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Employing novel animal models in the design of clinically efficacious GPCR ligands

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The headline success of targeting GPCRs in human diseases has masked the fact that many GPCR drug discovery programmes fail. This is despite a substantial increase in our understanding of GPCR pharmacology that has provided an array of ligands that target both orthosteric and allosteric sites as well as ligands that show stimulus bias. From this plethora of pharmacological possibilities, can we design ligand properties that would deliver maximal clinical efficacy with lowest toxicity? This may be achieved through animal models that both validate a particular GPCR as a target as well as revealing the signalling mechanisms that underlie receptor-mediated physiological and clinical responses. In this article, we examine recent novel transgenic models being employed to address this issue.

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Introduction

Given that G-protein coupled receptors (GPCRs) represent a large and diverse cell surface family that impact on nearly every physiological and pathophysiological scenario, coupled to the fact that small molecule ligands can be readily designed that either inhibit (antagonists) or activate (agonists) these receptors, the rational for targeting GPCRs in a range of human diseases appears well justified [1]. A cursory analysis would support this conclusion with approximately a quarter of the drugs currently on the market having modes of action via targeting GPCRs [2••,3,4]. Despite this apparent success, and the discovery of 'block-buster' drugs yielding many billions of dollars of annual sales [5], the promise held by GPCRs as targets in drug discovery has not fully materialised. Thus, of the >390 non-olfactory GPCRs in the human genome [6] only ~15% have been targeted successfully by the pharmaceutical industry [2••,4]. This is despite many decades of intense effort, which has seen a dramatic increase in our knowledge of the signalling mechanisms and molecular pharmacology of these receptors together with the recent revelation of the atomic structures and mechanisms of receptor ligand interactions revealed by molecular dynamic simulations. The question of why GPCRs have not been more fruitfully targeted is complex, but one important factor relates to the fact that many drugs fail in phase II and III clinical trials due to lack of clinical efficacy [7,8]. This raises questions not only about the suitability of the model systems used to validate GPCR targets but also about whether we know enough about the *in vivo* modes of action of GPCRs to design ligands with the pharmacological properties needed to deliver the desired physiological/therapeutic response? These questions are particularly relevant in an era that has seen an explosion in our understanding of molecular pharmacology, which has driven an increasing plethora of pharmacological possibilities from orthosteric ligands of various flavours to a complex array of allosteric modulators. In this article, we will examine one possible way forward, where, by bringing together molecular pharmacological approaches, structure based drug design and novel *in vivo* animal models an integrated knowledge base can be assembled that if correctly applied might inform more effective drug development aimed at improving the success rate of GPCR-based drug discovery programmes.

The sophistication of GPCR molecular pharmacology

One of the key characteristics of GPCRs is that small molecule ligands can be designed to interact with the natural ligand binding site, the so-called orthosteric site. The application of high-throughput screening (HTS) on recombinant receptors expressed *in vitro*, together with medicinal chemistry to develop hit molecules, has resulted in the generation of ligands with varied pharmacology, including full and partial agonists, antagonists and inverse agonists [9]. In many instances, the pharmacology has been extended to include ligands that interact with sites outside the orthosteric sites. Binding to these sites, termed allosteric sites, can directly drive the receptor into an active or inactive conformation (i.e. allosteric agonists or antagonists) [10•]. Alternatively, allosteric ligands can change the receptor conformation in a manner that influences the affinity of ligands for the orthosteric sites and/or that

change the coupling efficiency of the receptor to downstream signalling pathways. Thus, allosteric ligands can affect the activity of the natural ligand by being a positive allosteric modulator (PAMs), a negative allosteric modulator (NAMs) or silent allosteric modulators (SAMs) [10•].

