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Biased receptor functionality *versus* biased agonism in G-protein-coupled receptors

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Abstract: Functional selectivity is a property of G-protein-coupled receptors (GPCRs) by which activation by different agonists leads to different signal transduction mechanisms. This phenomenon is also known as biased agonism and has attracted the interest of drug discovery programs in both academy and industry. This relatively recent concept has raised concerns as to the validity and real translational value of the results showing bias; firstly biased agonism may vary significantly depending on the cell type and the experimental constraints, secondly the conformational landscape that leads to biased agonism has not been defined. Remarkably, GPCRs may lead to differential signaling even when a single agonist is used. Here we present a concept that constitutes a biochemical property of GPCRs that may be underscored just using one agonist, preferably the endogenous agonist. "Biased receptor functionality" is proposed to describe this effect with examples based on receptor heteromerization and alternative splicing. Examples of regulation of final agonist-induced outputs based on interaction with β-arrestins or calcium sensors are also provided. Each of the functional GPCR units (which are finite in number) has a specific conformation. Binding of agonist to a specific conformation, i.e. GPCR activation, is sensitive to the kinetics of the agonist-receptor interactions. All these players are involved in the contrasting outputs obtained when different agonists are assayed.

Keywords: conformational landscape; GPCR heteromer; cytocrin; effectors; dimer; oligomer; structure.

Introduction

Functional selectivity is triggered by binding of agonists interacting with residues at the orthosteric binding site that results in a conformational change in regions that are close to the intracellular domains. *In silico* modeling suggests that the second intracellular loop of the serotonin 5-HT_{2A} receptor is involved in functional selectivity. The binding of different agonists results in different conformations of the intracelular loops, ultimately resulting in contrasting functional outputs (1). Residues in transmembrane domain 3 are also proposed to be important for functional selectivity in muscarinic (2), cannabinoid (3) and β_2-adrenergic (4) receptors. Similarly, other regions within the G-protein-coupled receptor (GPCR) structure may participate specifically in activating a given signaling pathway. This mini-review focuses on the biased receptor functionality, a phenomenon that may be underscored by a single agonist leading to different signaling outputs in different cells or even in the same cell. This phenomenon seemingly involves similar mechanisms to those leading to biased agonism and to what is known as "system bias" (5–7), a concept whose definition and usefulness await general consensus.

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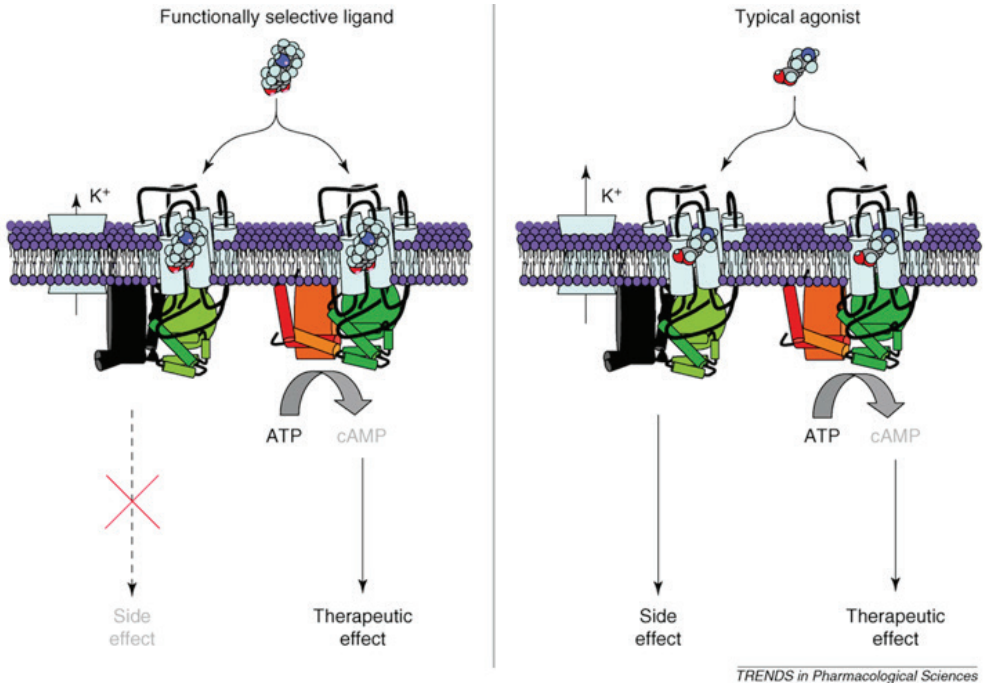

Figure 1: A scheme on functional selectivity based on differential agonist bias. Taken from (13)(fig. 2), with permission.

