affinity test coupled with a single functional test to assess the properties of compounds at each stage of the drug development. Functional testing should be extended to all signaling pathways available throughout the process. This approach may seem unrealistic, but has started to catch on [54–57].

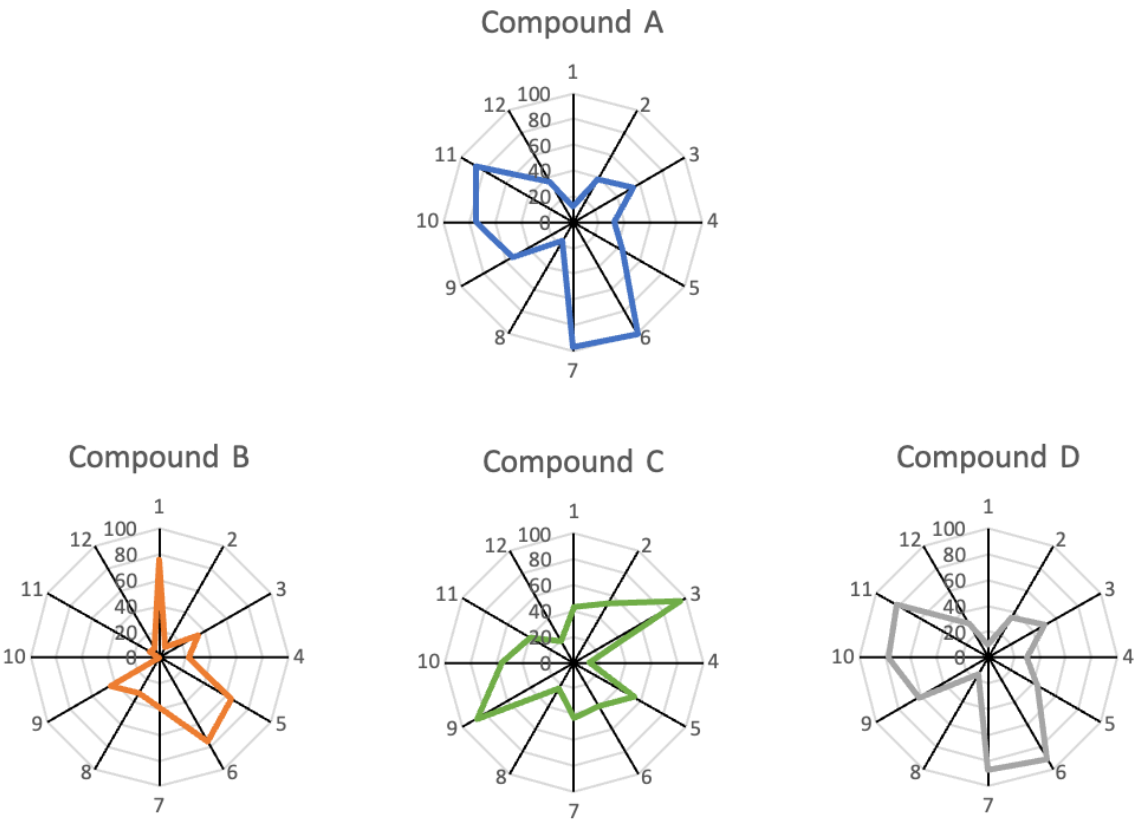

Figure 2. Radar plot representation of signaling bias. Radar plot representation of a functional parameter (arbitrary unit) describing the response of four compounds on 12 pathways of the GPCR of interest, to serve as a signature for each ligand.

5. The Remaining Key Questions

5.1. The Non-G Protein Signaling

At the practical level, one should have a way to categorize ligands as clear agonists, with their associated functional assay. This could become more achievable if—and only if—the biaism was strictly G protein-dependent. Indeed, due to the large but finished number of G protein subunit isoforms, one could classify the agonism by refereeing to the G protein that is activated in a given experiment, turning the notion of "agonism" into "α-agonism", for the agonism inducing a Gα protein. But then, what if the agonist induces several G protein-based functional responses, or, worse, if the agonist does not induce G protein-dependent functionality, but rather independent ones? In their reference recommendation review, Kolb et al. [3] made a short list of four G protein families (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$), but mentioned also the non-G protein functional pathways, such as the GPCR kinase and the arrestin pathways [58,59].

If one takes as a working example—but not necessarily as a model—the melatonin receptor MT_1 Jockers' group reported, using the two-hybrid system [60], more than 370 different proteins were able to be associated with this melatonin receptor [61]. This was exemplified also for the 5-HT_2 system [62,63]. Due to the way the association tests have been constructed, those associations concern the N-terminal part of the MT_1 receptor; a different one than the ones binding G protein(s). Therefore, the way these proteins fit into the receptor's biochemistry is currently poorly understood, but we might add one more factor of complexity, simply by modulating the functionality of the receptor upon activation (see below), including G protein-independent functional pathways. This leads, naturally, to another set of questions. Is the coupling of the GPCRs due only to G proteins—as in their definition: G protein-coupled receptors—or can we anticipate that there is more to the picture than meets the eye? Several coupling routes have been described as independent

from the G protein process. This would also indicate that other proteins, such as kinases, for instance—but possibly many others—are recruited upon agonist binding. In this context, let us mention the RAMPS accessory proteins that interact with class II GPCRs [64]. In the case of the calcitonin/CGRP peptide family, RAMPs determine the functional specificity of the calcitonin receptor [65], which switches from a CGRP to a adrenomedullin-activated receptor in the presence of RAMP1 or RAMP2/3, respectively. This would translate into the recruitment of protein(s) different from the G proteins and lead to unsuspected new functional pathways, leading to a new degree of complexity by recognition of those allegedly alternative pathways, if due to a protein/protein recognition in areas independent of the G protein-binding region.