This array of pharmacology is exemplified by ligands to the muscarinic receptor family that consists of five receptor subtypes that are all activated by the natural ligand acetylcholine [11]. These receptor subtypes are activated by full (acetylcholine and methacholine) and partial (arecoline and pilocarpine) orthosteric agonists in a manner that is not subtype selective due to the high level of conservation at the orthosteric site [12,13]. In addition, there is an array of allosteric modulators to this receptor family which target divergent sites which allows for subtype selectivity [14–16]. Thus, there are both positive and negative allosteric modulators that target selective muscarinic receptor subtypes. For example, benzyl quinolone carboxylic acid (BQCA) is a positive allosteric modulator to the M1-muscarinic receptor [17•]. This is one of the most robust PAMs described to date [18] for any GPCR subtype, increasing the affinity of acetylcholine specifically at the M1-muscarinic receptor by a factor of more than 100 [18]. Importantly, BQCA does not only show positive cooperativity towards acetylcholine binding but also appears to have intrinsic agonist activity which is revealed at high concentrations of BQCA in assay systems showing high sensitivity, such as pERK1/2 assays [18]. Thus, for the M1-muscarinic receptor, there is an array of both orthosteric and allosteric ligands with defined pharmacology which can be progressed in drug discovery programmes where targeting this receptor subtype has been determined as having potential therapeutic benefit [19–21]. Such programmes are currently aimed at treating cognitive impairment in neurodegenerative disease such as Alzheimer's disease and in psychiatric illness such as schizophrenia [19]. This situation is not, however, unique to the M_1-muscarinic receptor but can be played out for many other, if not all, receptor systems considered as targets in disease [10•,22].

In light of this explosion in pharmacological possibilities, pharmacologists face a fundamental problem in having to decide what type of pharmacology is most suitable to treat a particular disease. This is made more complex by the concept of functional selectivity, also termed ligand or stimulus bias [23•,24], whereby the signalling outcome of a receptor can be modulated differentially by different ligands. Thus, a receptor that couples to three signalling pathways such as calcium mobilisation, pERK signalling and Rho-activation might be stimulated by ligand 'A' that drives all three pathways in a manner equivalent to the endogenous ligand and hence shows no stimulus bias. In contrast, another ligand 'B' might preferentially drive calcium signalling and therefore be described as showing $G_{q/11}$-protein bias. Whereas a third ligand 'C' might show receptor phosphorylation/arrestin bias thereby preferentially activating pERK signalling (Figure 1). In this scenario, ligands A, B and C act at the same receptor but might have very different physiological impacts based on their different stimulus bias (Figure 1). This may be very important therapeutically since one signalling arm might lead to a therapeutically beneficial outcome and the other lead to an adverse response [25•,26]. Therefore, from a therapeutic point of view, one would want to design a ligand to be biased towards signalling that drives therapeutic benefit and away from pathways that mediate toxic/adverse outcomes.

Thus, we are left with a wide array of pharmacological possibilities, from full and partial orthosteric ligands, to positive and negative allosteric modulators; layered on to this is the fact that both orthosteric and allosteric ligands can show stimulus bias. The question now, therefore, is what type of pharmacology is desired to address any one therapeutic situation? One possible way of addressing this question is the use of novel animal models in combination with ligands with defined pharmacology.

Novel animal models to determine modes of action of GPCRs

Gene knockout studies have contributed hugely to our understanding of the physiological role of GPCRs [27,28]. However, it has become clear that more sophisticated transgenic animal models are required to determine with precision the receptor-mediated signalling pathways that underlie physiological GPCR responses. One recent advance in this area is the utilisation of chemical genetic approaches which involve the expression of a mutant form a receptor that is unable to be activated by the endogenous ligand but instead can be activated by an otherwise inert chemical; the first of such receptors was called a 'Receptor Activated Solely By Synthetic Ligand' (RASSLs). Although useful to define the potential physiological impact of receptors such as the kappa-opioid receptor [29••,30,31] and serotonin 5-HT4 [32,33] as well as the possible role of specific signalling pathways [34], these receptor mutants suffered from high constitutive activity and ligands which were able to interact with the endogenous receptor subtype [35]. The second generation RASSLs have initially focused on muscarinic receptors, where a high throughput yeast-based directed evolution strategy generated an M_3-muscarinic receptor mutant that was not activated by the endogenous ligand, acetylcholine, but was activated by clozapine-N-oxide (CNO), a compound that otherwise had very low activity at muscarinic receptors and other GPCR subtypes [36••] (Figure 2). By reverse engineering, Roth and colleagues were able to introduce analogous mutations into the other members of the muscarinic receptor family and generate RASSL mutants responsive to CNO for the M1-M5

muscarinic receptors [36••] (Figure 2). These second generation muscarinic receptor RASSLs were called 'Designer Receptors Exclusively Activated by Designer Drug' (DREADDs) and have proven useful in transgenic mouse models aimed at determining signalling pathways important in β-islet function [37••,38], neuronal networks involved in neurological responses such as locomotion [39••], learning and memory [40,41], limbic seizures [39••] and metabolism [42].