Functional selectivity or biased agonism: historical perspective

"Functional selectivity" and "biased agonism" can be used interchangeably and can be considered synonymous to one another. (1,8,9). The following sentence highlights GPCR conformational states as the reason for biased agonism: "*Different conformations adopted by receptors after associating with specific ligands can determine which intracellular signaling pathways get activated and which do not*"; this sentence appears in the article entitled "*Functional selectivity, ligand-directed trafficking, conformation-specific agonism: What's in a name*" (10). The link between biased agonism and stabilization of different receptor conformations leading to diverse signaling pathways is also reported in complementary reviews (11,12).

About a decade ago, functional selectivity was discussed by reputed scientists in the GPCR field from different points of view. Manuscripts related to the state of the question in 2007 were collected in a special issue in Trends in Pharmacological Science (Vol 28). One of these articles, "*GPCR functional selectivity has therapeutic impact*" (13), already envisaged their its potential in drug discovery. The term "functional selectivity" can be attributed to Mailman and coworkers from a publication in 1998 on dopamine receptor subtypes (14). Reports on the quantitative aspects of biased agonism appeared later. The effects of different adrenoceptor agonists on two signaling responses, namely regulation of cAMP levels and MAPK pathway activation, were determined, and the results were summarized as "*the results suggest that binding of different ligands promote distinct conformational changes leading to specific signaling outcomes. Our data therefore clearly illustrate that efficacy is a pluridimensional parameter that is not an intrinsic characteristic of a ligand/receptor couple*" (15). Since then the idea of biased agonism, which is related to therapeutic benefits of functional selectivity, has gained momentum both in academia and in drug discovery programs in the Pharmaceutical industry (Figure 1). Current drug discovery programs consider GPCR biased agonism and have developed strategies to select the most effective biased compound.

Biased agonism was first "measured" by ranking the potency of different signaling outputs (16,17). Later, the "operational model" was revisited to devise the "bias factor" that, for a given agonist, may be calculated by knowing the maximal effect and the concentration giving the half maximal effect, and taking both a compound and a given pathway as reference (7,18–21). Bias factors calculated in such a way do not provide all the information needed for pharmacological and physiological understanding of this singularity, i.e. they are not enough to select the most appropriate candidate to cure a given ailment. A summary

of the issues surrounding the interpretation of signaling results, via a given receptor using different agonists, is provided by (22) who present a guide to properly identify agonists that are really biased towards a given pathway. More tools are needed to properly address biased agonism research; nowadays the most successful are based on a variety of "signaling" biosensors (see (23) for review). The "pluridimensional" view is noted by (24) as the need to take into account different conformations as well as receptor trafficking and differential binding kinetics. We argue that this pluridimensional view must be enlarged by taking into account biochemical constraints related to GPCRs (receptor-receptor or receptor/protein interactions, membrane lipid composition and transmembrane environment) and kinetic constraints as well as cytocrin-related pathways (25). However, further studies into 'biased agonism' have suggested there could be complications. Among other confounding factors, (22) suggest that results on bias agonism may be skewed due to "cell specific effects". The example selected in their review is the case of cells overexpressing a GPCR kinase (GRK2) that increases β-arresting recruitment which can be a factor of distortion of results. Is cell specificity a confounding factor? Or does biased agonism depend on the intrinsic property of a GPCR in a given context? Furthermore, issues related to the actual usefulness of biased-agonism-guided drug development have been raised. These include detecting the signaling pathway of therapeutic interest, the cell type that should be targeted and how the signaling should be modulated to achieve the therapeutic effect (26).

Biased receptor functionality *versus* system bias

"System bias" (5–7), has been used intermittently in the literature and recently gained popularity (27,28). It stands to reason that any receptor-mediated response is conditioned by the system in which the signaling is assayed and that "system bias" likely means the bias due to a particular system.