5.2. The Weak Affinity Cases

As mentioned above (see Section 2), some endogenous agonists of GPCRs, such as dopamine to its five receptors, have an affinity (pKi) in the low µM range—from 4.3 to 7.6 [66]. This corresponds to concentrations that are not reachable in the bloodstream (~0.2 nM, 30 pg/mL), although they are locally accessible in the synaptic clefts [67]. Conversely, some receptors—the melatonin receptors, for instance—have an affinity for their natural agonist in the low nM range, in perfect line with its concentration in the bloodstream—at least during the night [68]. Since it is clear that dopamine receptors are strongly linked to each other, sequence-wise, it is easy to rule out the presence of an intruding receptor in this family. It should also be noted that histamine receptors also have poor pKi, e.g., between 3.7 and 6.7 for histamine [69]. Thus, how do these "so-called low affinity" systems actually work? What is hidden behind those "poor affinities"? Is the way we measured the affinity at receptors very artificial, in comparison to in situ situations, leading to this large underestimation? But then, why are only some families of GPCRs concerned, but not others, despite all of them being tested in similar experimental set ups? We can figure out that the native environment of the receptors at the cell membrane is so different than in artificial (experimental) conditions that it shifts the affinity of their natural ligand. As a matter of fact, we demonstrated, on the melatonin receptors, that their coupling status (with G protein) changes drastically their affinity for their natural ligands [70], assigning a dramatic influence to our way of analyzing those parameters. The recombinant systems are obviously artificial. But interestingly, once purified and inserted into a completely artificial environment (purified receptor protein, self-assembled lipid nanodisc and purified G protein), the melatonin receptor MT_1 displays characteristics identical to those measured in recombinant system [71,72], suggesting that oversimplified systems, as well as recombinant systems, while similar, might lack other regulating components at the smallest biochemical level of the receptors. Multi homo- or hetero-protein complexes at the plasma membrane might have a major influence on those parameters. The homo-multimerization phenomenon has been described for some ion channels [73], but this literature on GPCRs is rather silent, although dimerization—between homologous or heterologous receptors—has been studied on several occasions [74–76]. However, the influence of the formation of homo-dimeric complexes is certainly still misunderstood. As any receptor could dimerize with any other, as long as they are both expressed in the same cell and the same membrane microdomain, it is very difficult to decipher the functional intracellular pathways generating additional complexity.

Finally, as reported for taste receptors [34], alternative, endogenous, small molecules defined as allosteric modulators might enhance the affinity of the receptors for their supposedly endogenous ligands/agonists. This would be biaism in plain sight, with endogenous compounds regulating the affinity of some of the most basic endobiotes, which are key components of physiology. This is a very important and interesting hypothesis that could shed new light in our understanding of receptor physiology, particularly taking these data as a general model in which alternative molecules participate in the general activity of the receptors via non-orthosteric sites.

6. Using a Biased Profile as a Model for New Drug Discovery

The whole process is in fact based on a key assumption: one wants to find a compound mimicking a known drug, active against a disease. The profile of this drug will be used as a standard model for the search of a new drug with similar profile on the several pathways defining the biaism profile (Figure 2). Alternatively, one might want to undertake a more refined job by selecting only the relevant pathway(s) responsible for the desired activity.

This presumes that one knows that the model drug has no other target(s) through which at least part of its reported activity against the disease could be assigned to. It is also important to assume that no other pathway(s) at this precise receptor is responsible for any facet of the activity. These demonstrations are of course key in the understanding of the disease and its therapy and could be solved using an animal model for which the target GPCR would have been knocked out, and showing that, on this model, the compound has no therapeutical potential.

Defining a compound by its profile of pathways at a given receptor is of course a major simplification of the way the compound actually works in situ. It must be understood that this profile is a signature, and does not preclude the final result (i.e., if the compound will have a similar therapeutical effect than the parent compound). Furthermore, if one can identify an unbalanced profile, from which a particular pathway activation is not desired, one can indeed search for related compounds acting following the desired profile, with the same restrictions as above: no preclusion of the final result in the therapeutical situation.

Additionally, one also can see that the accumulation of knowledge following these descriptions of biased pathway profiles will serve the general understanding first of the compound, then of the receptor in question and finally of the disease. Ultimately, these data will also serve to qualify the disease from a molecule/drug prospect.