In tandem with this chemical genetic approach, have been approaches that have generated mutant receptors designed to determine the physiologically relevant signalling pathways that lay downstream of receptor activation. In these experiments, GPCR signalling is considered to be bimodal, where signalling progresses via heterotrimeric G-protein coupling or via receptor phosphorylation and the recruitment of arrestin scaffolding proteins [43]. By removal of receptor phosphorylation sites, the receptor is less efficiently coupled to receptor phosphorylation/arrestin dependent pathways relative to coupling to G-protein activated pathways. In this way, the receptor mutants lacking phospho-acceptor sites can be considered as being G-protein biased [44,45••]. Experiments conducted in the author's laboratory have focused on the generation of a G-protein biased M_3-muscarinic receptor by the removal of 15 serine phospho-acceptor sites in the third intracellular loop. This receptor was seen to be reduced in its phosphorylation status and uncoupled from arrestin and arrestin-dependent processes such as receptor internalization [44,45••]. The phosphorylation-deficient receptor mutant was, however, seen to be robustly coupled to $G_{q/11}$-protein pathways such as phosphoinositide hydrolysis and calcium signalling. By knocking in this mutant sequence into the gene locus of the wild type M_3-muscarinic receptor a mutant mouse strain was generated whereby the wild type M_3-muscarinic receptor was replaced by a mutant receptor [44,45••]. Thus, as opposed to generating biased ligands and testing the impact of these ligands on physiological responses, as has been done for the angiotensin II type 1 receptor for example [46–50], this approach generates a biased receptor. By monitoring the physiological phenotype of these mutant mice it was possible to determine the signalling modality employed in delivering a given receptor-mediated physiological response.

Employing transgenic animals expressing a G-protein biased GPCR to determine physiological signalling pathways

Thus, having generated a mouse mutant that expressed a phosphorylation deficient version of the M_3-muscarinic receptor, the question was what impact did this mutation have on muscarinic receptor-mediated physiological responses? One response that was investigated was glucose-dependent insulin secretion from β-cells of pancreatic islets. Previous work, primarily from Jurgen Wess' laboratory, had determined that muscarinic receptor-mediated augmentation of glucose dependent insulin release was mediated by the M_3-muscarinic receptor [51–54]. Importantly, this augmentation occurs in both the early and sustained phases of insulin release and appears to have a number of regulatory elements. Certainly, there is an involvement of $G_{q/11}$-mediated calcium mobilisation [55,56] which appears to be controlled, at least in part, by spinophilin/RGS4 complex that limits the lifetime of the activated $G\alpha_{q/11}$-subunit [57]. The prolonged phase of insulin release was, however, significantly diminished in β-cells isolated from mice expressing the phosphorylation-deficient version of the M_3-muscarinic receptor [44]. This occurred despite the fact that both the early and prolonged phases of calcium mobilisation in these islets were robustly maintained [44]. Further examination revealed that the M_3-muscarinic receptor in β-cells was coupled to the atypical PKC-isomer, PKD1 [44]. This protein kinase was known from previous studies to be important in mediating insulin vesicle fusion [58]. Removal of the phosphorylation sites on the M_3-muscarinic receptor significantly decreased the coupling of the receptor to PKD1 [44]. Furthermore, observations made in other studies had implicated arrestin as important for PKD1 activity [59]. Thus, it appears that the sustained phase of insulin release observed in response to glucose challenge can be augmented by M_3-muscarinic receptors in a manner that is mediated certainly by receptor phosphorylation mediated activation of PKD1 via a mechanisms that is possibly dependent on the recruitment of arrestin [44] (Figure 3). In this way, the use of a G-protein biased receptor expressed in mice has been used to dissect the physiological functions of the bimodal signalling pathways of the M_3-muscarinic receptor.