Obviously, the system "defines" the conditions, thus affecting signaling (qualitative and/or qualitatively). It appears that the final result(s) when a GPCR is activated by the endogenous ligand varies from cell to cell but also from organism to organism. For example, let us take epinephrine that, via cognate adrenoceptors, provokes an increase in the mammalian heart rate. The same hormone and the equivalent receptor may be expressed in organisms lacking a heart thus leading to other functions by similar and/or dissimilar mechanisms. Even in mammals, epinephrine and the receptor mediating the increase of heart rate surely have another functionality in another organ. For instance, in the brain, epinephrine regulates neurotransmission whereas in skeletal muscles it leads to glycogen mobilization for glucose formation and disposal. Actually, it is remarkable that the same molecular mechanism of signal transduction (increases in cAMP levels) may lead to different cell outputs (contractility, differentiation, neurotransmitter regulation, etc.) due to a differential cell machinery (Figure 2). Hence, a more general concept must be considered, one that stays closer to the biochemistry of GPCRs and to the proximal components of the signaling machinery. For that reason, we would like to concentrate on signaling provided by a given receptor and a given agonist (preferably the endogenous one). In other words, we consider it relevant to highlight how a given receptor and a given hormone may lead to differential signaling (for this purpose we shall ignore differences in receptor functions in different organisms). To do so, a novel concept is needed that, in our opinion, should be both user-friendly and instrumental. We propose to name it as *biased receptor functionality*.

Biased receptor functionality would be defined as the property by which the activation of GPCRs by a single agonist may lead to differential engagement of signaling pathways. By definition, biased receptor functionality should be underscored by a given agonist acting on a given receptor but in different contexts, even in the same system. Two examples of "context" are shown in Figure 2; the first is the receptor in two different locations of the same cell. The possibility of different activities for the same receptor, in two different locations of the same cell, would be the result of the occurrence of multiple conformations. Relevant but often forgotten, conformations are not infinite but each conformation derives from one particular receptor context, i.e. different conformations of a given GPCR in a cell reflect different contexts of the receptor (see below for examples).

Mechanisms underlying biased receptor functionality

In our opinion, the following sentence in a seminal manuscript (6) encompasses biased agonism: "*Seven-transmembrane receptors are pleiotropic with respect to the signaling protein to which they couple in a cell, and many conformations of the receptor can be formed; this leads to*

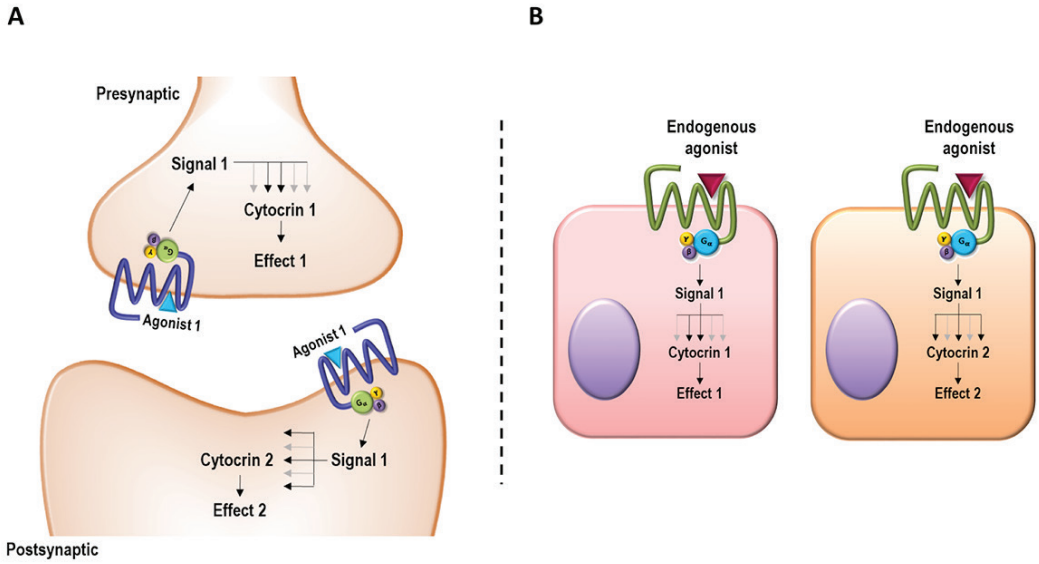

Figure 2: Scheme of differential effects underscored by a given receptor and a given agonist in two locations of a cell or in two different cells. Depending on the cytocrin pathways/mechanisms (see (25)) engaged after activation of a single receptor and a single pathway (e.g. the cAMP-dependent pathway), the effect may be different. Panel A: Biased receptor functionality in a neuron taking into account the same agonist and the same receptor located pre- *versus* postsynaptically. Panel B: Biased receptor functionality in two different cells in which the same agonist, the same receptor and the same pathway (e.g. the cAMP-dependent pathway) engages two different cytocrin pathways/mechanisms.

systems where ligands can stabilize unique conformations that go on to selectively activate signaling pathways".