7. Context of Complexity in Receptology

The complexity of GPCRs comprise at least one more dimension than the five we previously listed [1], i.e., (i) expression and co-expression, (ii) the receptor interactome and its activation upon agonist binding, (iii) G protein recruitment and processing of the further downstream functional signal, (iv) the biaism of the ligand, as it might change the previous pathway (see item 3), (v) homo- and hetero-dimerization, as it seems to be universal and might concern all the GPCRs' subfamilies and (vi) the influence of extra molecules that bind to the receptor at a non-orthosteric site (PAM or NAM). Nevertheless, the real impact of biaism in drug research [77], even for antagonist research, seems to be far from day-to-day/systematic use, because basic and simplistic coupling systems are still used to characterize new drug candidates. Beyond the feasibility of the classical coupling approach, which is essentially G protein-based, questions may begin to arise on non-G protein coupling systems. It does not change our view of the analyses of those more complex systems, but it might change our view on what is currently done and should be done to characterize such molecules—essentially agonists, although antagonists are studied, as opponents to an agonist, for which the questions remain. As pure antagonists are compounds competing with agonist binding, it becomes clear that antagonism is a universal notion, independent of the agonistic pathway by which agonists are revealed. What is not clear for us is the way biaism can influence the discovery of antagonists. Furthermore, the observation of antagonism can be distorted by the presence of an allosteric modulator, for the description of which complete biochemical studies would be necessary, in particular its influence on the natural ligand binding at its receptor. Therefore, one can question how these model experiments are influencing the receptor biochemistry, the G protein(s) and the machinery responsible for the post-activation behavior of the receptor. This question has been haunting the discoveries of receptor pharmacology for decades, and the only way out is certainly the partial confirmation of the behavior of the ligands in living animals, including humans, for the drugs issued from receptor pharmacology predictions.

As a prospective comment, one should also highlight that the use of the cutting-edge technologies (i.e., AI) will be necessitated to feed algorithms with strong data, meaning

that the structural details of the molecular association of ligands with GPCR, on one hand, and effector(s)—mostly proteins—as well as allosteric modulators with GPCR, on the other hand, will be required. These data are, for the time being, far from complete, as the majority of the structural data available has been obtained from crystallization and X-ray diffraction experiments; that is to say, from the inactive state of the receptor, as we discussed elsewhere [78].

A consensus on the use of the term "agonist" dependent on the functional pathway measured becomes an absolute necessity, to be able to benefit from the discovery of other laboratories, particularly on synthetic agonists [18]. The International Union of Basic and Clinical Pharmacology (IUPHAR) should deliberate on this issue.

It is clear, now, that the agonist-induced receptor response is influenced by its immediate surroundings, at the membrane levels, including (i) the presence of other receptors that might heterodimerize with the target, (ii) the presence of G proteins and the absence of other ones, leading, already, to some sources of bias, (iii) the very existence of endogenous modulator(s)—proteins or small molecules—that could be positive or negative modulators at this and (iv) the difficult question of the relative concentrations of each previously mentioned actor of the receptor biochemistry (Figure 3). Despite these complexities, the dimension of biaism has started to be integrated into the therapeutical research, such as in osteoporosis therapy [79] or in neurological disease therapies [80], among others.

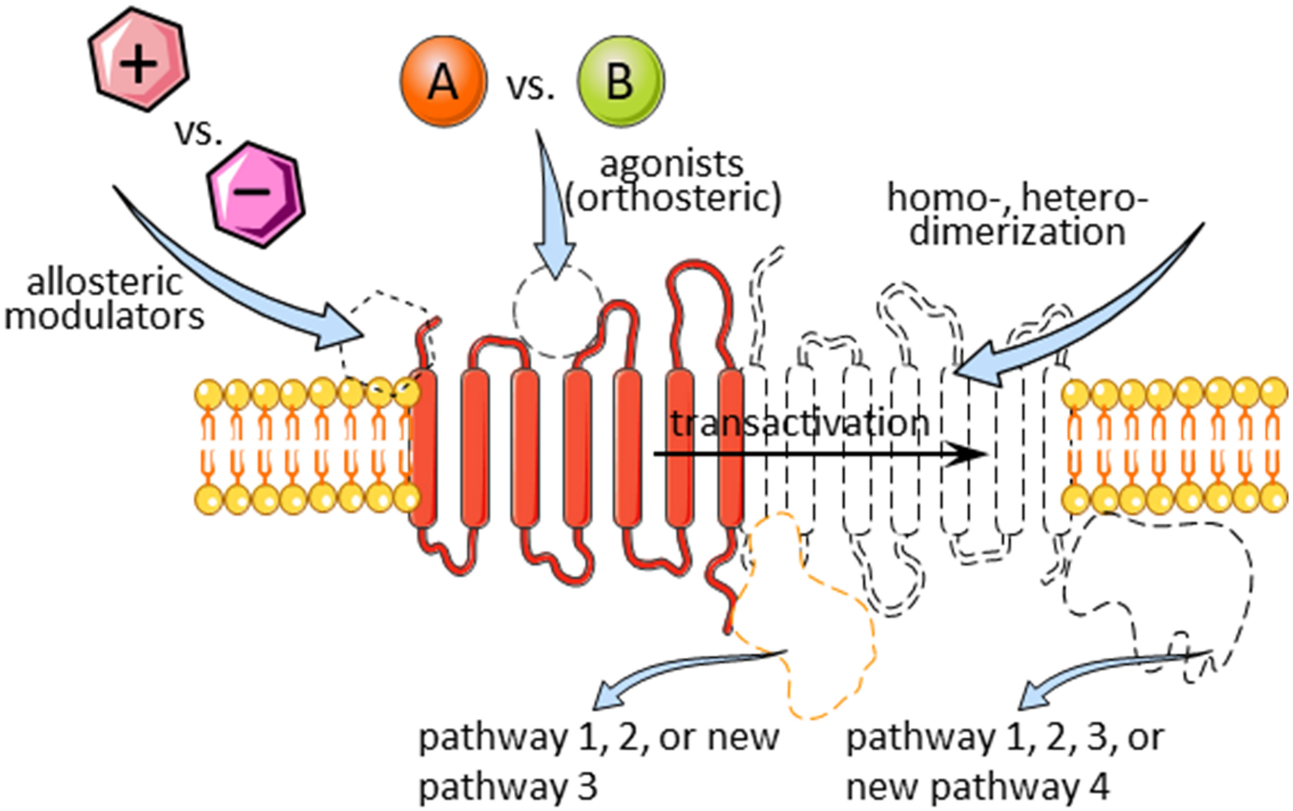

Figure 3. Schematic representation of the complexity of biaism. Partial representation of the complexity of ligand, system, and observational biaisms of a receptor at a membrane microdomain, leading to the recruitment of multiple transducers. Ligand and system biaisms both determine the functional selectivity. Observational biaism is not of biological origin but is a consequence of assay sensitivity/non-linearity and of the experimental set-up. Each type of biaism is measured relative to a reference.