Combining animal models to determine the functional role of GPCRs and the physiologically relevant signalling pathways

As described above, we now have a number of animal models including transgenic knockouts, chemical genetics and biased receptors that can be combined to not only establish the physiological role and therapeutic potential of any individual GPCR subtype, but also the relevant signalling modality to be targeted. Can these model animals therefore help in the design of the pharmacological properties of ligands to maximize clinical efficacy? It appears that the answer to this question may be yes. Consider again the example of the M_3-muscarinic receptor where gene knockout studies have determined a role in the control of insulin release [53] and chemical genetics (i.e. an M_3-muscarinic receptor DREADD mutant) has defined the importance of $G_{q/11}$-signalling in insulin release which [37••], together with mutant mice expressing a G-protein biased receptor, has determined that the early phase of insulin release is primarily mediated via $G_{q/11}$ signalling and the prolonged phase via phosphorylation/arrestin signalling [44]. Based on

Figure 1

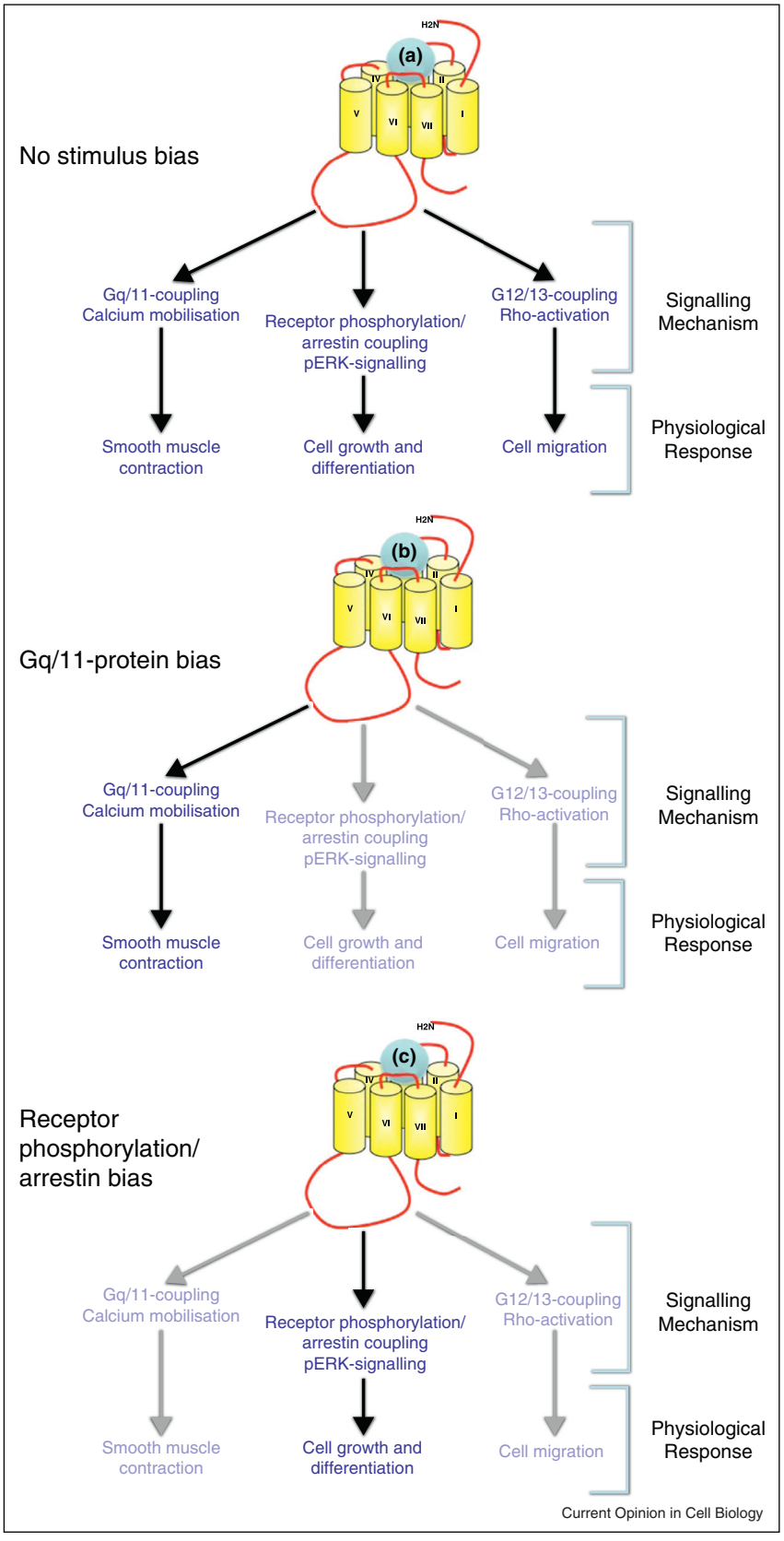
No stimulus bias
(a)
H2N
IV
II
V
VI
VII
I
Gq/11-coupling
Calcium mobilisation
Receptor phosphorylation/
arrestin coupling
pERK-signalling
G12/13-coupling
Rho-activation
Signalling
Mechanism
Smooth muscle
contraction
Cell growth and
differentiation
Cell migration
Physiological
Response
Gq/11-protein bias
(b)
Gq/11-coupling
Calcium mobilisation
Receptor phosphorylation/
arrestin coupling
pERK-signalling
G12/13-coupling
Rho-activation
Signalling
Mechanism
Smooth muscle
contraction
Cell growth and
differentiation
Cell migration
Physiological
Response
Receptor
phosphorylation/
arrestin bias
(c)
Gq/11-coupling
Calcium mobilisation
Receptor phosphorylation/
arrestin coupling
pERK-signalling
G12/13-coupling
Rho-activation
Signalling
Mechanism
Smooth muscle
contraction
Cell growth and
differentiation
Cell migration
Physiological
Response
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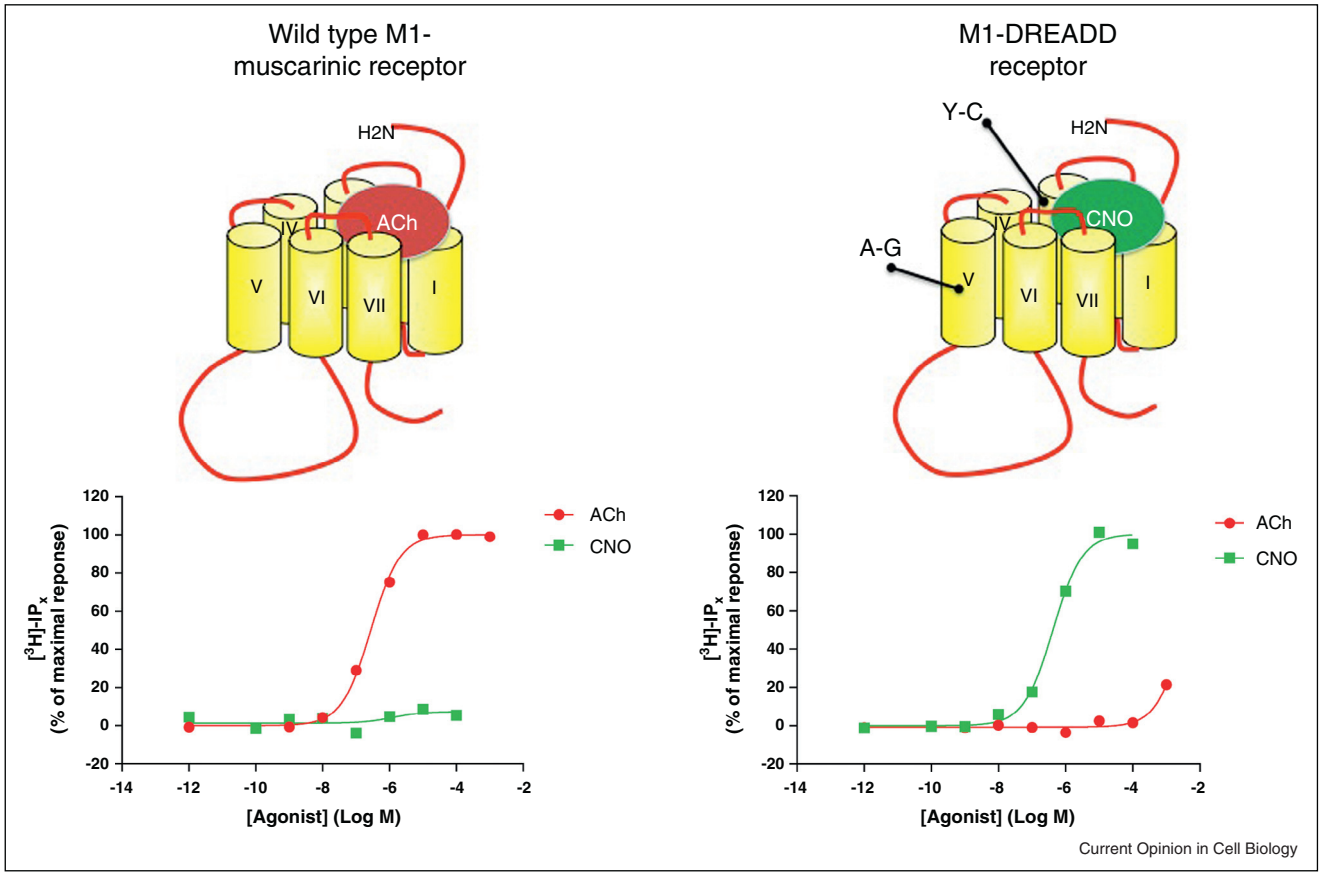
Figure 2