The first part of the sentence is a straightforward expression of the mechanism underlying functional selectivity that implies the vague term: "signaling protein". The second part assumes that any cell could display many conformations of the receptor. In our opinion, there is consensus in that the mechanisms explaining bias due to the system and biased agonism are based on different conformations that once "stabilized" by an agonist lead to specific cytocrin pathways (23–25). Cytocrin is defined as "*the (intracellular) top-down effect of the different molecules impacting a unicellular organism or a single cell*" (25). The suffix "crin" as opposed to the suffix "crine" (as in endocrine) was coined to define the chain of events occurring inside a cell after a signal is transduced by a given receptor in a cell membrane. "Crin" can, therefore, be used to describe events that start near a membrane but go deep into the cell –cytocrin- (or into a cell organelle, e.g. nucleus –nucleocrin-). In terms of cytosolic components, the cytocrin system is constituted mainly by effector proteins in such a way that biased receptor functionality translates into different cytocrin-mediated outputs (Figures 2-3). The underlying question is whether the number of conformations is limited and whether they are dependent on specific interactions of the GPCR with other proteins.

One straightforward way to look for differential functional selectivity is the use of different agonists for a given receptor and the measure of their potency and maximal effect in different signaling pathways (G-protein-dependent, β-arrestin-dependent, etc.) using a cell heterologously expressing the receptor, i.e. after transient or stable cDNA transfection in CHO or HEK-293T cells. The outcome would give information on different conformational states that occur upon agonist stabilization which may lead to signaling via a preferential pathway (Figures 1). The output signaling pattern for a given GPCR is, therefore, biased/skewed depending on the chemical nature of the agonist used. Collectively, this idea is known as biased agonism and one may verbalize the concept by stating that agonist A is Gs biased, agonist B is MAPK biased, etc. In an extreme example, if five agonists are used and each stabilizes a different conformation, these five conformationally different receptors may (each) lead to a different signaling signature. However, we are more interested here in taking into account first what a single agonist may disclose in terms of receptor functionality.