As a conclusive remark, we would like to insist on the following two aspects of the present commentary: (i) it is not meant to be extensive, as an in-depth review on the

subject can be and (ii) we found the landscape extremely complex, as might be predicted for living systems which have evolved for several million years, due to our tunnel vision of GPCR biology, biochemistry and pharmacology. We have been working for decades on "simplified systems" that have led to wonderful progress in therapeutical approach, and this, despite our extremely partial knowledge of the actual systems we were working on, altered because we cloned/expressed proteins with endpoints that we did not realize were changing the system we created (as opposed to its very existence in physiological or pathological situations). We saw the progress made in the community understanding of receptology and realized that our successes were often due to reasons we did not even know existed. Nowadays, one might look at the whole picture and find a way throughout this jungle to design new ligands with predicted and oriented signaling pathway(s). The picture of receptor biochemistry can be complexified *ad nauseum* by adding the influence of perturbators we do not even know exist, by the lipid surrounding of receptors that are key to their stability and dynamics, and so on. We need a lot of humility today, when facing our ambition to decipher the way receptors function. This commentary is more a warning sign to the involved reader that the picture is far more complex than the one we are used to meeting the eye.

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References

1. Boutin, J.A.; Legros, C. The five dimensions of receptor pharmacology exemplified by melatonin receptors: An opinion. *Pharmacol. Res. Perspect.* **2020**, *8*, e00556. [CrossRef]
2. Wingler, L.M.; Skiba, M.A.; McMahon, C.; Staus, D.P.; Kleinhenz, A.L.W.; Suomivuori, C.-M.; Latorraca, N.R.; Dror, R.O.; Lefkowitz, R.J.; Kruse, A.C. Angiotensin and biased analogs induce structurally distinct active conformations within a GPCR. *Science* **2020**, *367*, 888–892. [CrossRef]
3. Kolb, P.; Kenakin, T.; Alexander, S.P.H.; Bermudez, M.; Bohn, L.M.; Breinholt, C.S.; Bouvier, M.; Hill, S.J.; Kostenis, E.; Martemyanov, K.A.; et al. Community guidelines for GPCR ligand bias: IUPHAR review 32. *Br. J. Pharmacol.* **2022**, *179*, 3651–3674. [CrossRef] [PubMed]
4. Breit, A.; Wolff, K.; Kalwa, H.; Jarry, H.; Büch, T.; Gudermann, T. The natural inverse agonist agouti-related protein induces arrestin-mediated endocytosis of melanocortin-3 and -4 receptors. *J. Biol. Chem.* **2006**, *281*, 37447–37456. [CrossRef] [PubMed]
5. Yang, Z.; Tao, Y.-X. Biased signaling initiated by agouti-related peptide through human melanocortin-3 and -4 receptors. *Biochim. Biophys. Acta* **2016**, *1862*, 1485–1494. [CrossRef] [PubMed]
6. Yang, Z.; Liang, X.-F.; Li, G.-L.; Tao, Y.-X. Biased signaling in fish melanocortin-4 receptors (MC4Rs): Divergent pharmacology of four ligands on spotted scat (*Scatophagus argus*) and grass carp (*Ctenopharyngodon idella*) MC4Rs. *Mol. Cell. Endocrinol.* **2020**, *515*, 110929. [CrossRef] [PubMed]
7. Swaminath, G.; Deupi, X.; Lee, T.W.; Zhu, W.; Thian, F.S.; Kobilka, T.S.; Kobilka, B. Probing the beta2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. *J. Biol. Chem.* **2005**, *280*, 22165–22171. [CrossRef] [PubMed]
8. Gregorio, G.G.; Masureel, M.; Hilger, D.; Terry, D.S.; Juette, M.; Zhao, H.; Zhou, Z.; Perez-Aguilar, J.M.; Hauge, M.; Mathiasen, S.; et al. Single-molecule analysis of ligand efficacy in β2AR-G-protein activation. *Nature* **2017**, *547*, 68–73. [CrossRef] [PubMed]
9. Xia, Z.; Gray, J.A.; Compton-Toth, B.A.; Roth, B.L. A direct interaction of PSD-95 with 5-HT2A serotonin receptors regulates receptor trafficking and signal transduction. *J. Biol. Chem.* **2003**, *278*, 21901–21908. [CrossRef]