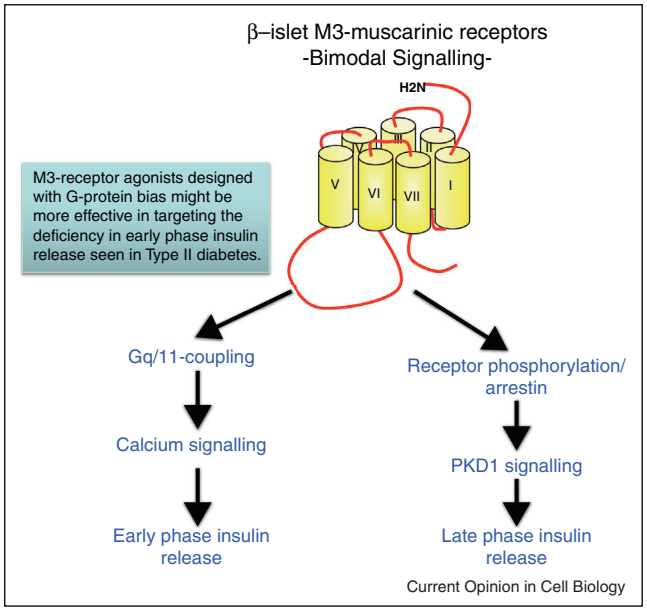
Introducing two specific orthosteric site mutations, one in transmembrane domain III and the other in transmembrane domain V, of muscarinic receptors results in a reduced affinity for the natural ligand acetylcholine (ACh) and an increase in affinity for the otherwise inert chemical ligand clozapine-n-oxide (CNO). This mutant receptor is called a DREADD. Shown is the example of the signalling of the wild type M1-muscarinic receptor coupled to phosphoinositide pathway (measured as the accumulation of inositol phosphates (IPx)) following stimulation with acetylcholine or CNO compared to the signalling of the M1-DREADD receptor mutant generated by an A–G and Y–C substitution. As can be seen, the M1-DREADD is responsive to CNO and lacks a response to acetylcholine, whereas the wild type receptor shows no response to CNO but is fully activated by acetylcholine.

these studies we can make the following predictions; that an M_3-muscarinic receptor ligand that shows stimulus bias towards G-protein coupling would preferentially promote the early phase of insulin release and not the prolonged phase. Conversely, an M_3-muscarinic receptor ligand that was biased towards receptor phosphorylation/arrestin signalling would be expected to promote the prolonged phase of insulin release over the early phase. This observation is very important since one of the features of the early stages of type 2 diabetes is the reduction in the early phase of insulin release [60,61]. Thus, an M_3-muscarinic receptor specific ligand showing bias to the G-protein signalling might be the desired ligand to treat the dysfunction in the early phase of insulin release observed in type 2 diabetes.

These mouse models can be extended further to predict toxicity and adverse drug reaction. Knockout mice of the M_3-muscarinic receptor subtype have been used to establish an unexpected role for this receptor in learning and

(Figure 1 Legend) Shown is a hypothetical example of a GPCR that is able to mediate three physiological responses via the coupling to three distinct signalling pathways. In the first example, ligand A is able to activate all three pathways with the same coupling efficiency as that seen for the natural ligand. In this case, ligand A shows no stimulus bias. In the second example, ligand B induces a receptor state that couples preferentially to $G_{q/11}$-protein, which drives calcium mobilisation and subsequent smooth muscle contraction. This ligand shows $G_{q/11}$-protein bias. In the final example, ligand C drives signalling primarily via the pERK pathway, which leads to cell growth. From a therapeutic perspective, it might be clinically beneficial to activate the $G_{q/11}$-protein pathway, whereas targeting the other two pathways might lead to toxicity or adverse drug affects. If this were the case, then a $G_{q/11}$-protein biased ligand (i.e. ligand B) might represent the desired pharmacological properties of a drug to deliver maximal clinical efficacy with lowest toxicity.