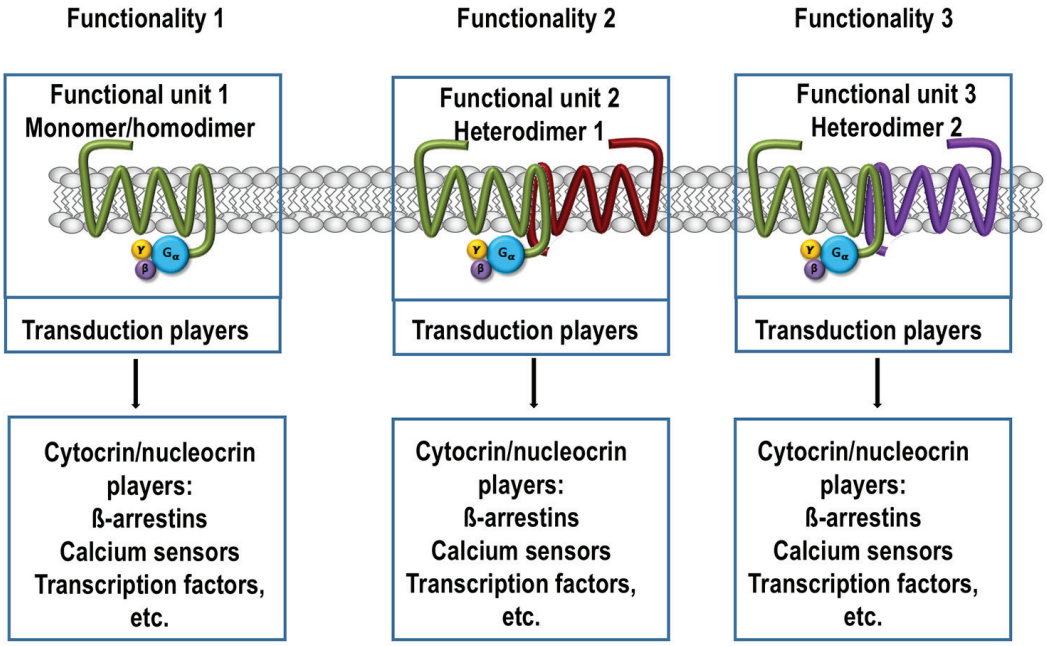

Figure 3: Scheme of biased receptor functionality. A same receptor (in green) in three different heteromeric contexts in a given system may lead to different signaling events using the same agonist. By definition (34), the function of a given heteromer is different of that displayed by the constituting receptors when expressed alone (i.e. in monomeric form). Different heteromeric contexts correlate with different conformations and may occur even in the same system (a given cell). Conformation of the green receptor depends on interactions with other receptors but also on membrane lipid composition, adapter proteins and cellular transmembrane environment. Proteins that interact with the GPCR once it is in active form are considered members of the cytocrin machinery.

As already mentioned, we think that before searching/developing biased agonists it is crucial to understand what happens when we look at a single receptor and a single agonist. The cell response may be different in a cardiomyocyte and in a neuron but, is it the signaling machinery the same that is engaged in both cell types? There is cumulative evidence showing that the endogenous agonist (or any standard synthetic agonist) may underscore different functional outputs. The scenario consists of i) diverse receptor structures/conformations, ii) diverse coupling to proteins of the proximal signal transduction machinery and iii) diverse cytocrin players. In summary, the common mechanisms of biased receptor functionality and of biased agonism rely on different conformations, on diverse coupling to effector proteins and on which are the components of the cytocrin machinery. However, biased receptor functionality is a property of GPCRs. A given GPCR in a given context has a given functionality which is likely expressed by engagement of a specific signal transduction component. A single receptor in a given cell may be expressed in different structural contexts thus giving rise to differential functionalities disclosed by the same agonist. A classic example is the expression of a receptor in a neuron that may be located in the cell body as well as pre- and/or post-synaptically (Figure 2). In each location, the endogenous ligand acts on a given conformation and leads to different functionalities that may or may not have several common components of the signaling/cytocrin machineries. A further relevant aspect to consider is whether the transduction machinery is precoupled with a given conformation or whether it is the stabilization of the conformation that forces the engagement of a specific signal transduction. In our recent experience, there are GPCRs that are already precoupled and just "waiting" for the appropriate agonist that stabilizes the overall macromolecular complex and leads to a particular signaling output (29).

Indeed, biased agonism is a consequence of the diversity of receptor functionalities. Using different agonists, one may be able to detect a specific conformation of a given receptor in a given context. Also, drug discovery programs rely on the biased agonism concept to identify drugs with more efficacy and less side effects (Figure 1). Gilchrist (2007) was able to concisely convey the idea in this sentence written more than a decade ago: "*a GPCR could have a different pharmacological profile depending on which G protein is activated and that the same GPCR could have different roles depending on the activating molecule as well as the G proteins present in the local environment*" (30). The statement that the same GPCR

could have different roles depending on the G proteins present in the local environment, may be elucidated by a single agonist and would be holistically considered as a biochemical property of GPCRs in a given context, i.e. forming part of a functional unit (Figure 3). We favor the idea that functional units are supported by specific conformations. Conformation of a receptor in a given context depends on a variety of factors that include membrane lipid composition, adapter proteins and cellular transmembrane environment.

The explanation of GPCR-related signaling bias via conformation-based mechanism is very robust. We will provide some examples to be added to those described above. However, a detailed review on the studies of this subject is out of the scope of the present article. Consistent with structural data on GPCRs in complex with G proteins, mutations in intracellular loops lead to conformational changes that affect agonist actions. Indeed, alanine substitution of amino acids in the IL3 loop of the melanocortin-3 receptor (MC3R) does not provide constitutive activity while alters receptor occupancy. Interestingly, some mutants enhance it and others decrease occupancy. Furthermore, mutations also modify maximal responses and EC_{50} values (31). The same laboratory demonstrated that a motif, DPLIY in helix 8, is important for obtaining a biased response in terms of balance between cAMP-related responses *versus* MAPK pathway activation (32). Mutants of the muscarinic acetylcholine M2 receptor in a model in which the cell context is nullified and the effect of the endogenous ligand is minimized, provided "*evidence that downstream signaling pathways previously considered to be related to each other (i.e. receptor phosphorylation, internalization, and activation of ERK1/2) can act independently*" (33). These results constitute proof of constraints that limit the active conformation(s) of the receptor.