10. Sutkeviciute, I.; Vilardaga, J.-P. Structural insights into emergent signaling modes of G protein-coupled receptors. *J. Biol. Chem.* **2020**, *295*, 11626–11642. [CrossRef]
11. Huang, S.K.; Pandey, A.; Tran, D.P.; Villanueva, N.L.; Kitao, A.; Sunahara, R.K.; Sljoka, A.; Prosser, R.S. Delineating the conformational landscape of the adenosine A2A receptor during G protein coupling. *Cell* **2021**, *184*, 1884–1894.e14. [CrossRef]
12. Konermann, L.; Tong, X.; Pan, Y. Protein structure and dynamics studied by mass spectrometry: H/D exchange, hydroxyl radical labeling, and related approaches. *J. Mass Spectrom.* **2008**, *43*, 1021–1036. [CrossRef]
13. Wang, L.; Chance, M.R. Protein Footprinting Comes of Age: Mass Spectrometry for Biophysical Structure Assessment. *Mol. Cell. Proteom.* **2017**, *16*, 706–716. [CrossRef] [PubMed]
14. Erdem Tuncdemir, B. Gαs and Gαq/11 protein coupling bias of two AVPR2 mutants (R68W and V162A) that cause nephrogenic diabetes insipidus. *J. Recept. Signal Transduct. Res.* **2022**, *42*, 573–579. [CrossRef] [PubMed]
15. Rajagopal, S.; Ahn, S.; Rominger, D.H.; Gowen-MacDonald, W.; Lam, C.M.; Dewire, S.M.; Violin, J.D.; Lefkowitz, R.J. Quantifying ligand bias at seven-transmembrane receptors. *Mol. Pharmacol.* **2011**, *80*, 367–377. [CrossRef]
16. Legros, C.; Dupré, C.; Brasseur, C.; Bonnaud, A.; Bruno, O.; Valour, D.; Shabajee, P.; Giganti, A.; Nosjean, O.; Kenakin, T.P.; et al. Characterization of the various functional pathways elicited by synthetic agonists or antagonists at the melatonin MT1 and MT2 receptors. *Pharmacol. Res. Perspect.* **2020**, *8*, e00539. [CrossRef]
17. Wingler, L.M.; Lefkowitz, R.J. Conformational Basis of G Protein-Coupled Receptor Signaling Versatility. *Trends Cell Biol.* **2020**, *30*, 736–747. [CrossRef] [PubMed]
18. Boutin, J.A.; Leprince, J. Why Search for Alternative GPCR Agonists? *Receptors* **2023**, *2*, 16–33. [CrossRef]
19. Wootten, D.; Christopoulos, A.; Marti-Solano, M.; Babu, M.M.; Sexton, P.M. Mechanisms of signalling and biased agonism in G protein-coupled receptors. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 638–653. [CrossRef]
20. Plante, A.; Shore, D.M.; Morra, G.; Khelashvili, G.; Weinstein, H. A Machine Learning Approach for the Discovery of Ligand-Specific Functional Mechanisms of GPCRs. *Molecules* **2019**, *24*, 2097. [CrossRef]
21. Plante, A.; Weinstein, H. Ligand-Dependent Conformational Transitions in Molecular Dynamics Trajectories of GPCRs Revealed by a New Machine Learning Rare Event Detection Protocol. *Molecules* **2021**, *26*, 3059. [CrossRef] [PubMed]
22. Costanzi, S.; Stahr, L.G.; Trivellin, G.; Stratakis, C.A. GPR101: Modeling a constitutively active receptor linked to X-linked acrogigantism. *J. Mol. Graph. Model.* **2023**, *127*, 108676. [CrossRef] [PubMed]
23. Xia, A.; Yong, X.; Zhang, C.; Lin, G.; Jia, G.; Zhao, C.; Wang, X.; Hao, Y.; Wang, Y.; Zhou, P.; et al. Cryo-EM structures of human GPR34 enable the identification of selective antagonists. *Proc. Natl. Acad. Sci. USA* **2023**, *120*, e2308435120. [CrossRef]