Figure 3



The M_3-muscarinic receptor expressed in β-islets of the pancreas is involved in the augmentation of insulin release in response to increasing concentrations of glucose. This response appears to be mediated by two signalling pathways. The first is M_3-receptor mediated signalling to $G_{q/11}$-protein and calcium mobilisation, driving the early phase of insulin release. The second sustained phase of insulin release phase is dependent on receptor phosphorylation/arrestin mediated activation of the atypical PKC, PKD1. Since type II diabetes is characterised by a deficiency in the early phase of insulin release, it would seem that a compound that targeted the M_3-muscarinic receptor in a manner that preferentially drove signalling through the $G_{q/11}$-protein pathway would provide greatest clinical efficacy.

memory [45••]. Furthermore, use of the mutant mouse expressing a G-protein biased receptor determined an important role for receptor phosphorylation, and possibly arrestin signalling in this response [45••]. Integrating this information with the features of the M_3-muscarinic receptor mediated insulin release described above, one might make the following prediction: that an M_3-muscarinic receptor specific ligand displaying stimulus bias towards receptor phosphorylation/arrestin signalling might have properties that were beneficial in promoting learning and memory. This same compound would likely engage M_3-muscarinic receptors expressed on β-islets, but importantly, would not be expected to promote M_3-muscarinic receptor mediated early phase insulin secretion since this response is largely mediated by coupling to $G_{q/11}$ signalling. Thus, an M_3-muscarinic receptor ligand designed for maximal efficacy in learning and memory, and thus showing stimulus bias towards receptor phosphorylation/arrestin, would not be expected to show a toxic/adverse response via muscarinic-mediated early phase insulin release. However, any clinical trial with such a molecule would have to determine that the molecule did not have adverse effects by promoting late phase insulin release since this would be promoted by an M_3-muscarinic receptor ligand that was biased towards receptor phosphorylation/arrestin signalling.

Conclusion: How might the animal models be integrated with modern molecular pharmacology to deliver on clinical efficacy

It is now the case that drug discovery programmes are beginning to apply the sophisticated approaches described here in the design of therapeutically active GPCR ligands. This is most notable in heart failure where chronic stimulation of G-protein signalling is considered to be one of the contributing factors leading to cardiopathology [62,63]. Hence, antagonists of two key GPCRs implicated in heart failure, namely the β_1-adrenoceptor and the angiotensin II type 1 receptor, have traditionally been pursued with undoubted clinical success [64,65]. However, it is now clear that in addition to the inhibition of G-protein signalling, it is desirable that ligands to these two receptors are also able to activate arrestin signalling which appears to provide cardioprotection [66]. Thus, biased ligands to both the β_1-adrenoceptor and the angiotensin II type 1 receptor, where the ligands act as an antagonists (or even inverse agonists) at G-protein signalling but are agonists at arrestin signalling is now considered the ideal ligand to provide maximum therapeutic efficacy in the chronic treatment of heart failure [49,64,67••].

Thus, in heart failure, and other diseases, it will be important to revisit the screening of ligands to develop novel ligands that have newly defined, desirable, pharmacological properties. An important factor in the search for these novel ligands is the growing list of crystal structures [2••] for GPCRs which provides the opportunity to incorporate *in silico* screening and structure-based drug design as an approach to screen and develop GPCR ligands[2••,68]. These approaches have certainly been applied to a number of commercial screening programmes as well as screening programmes in academic laboratories [69,70,71••]. However, structure-based screening methods are still restricted as most of the GPCR structures currently available are in an inactive conformation and have been resolved with orthosteric and not allosteric ligands. Nonetheless, the publication of the first active structures of non-visual GPCRs [72,73,74••] and the development of mutant receptors stabilised in the partially active conformation [75], together with the application of molecular dynamics to map the interaction of ligands at both orthosteric and allosteric sites [72,76–78], means that we are racing towards a time where *in silico* docking and structure-based methods can be readily applied to the development of pharmacological ligands. These new approaches will undoubtedly be used in combination with sophisticated transgenic and chemical genetic animal models to facilitate design of ligands with the

desired pharmacological properties required to deliver a therapeutic response. By combining these approaches, the next generation of GPCR ligands will unquestionably be more sophisticated, employing rational design principles to deliver GPCR ligands with low toxicity with maximal clinical efficacy.

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