Biased receptor functionality: examples based on heteromer formation

From a rigorous biochemical point of view, a given GPCR conformation is due to the constraints imposed by the local context, in general terms by the lipid bilayer and, in a more specific way, by direct molecular interactions with diverse proteins. It is worth repeating that any unbound GPCR conformation reflects the context of the receptor, i.e. the dependence on the interaction with the cognate G protein and on stable interactions with other proteins.

GPCRs are able to form a variety of heteromers that are characterized by properties different from those of the constituting receptor protomers (34,35). By definition, and importantly, by existing evidence, the functionality of a GPCR in a heteromeric context is different of the functionality in another heteromeric context, i.e. two different heteromers in the same cell or in different cells display different functionalities in response to the same agonist (Figure 3). In summary, one relevant property displayed by GPCRs is the coupling to different transduction machineries depending on the heteromeric context and/or on interactions with other proteins such as calcium sensors. It may be such that A receptor or AA homodimer couples to a G protein, whereas the AB heteromer couples to a different G protein. There is also the possibility that a heterotetramer may couple to two G proteins (see below).

There are multiple examples of differential functionality due to receptor heteromerization. To us one of the most relevant was reported by (36). They used the dopamine D_2 receptor long form and specific mutants to demonstrate that the functionality of the D_2 receptor depended on the interaction with the cannabinoid type 1 receptor (CB_1R). Further data on this interaction was provided years later by our laboratory (37). Relevant in this example is the differential functionality due to isoforms of the receptors; for the D_2 receptors two forms have been identified and characterized, the short and the long forms, whose mature mRNAs differ in one exon which results in a different length of the 3rd intracellular loop of the receptor (38-40). It is likely that, if not all, many members of the GPCRs family display isoforms whose functionality in response to the endogenous agonist will be, quite likely, different. Another striking example is reflected by the differential functionality when dopamine D_1 and D_2 receptors do form heteroreceptor complexes. Whereas dopamine leads to increases in cAMP, via D_1 receptors, and to decreases in cAMP, via D_2 receptors, the neurotransmitter leads to increases in cytosolic calcium via D_1-D_2 heteroreceptor complexes. These results indicate that the D_1 receptor couples to Gs, the D_2 receptor couples to Gi and the D_1-D_2 heteromer to Gq (41–43).

The ghrelin receptor is among the few for which a full-length functional form and a truncated non-functional (likely regulatory) form result from alternative splicing. Both have been identified and characterized under different physiological conditions and also in cancer (44–48). Pathogenicity mechanisms may be mediated by functionality regulation via heteromers formed by the two proteins resulting from alternative splicing. Angiotensin II type 1 and CB_1 receptor heteromerization is

involved in the abnormal function of angiotensin II after ethanol consumption. Authors conclude that their results "*provide a molecular basis for the pivotal role of heteromer-dependent signal integration in pathology*" (49).

Another striking example is provided in a recent report that demonstrates that binding and signaling of adenosine A_{2A} receptors are regulated by expression of adenosine A_{2B} receptors. Remarkably, the full signaling of the A_{2A} receptor is progressively decreased and becomes virtually negligible when the A_{2B} receptor is expressed and A_{2A}-A_{2B} heteroreceptor complexes are formed (50). In practice, A_{2A} receptor-mediated signaling is blocked by the presence of the A_{2B} receptor and the formation of A_{2A}-A_{2B} receptor heteromers.

Years ago, it was demonstrated that signaling properties of a given GPCR heteromer depend on its quaternary structure (37). We have recently proposed the quaternary structure of a GPCR heteromer in complex with G proteins (51,52). The most abundant species in receptors co-expressed in a heterologous system is the A_1-A_{2A} heterotetramer (2:2 stoichiometry leading to a heterotetramer formed by two homodimers). The GPCRs arrange into a rhombus-shaped tetramer coupled to one Gs and one Gi protein. In summary, the differential receptor functionality in this specific case results from Gi-directed signaling at low concentrations of endogenous agonist, adenosine, and a Gs-directed signaling at high concentrations of the purine nucleoside. The mechanisms underlying these completely opposed signaling (and regulatory) outputs are quite noteworthy. While the structure of the macromolecular GPCR/G protein complex is fairly symmetric, the signal arising from this adenosine concentration sensor is asymmetric (either Gi- or Gs-mediated). The adenosine A_1 receptor has higher affinity for adenosine than the A_{2A} receptor. Then, at low concentrations A_{2A} receptor remains inactive, while the A_1 receptor is activated and Gi signaling occurs; the A_{2A} receptor only gets activated when adenosine levels rise. Thus, enhanced adenosine production is able to theoretically activate the two receptors. However, in such conditions only A_{2A}-mediated Gs signaling occurs. This fact is due to intratetramer-mediated blockade of Gi function. Such unique mechanism by which Gi is unable to activate occurs because there is an "asymmetry" imposed by the 122-amino-acid long C-terminal tail of the human A_{2A} receptor (the C terminal domain of the A_1 receptor is very short). Receptor functionality relates to agonist concentration and only depends on two parameters: the particular structure of the GPCR heteromer and the A_1 *versus* A_{2A} differential affinity for adenosine. It should be noted that biased functionality disclosed by receptor activation in the A_1-A_{2A} GPCR heteromer does not require different agonists leading to diversified signals. Surely, biased agonism may be tested on individual receptors or in tetrameric functional units to provide new molecules to combat specific diseases, which constitutes the pharmacology therapy-oriented consequence of a biochemical/structural property.