24. Liu, H.; Zhang, Q.; He, X.; Jiang, M.; Wang, S.; Yan, X.; Cheng, X.; Liu, Y.; Nan, F.-J.; Xu, H.E.; et al. Structural insights into ligand recognition and activation of the medium-chain fatty acid-sensing receptor GPR84. *Nat. Commun.* **2023**, *14*, 3271. [CrossRef] [PubMed]
25. Pei, Y.; Wen, X.; Guo, S.-C.; Yang, Z.-S.; Zhang, R.; Xiao, P.; Sun, J.-P. Structural insight into the selective agonist ST1936 binding of serotonin receptor 5-HT6. *Biochem. Biophys. Res. Commun.* **2023**, *671*, 327–334. [CrossRef] [PubMed]
26. Liang, Y.-L.; Khoshouei, M.; Glukhova, A.; Furness, S.G.B.; Zhao, P.; Clydesdale, L.; Koole, C.; Truong, T.T.; Thal, D.M.; Lei, S.; et al. Phase-plate cryo-EM structure of a biased agonist-bound human GLP-1 receptor-Gs complex. *Nature* **2018**, *555*, 121–125. [CrossRef] [PubMed]
27. Seufert, F.; Chung, Y.K.; Hildebrand, P.W.; Langenhan, T. 7TM domain structures of adhesion GPCRs: What's new and what's missing? *Trends Biochem. Sci.* **2023**, *48*, 726–739. [CrossRef]
28. Marti-Solano, M.; Crilly, S.E.; Malinverni, D.; Munk, C.; Harris, M.; Pearce, A.; Quon, T.; Mackenzie, A.E.; Wang, X.; Peng, J.; et al. Combinatorial expression of GPCR isoforms affects signalling and drug responses. *Nature* **2020**, *587*, 650–656. [CrossRef]
29. Heydenreich, F.M.; Plouffe, B.; Rizk, A.; Milić, D.; Zhou, J.; Breton, B.; Le Gouill, C.; Inoue, A.; Bouvier, M.; Veprintsev, D.B. Michaelis-Menten Quantification of Ligand Signaling Bias Applied to the Promiscuous Vasopressin V2 Receptor. *Mol. Pharmacol.* **2022**, *102*, 139–149. [CrossRef] [PubMed]
30. Kenakin, T. Biased Receptor Signaling in Drug Discovery. *Pharmacol. Rev.* **2019**, *71*, 267–315. [CrossRef]
31. Kenakin, T. Emergent Concepts of Receptor Pharmacology. *Handb. Exp. Pharmacol.* **2019**, *260*, 17–41. [CrossRef] [PubMed]
32. Kenakin, T. Prescient Indices of Activity: The Application of Functional System Sensitivity to Measurement of Drug Effect. *Trends Pharmacol. Sci.* **2019**, *40*, 529–539. [CrossRef] [PubMed]
33. Kenakin, T. Biased signaling as allosteric probe dependence. *Cell. Signal.* **2021**, *79*, 109844. [CrossRef] [PubMed]
34. Servant, G.; Kenakin, T.; Zhang, L.; Williams, M.; Servant, N. The function and allosteric control of the human sweet taste receptor. *Adv. Pharmacol.* **2020**, *88*, 59–82. [CrossRef]
35. Li, A.; Liu, S.; Huang, R.; Ahn, S.; Lefkowitz, R.J. Loss of biased signaling at a G protein-coupled receptor in overexpressed systems. *PLoS ONE* **2023**, *18*, e0283477. [CrossRef]
36. Cabaniols, J.-P.; Ouvry, C.; Lamamy, V.; Fery, I.; Craplet, M.-L.; Moulharat, N.; Guenin, S.-P.; Bedut, S.; Nosjean, O.; Ferry, G.; et al. Meganuclease-driven targeted integration in CHO-K1 cells for the fast generation of HTS-compatible cell-based assays. *J. Biomol. Screen.* **2010**, *15*, 956–967. [CrossRef]
37. Legros, C.; Rojas, A.; Dupré, C.; Brasseur, C.; Riest-Fery, I.; Muller, O.; Ortuno, J.-C.; Nosjean, O.; Guenin, S.-P.; Ferry, G.; et al. Approach to the specificity and selectivity between D2 and D3 receptors by mutagenesis and binding experiments Part I: Expression and characterization of D2 and D3 receptor mutants. *Protein Sci.* **2022**, *31*, e4459. [CrossRef]