Biased receptor functionality is also detectable as a link to the MAPK pathway when heteromerization of dopamine D_1 and histamine H_3 receptors occurs. This results, in both a heterologous system and primary cultures of neurons, have shown that D_1-H_3 receptor heteromers are needed for histamine to produce the activation of the MAPK pathway. Activation of individual receptors does not lead to significant stimulation of the pathway by histamine. This specific biased receptor functionality occurring in a motor-control area of the brain, striatum, has a relevant physiological consequence as it happens in D_1-containing but not in D_2-containing neurons, i.e. it is specific for cells expressing D_1, H_3 and D_1-H_3 heteromers (53,54). Accordingly, histamine activates the MAPK pathway in the so-called direct pathway (of motor control) but not in the indirect pathway; D_1 and D_1-H_3 receptor heteromers are expressed in neurons of the direct but not of the indirect pathway. Opioid receptors are also able to heteromerize and, importantly, desensitization of opioid receptors in an heteromeric context is completely different than that in absence of heteromerization (55). The result is relevant because opioids are drugs whose efficacy depends on receptor desensitization.

Dopamine D_1 receptors may heteromerize with ghrelin GHS-R1a receptors and such heteromerization leads to a shift in the signaling induced by the *hunger* hormone, ghrelin. In fact, the canonical ghrelin activation leads to the engagement of Gq signaling and, therefore, to intracellular calcium mobilization. In some circumstances, it has been described that ghrelin receptors may couple to Gi (56). However, in an heteromeric context ghrelin may couple to Gs. The most reasonable hypothesis of such finding is that individual ghrelin receptors couple to Gq or Gi, but that in cells expressing D_1-GHS-R1a heteroreceptor complexes the hormone produces an increase in cAMP levels. There is a Gq/Gi bias of the ghrelin receptor that in the presence of the dopamine receptor is converted into a Gs bias. It is likely that the D_1-GHS-R1a receptor heteromer is only coupled to Gs. In summary, depending on the heteromeric context a given hormone may lead to increases in $[Ca^{2+}]$ or to increases in [cAMP]. Importantly, the Gs coupling to the ghrelin receptor was identified in primary cultures of striatal neurons (56), i.e. it has physiological relevance. A further proof of physiological meaning was the absence of

any Gs-mediated signal when neurons from another brain area (hippocampus) were treated with ghrelin (56).

So-far the number of identified GPCR heteromers is >500; a platform that displays all of them also showing the *ad hoc* references is found in: www.gpcr-hetnet.com (see description of the platform in (57)).

Biased receptor functionality and biased agonism: mechanisms based on cytocrin pathways, β-arrestin recruitment and signaling regulation by calcium

As commented above, activation of a given receptor may lead to differential cytocrin outputs, even in the same cell. Although from a pharmacological point of view it is convenient to assume that functional selectivity is disclosed using different agonists, the root of biased receptor functionality is less pharmacological and more biological/biochemical. The result of receptor activation by an endogenous agonist is not only a consequence of signal transduction but of the components of the cytocrin machinery, which are not directly related to GPCRs themselves (Figures 2-3). Actually, β-arrestins cannot be considered members of the signal transduction machinery but of the cytocrin machinery. β-arrestins may be more pleiotropic than other cytocrin players but they do not directly interact with "resting" GPCRs. It is not until the receptor is activated that these proteins are recruited and participate in the overall output. Just taking into account the kinetics of recruitment (e.g. due to different affinity for the same receptor conformation), biased agonism occurs. Indeed, it is often forgotten that affinities depend on association and dissociation constants and they indeed affect the kinetics of overall signal transduction. Kinetics of β-arrestin recruitment is a variable that not only depends on the receptor and the agonist but on the cytocrin components/effectors.