38. Lin, Y.-C.; Boone, M.; Meuris, L.; Lemmens, I.; van Roy, N.; Soete, A.; Reumers, J.; Moisse, M.; Plaisance, S.; Drmanac, R.; et al. Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations. *Nat. Commun.* **2014**, *5*, 4767. [CrossRef]
39. O'Hayre, M.; Vázquez-Prado, J.; Kufareva, I.; Stawiski, E.W.; Handel, T.M.; Seshagiri, S.; Gutkind, J.S. The emerging mutational landscape of G proteins and G-protein-coupled receptors in cancer. *Nat. Rev. Cancer* **2013**, *13*, 412–424. [CrossRef] [PubMed]
40. Tihanyi, B.; Nyitray, L. Recent advances in CHO cell line development for recombinant protein production. *Drug Discov. Today Technol.* **2020**, *38*, 25–34. [CrossRef] [PubMed]
41. Hsu, T.C.; Zenzes, M.T. Mammalian chromosomes in vitro. XVII. Idiogram of the Chinese hamster. *J. Natl. Cancer Inst.* **1964**, *32*, 857–869.
42. Tharmalingam, T.; Barkhordarian, H.; Tejeda, N.; Daris, K.; Yaghmour, S.; Yam, P.; Lu, F.; Goudar, C.; Munro, T.; Stevens, J. Characterization of phenotypic and genotypic diversity in subclones derived from a clonal cell line. *Biotechnol. Prog.* **2018**, *34*, 613–623. [CrossRef]
43. Delage, C.; Breard-Mellin, L.; Thérésine, C.; Simioneck, S.; Lefranc, B.; Leprince, J.; Bénard, M.; Vaudry, D. The Heterogeneity of Response of PC12 Cells from Different Laboratories to Nerve Growth Factor and Pituitary Adenylate Cyclase-Activating Polypeptide Questions the Reproducibility of Studies Carried Out with Tumor Cell Lines. *Neuroendocrinology* **2021**, *113*, 216–230. [CrossRef]
44. Omasa, T.; Onitsuka, M.; Kim, W.-D. Cell engineering and cultivation of chinese hamster ovary (CHO) cells. *Curr. Pharm. Biotechnol.* **2010**, *11*, 233–240. [CrossRef]
45. Hamann, A.; Pannier, A.K. Innovative nonviral gene delivery strategies for engineering human mesenchymal stem cell phenotypes toward clinical applications. *Curr. Opin. Biotechnol.* **2022**, *78*, 102819. [CrossRef]
46. Lee, C.-W.; Rivera, R.; Gardell, S.; Dubin, A.E.; Chun, J. GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J. Biol. Chem.* **2006**, *281*, 23589–23597. [CrossRef]
47. Huang, X.-P.; Kenakin, T.P.; Gu, S.; Shoichet, B.K.; Roth, B.L. Differential Roles of Extracellular Histidine Residues of GPR68 for Proton-Sensing and Allosteric Modulation by Divalent Metal Ions. *Biochemistry* **2020**, *59*, 3594–3614. [CrossRef]
48. Yu, X.; Huang, X.-P.; Kenakin, T.P.; Slocum, S.T.; Chen, X.; Martini, M.L.; Liu, J.; Jin, J. Design, Synthesis, and Characterization of Ogerin-Based Positive Allosteric Modulators for G Protein-Coupled Receptor 68 (GPR68). *J. Med. Chem.* **2019**, *62*, 7557–7574. [CrossRef]
49. Nguyen, T.; Gamage, T.F.; Decker, A.M.; German, N.; Langston, T.L.; Farquhar, C.E.; Kenakin, T.P.; Wiley, J.L.; Thomas, B.F.; Zhang, Y. Diarylureas Containing 5-Membered Heterocycles as CB1 Receptor Allosteric Modulators: Design, Synthesis, and Pharmacological Evaluation. *ACS Chem. Neurosci.* **2019**, *10*, 518–527. [CrossRef]
50. Lind, S.; Holdfeldt, A.; Mårtensson, J.; Sundqvist, M.; Kenakin, T.P.; Björkman, L.; Forsman, H.; Dahlgren, C. Interdependent allosteric free fatty acid receptor 2 modulators synergistically induce functional selective activation and desensitization in neutrophils. *Biochim. Biophys. Acta Mol. Cell Res.* **2020**, *1867*, 118689. [CrossRef]
51. Cvetkovic, C.; Patel, R.; Shetty, A.; Hogan, M.K.; Anderson, M.; Basu, N.; Aghlara-Fotovat, S.; Ramesh, S.; Sardar, D.; Veiseh, O.; et al. Assessing Gq-GPCR-induced human astrocyte reactivity using bioengineered neural organoids. *J. Cell Biol.* **2022**, *221*, e202107135. [CrossRef]
52. Wang, Q.; Dong, X.; Hu, T.; Qu, C.; Lu, J.; Zhou, Y.; Li, J.; Pei, G. Constitutive Activity of Serotonin Receptor 6 Regulates Human Cerebral Organoids Formation and Depression-like Behaviors. *Stem Cell Rep.* **2021**, *16*, 75–88. [CrossRef]
53. Vijftigschild, L.A.W.; Berkers, G.; Dekkers, J.F.; Zomer-van Ommen, D.D.; Matthes, E.; Kruisselbrink, E.; Vonk, A.; Hensen, C.E.; Heida-Michel, S.; Geerdink, M.; et al. β2-Adrenergic receptor agonists activate CFTR in intestinal organoids and subjects with cystic fibrosis. *Eur. Respir. J.* **2016**, *48*, 768–779. [CrossRef]
54. Quoyer, J.; Janz, J.M.; Luo, J.; Ren, Y.; Armando, S.; Lukashova, V.; Benovic, J.L.; Carlson, K.E.; Hunt, S.W.; Bouvier, M. Pepducin targeting the C-X-C chemokine receptor type 4 acts as a biased agonist favoring activation of the inhibitory G protein. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, E5088–E5097. [CrossRef]
55. Pedersen, M.F.; Wróbel, T.M.; Märcher-Rørsted, E.; Pedersen, D.S.; Møller, T.C.; Gabriele, F.; Pedersen, H.; Matosiuk, D.; Foster, S.R.; Bouvier, M.; et al. Biased agonism of clinically approved μ-opioid receptor agonists and TRV130 is not controlled by binding and signaling kinetics. *Neuropharmacology* **2020**, *166*, 107718. [CrossRef]
56. Paradis, J.S.; Feng, X.; Murat, B.; Jefferson, R.E.; Sokrat, B.; Szpakowska, M.; Hogue, M.; Bergkamp, N.D.; Heydenreich, F.M.; Smit, M.J.; et al. Computationally designed GPCR quaternary structures bias signaling pathway activation. *Nat. Commun.* **2022**, *13*, 6826. [CrossRef]
57. Hauser, A.S.; Avet, C.; Normand, C.; Mancini, A.; Inoue, A.; Bouvier, M.; Gloriam, D.E. Common coupling map advances GPCR-G protein selectivity. *Elife* **2022**, *11*, e74107. [CrossRef]
58. Luttrell, L.M. Arrestin pathways as drug targets. *Prog. Mol. Biol. Transl. Sci.* **2013**, *118*, 469–497. [CrossRef]
59. Maginnis, M.S. β-arrestins and G protein-coupled receptor kinases in viral entry: A graphical review. *Cell. Signal.* **2023**, *102*, 110558. [CrossRef]
60. Snider, J.; Kittanakom, S.; Damjanovic, D.; Curak, J.; Wong, V.; Stagljar, I. Detecting interactions with membrane proteins using a membrane two-hybrid assay in yeast. *Nat. Protoc.* **2010**, *5*, 1281–1293. [CrossRef]

61. Benleulmi-Chaachoua, A.; Chen, L.; Sokolina, K.; Wong, V.; Jurisica, I.; Emerit, M.B.; Darmon, M.; Espin, A.; Stagljar, I.; Tafelmeyer, P.; et al. Protein interactome mining defines melatonin MT1 receptors as integral component of presynaptic protein complexes of neurons. *J. Pineal Res.* **2016**, *60*, 95–108. [CrossRef]
62. Gavarini, S.; Bécamel, C.; Chanrion, B.; Bockaert, J.; Marin, P. Molecular and functional characterization of proteins interacting with the C-terminal domains of 5-HT2 receptors: Emergence of 5-HT2 "receptosomes". *Biol. Cell* **2004**, *96*, 373–381. [CrossRef]
63. Bohn, L.M.; McDonald, P.H. Seeking ligand bias: Assessing GPCR coupling to β-arrestins for drug discovery. *Drug Discov. Today Technol.* **2010**, *7*, e37–e42. [CrossRef]
64. Serafin, D.S.; Harris, N.R.; Nielsen, N.R.; Mackie, D.I.; Caron, K.M. Dawn of a New RAMPage. *Trends Pharmacol. Sci.* **2020**, *41*, 249–265. [CrossRef]
65. Alexander, S.P.H.; Christopoulos, A.; Davenport, A.P.; Kelly, E.; Mathie, A.A.; Peters, J.A.; Veale, E.L.; Armstrong, J.F.; Faccenda, E.; Harding, S.D.; et al. The Concise Guide to Pharmacology 2023/24: G protein-coupled receptors. *Br. J. Pharmacol.* **2023**, *180* (Suppl. S2), S23–S144. [CrossRef]
66. Beaulieu, J.-M.; Borrelli, E.; Carlsson, A.; Caron, M.G.; Civelli, O.; Espinoza, S.; Fisone, G.; Gainetdinov, R.R.; Grandy, D.K.; Kebabian, J.W.; et al. Dopamine Receptors (Version 2023.1 in the IUPHAR/BPS Guide to Pharmacology Database. 2023. Available online: http://journals.ed.ac.uk/gtopdb-cite/article/view/8673 (accessed on 1 November 2023).
67. Garris, P.A.; Ciolkowski, E.L.; Pastore, P.; Wightman, R.M. Efflux of dopamine from the synaptic cleft in the nucleus accumbens of the rat brain. *J. Neurosci.* **1994**, *14*, 6084–6093. [CrossRef]
68. Kennaway, D.J. Measuring melatonin by immunoassay. *J. Pineal Res.* **2020**, *69*, e12657. [CrossRef]
69. Panula, P.; Chazot, P.L.; Cowart, M.; Gutzmer, R.; Leurs, R.; Liu, W.L.S.; Stark, H.; Thurmond, R.L.; Haas, H.L. International Union of Basic and Clinical Pharmacology. XCVIII. Histamine Receptors. *Pharmacol. Rev.* **2015**, *67*, 601–655. [CrossRef]
70. Legros, C.; Devavry, S.; Caignard, S.; Tessier, C.; Delagrange, P.; Ouvry, C.; Boutin, J.A.; Nosjean, O. Melatonin MT_1 and MT_2 receptors display different molecular pharmacologies only in the G-protein coupled state. *Br. J. Pharmacol.* **2014**, *171*, 186–201. [CrossRef]
71. Logez, C.; Berger, S.; Legros, C.; Banères, J.-L.; Cohen, W.; Delagrange, P.; Nosjean, O.; Boutin, J.A.; Ferry, G.; Simonin, F.; et al. Recombinant human melatonin receptor MT1 isolated in mixed detergents shows pharmacology similar to that in mammalian cell membranes. *PLoS ONE* **2014**, *9*, e100616. [CrossRef]
72. Logez, C.; Damian, M.; Legros, C.; Dupré, C.; Guéry, M.; Mary, S.; Wagner, R.; M'Kadmi, C.; Nosjean, O.; Fould, B.; et al. Detergent-free Isolation of Functional G Protein-Coupled Receptors into Nanometric Lipid Particles. *Biochemistry* **2016**, *55*, 38–48. [CrossRef] [PubMed]
73. Cebada, J.; García, U. Histamine operates Cl -gated channels in crayfish neurosecretory cells. *J. Exp. Biol.* **2007**, *210*, 3962–3969. [CrossRef] [PubMed]
74. Tse, L.H.; Wong, Y.H. Modeling the Heterodimer Interfaces of Melatonin Receptors. *Front. Cell. Neurosci.* **2021**, *15*, 725296. [CrossRef] [PubMed]
75. Cecon, E.; Liu, L.; Jockers, R. Melatonin receptor structures shed new light on melatonin research. *J. Pineal Res.* **2019**, *67*, e12606. [CrossRef] [PubMed]
76. Jockers, R.; Maurice, P.; Boutin, J.A.; Delagrange, P. Melatonin receptors, heterodimerization, signal transduction and binding sites: What's new? *Br. J. Pharmacol.* **2008**, *154*, 1182–1195. [CrossRef]
77. Kim, D.; Tokmakova, A.; Woo, J.-A.A.; An, S.S.; Goddard, W.A.; Liggett, S.B. Selective Signal Capture from Multidimensional GPCR Outputs with Biased Agonists: Progress Towards Novel Drug Development. *Mol. Diagn. Ther.* **2022**, *26*, 383–396. [CrossRef]
78. Vénien-Bryan, C.; Li, Z.; Vuillard, L.; Boutin, J.A. Cryo-electron microscopy and X-ray crystallography: Complementary approaches to structural biology and drug discovery. *Acta Crystallogr. F Struct. Biol. Commun.* **2017**, *73*, 174–183. [CrossRef]
79. Kalinkovich, A.; Livshits, G. Biased and allosteric modulation of bone cell-expressing G protein-coupled receptors as a novel approach to osteoporosis therapy. *Pharmacol. Res.* **2021**, *171*, 105794. [CrossRef]
80. Franco, R.; Castelló, J.; Canela, E.I. The Kinetic Component in Drug Discovery: Using the Most Basic Pharmacological Concepts to Advance in Selecting Drugs to Combat CNS Diseases. *Curr. Neuropharmacol.* **2020**, *18*, 250–257. [CrossRef]