Biased receptor functionality displayed by a given cell (preferentially the cell targeted in a given disease) may provide clues on underlying mechanisms and/or to correlate stabilization of particular GPCR conformations with particular cytocrin pathways and signaling outputs. Biased receptor functionality may be disclosed by a given agonist (preferentially the endogenous one) when tested in different cells and/or in a given cell type in different locations/contexts. How may this be possible? It could be that, different affinity states may exist for a given receptor, each of them leading to a different transduction outputs. One example of GPCR with different affinity states in binding of a given radioligand agonist is provided by the adenosine A_1 receptor (58–61). Interestingly, the high affinity state was assumed to be G-protein-coupled while the low affinity one was assumed to be G-protein-uncoupled. A second, more interesting possibility, is differential functionality of a given GPCR derived from interactions engaged by second messengers not directly related to the signaling of the GPCR that is being studied. It should be added that affinity may also vary in a heteromeric context; the binding of agonists and the G-protein-coupling vary when the A_1 receptor interacts with β-adrenergic receptors (62). It is relatively common that the affinity of ligand binding to a given receptor varies in a heteromeric context, for instance in the case of opioid receptor heteromers (63).

Not only the interaction with components of the signaling machinery but the intracellular concentration of relevant compounds, second messengers in the case of GPCRs, may lead to diverse receptor functionality. A recent example is provided by the CB_1R which is canonically coupled to Gi and, therefore, its activation leads to a decrease in the level of intracellular cAMP. This receptor is the most abundant GPCR in neurons of the central nervous system in which calcium is a key factor in neurotransmission. Upon interacting with calcium binding proteins and upon increases of intracellular calcium, activation of the CB_{1R} does not lead to Gi engagement and the output may even seem as if the GPCR is coupled to Gs (64). In this case, a given agonist could cause decreases or increases of cAMP depending on the intracellular calcium levels. Once more, biased receptor functionality is disclosed by using a single agonist. Summing up, GPCR functionality depends on the overall context involving calcium concentration and calcium binding protein expression. In fact, GPCR functionality is regulated by the calcium sensors expressed in a given cell and by the dynamic GPCR/calcium sensor interactions that are dependent in turn on the concentration of calcium (64). Another example would be the case in which calcium sensor expression changes. Thus, GPCR functionality will be different in resting *versus* reactive microglial cells due to differential GPCR-protein interactions (64,65). This type of regulation may occur in the case of heteromers. In fact, the first report on regulation of GPCR heteromers by calcium and calcium sensors showed an interaction between calmodulin and the C-terminal domain of the adenosine A_{2A} receptor. Via calmodulin, conformational changes triggered by calcium modulate MAPK signaling induced by activation of adenosine A_{2A} and dopamine D_2

receptors within the "context" of A_{2A}-D_2 heteromerization (66).

Conclusion

Biased agonism has a clear objective, which is of pharmacological nature, i.e. given a therapeutic target (a GPCR), one ligand may be better than another and differences may be disclosed by measuring the bias factor. However, by the new concepts presented in this mini-review, the right model and the right conditions must be carefully selected for making the selection of the right biased ligand. In particular, the well-known but often forgotten biased receptor functionality should be taken into account. In practice, the GPCR drug development field must consider that finite conformations exist in a given cell, that each conformation reflects a given structure involving the receptor and directly interacting partners, and that cytocrin components must be taken into consideration. The appropriate therapeutic action likely depends on the biased receptor functionality in the target cell whereas detection of the *ad hoc* biased agonist comes later. Hence, agonist bias factors calculated using heterologous cells may not be as successful in drug discovery as *a priori* hypothesized.

We argue that conformations are finite and that the cell surface cannot be observed as a monotonous landscape allowing multiple GPCR conformations. Therefore, we consider that biased receptor functionality could be first disclosed by a single agonist (preferably the endogenous one) and that the landscape is constituted by different conformational receptors each having its own functionality. A corollary would be that receptor functionality will condition the results of biased agonism, both in terms of the value of bias factors and of engagement of the most appropriate signaling pathway.

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