The state of GPCR research in 2004 *(20 Questions)*

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Tamas Bartfai 578

Jeffrey L. Benovic 582

Joël Bockaert 584

Richard A. Bond 590

Michel Bouvier 591

Arthur Christopoulos 595

Olivier Civelli 598

Lakshmi A. Devi 601

Susan R. George 602

Akio Inui 603

Brian Kobilka 608

Rob Leurs 610

Rick Neubig 612

Jean-Philippe Pin 614

Rémi Quirion 615

Bernard P. Roques 618

Thomas P. Sakmar 618

Roland Seifert 619

Ronald E. Stenkamp 622

Philip G. Strange 623

1 What new technologies are having the greatest impact on GPCR research, and why? **578**

2 What tools and technologies for GPCR research would you most like to be available in the future, and why? **583**

3 What are the main pitfalls and advantages of currently used cell-based assay systems for GPCRs? **585**

4 How will changing views of agonist and antagonist behaviour at GPCRs change the way we view these receptors as therapeutic targets? **588**

5 Many predictions for heptahelical receptors have been based on using the rhodopsin crystal structure as a template. How successful have these been? **592**

6 How close are we to having further GPCR structures, and what are the main barriers to obtaining these? **594**

7 What big questions remain for GPCRs at the structure/function interface? **597**

8 How widespread do you think GPCR heteromerization will turn out to be, and how great a functional significance will it have? **599**

9 Almost two-thirds of drugs on the market are thought to interact with GPCRs, by far the largest family of targets, and yet new drugs targeting GPCRs are few and far between. Why? **603**

10 What are the barriers to achieving specificity using GPCR ligands? **606**

11 How will our increasing knowledge of interacting protein partners for GPCRs change the way that we think about achieving selective GPCR modulation? **607**

12 Could there be an approach to GPCR pharmacology based around monoclonal antibodies or proteins, rather than small molecules? **609**

13 How close are we to being able to integrate understanding of how individual GPCRs function with their role in disease states? **612**

14 How successful are the animal models that seek to recapitulate diseases linked to the aberrant function of peptide-activated GPCRs? **614**

15 In your opinion, which diseases are most strongly associated with aberrant function of peptide-activated GPCRs? **615**

16 What are the main hurdles that will need to be overcome to create further successful therapeutic approaches based around targeting GPCRs? **617**

17 What would you describe as the major concerns surrounding the continued development of GPCR-targeted therapies? **619**

18 What effect is the deorphanizing of GPCRs likely to have on the field? **620**

19 What outstanding questions in GPCR research are likely to be clarified in the next few years? **622**

20 What areas are unlikely to be clarified by research over the next few years? **623**

Glossary **611**

References **624**

1

What new technologies are having the greatest impact on GPCR research, and why?

Olivier Civelli. A technology that has had a major impact is the use of fluorescence detection as a tool to monitor G-protein-coupled receptor (GPCR) reactivity. This has led to studies of receptor–receptor and receptor–ligand interactions, and to real-time measurement of signal transduction. Practically every report dealing with GPCR expression at the membrane, or with GPCR interactions with intracellular proteins, relies on fluorescence detection, including techniques such as fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) (FIG. 1; page 579).

Michel Bouvier. Many new technologies are turning out to be extremely useful for analysing GPCR functions. Among those, I would cite fluorescence-based techniques, such as INTERNAL FLUORESCENCE REFLECTION and single-molecule fluorescence, and resonance energy transfer approaches, including FRET, BRET and FLUORESCENCE LIFETIME IMAGING (FLIM). These new approaches should allow the real-time probing of conformational changes, and of the dynamic protein–protein interactions that are involved in GPCR activation and regulation in the environment in which they normally occur; that is, living cells.

Lakshmi A. Devi. In recent years, high-throughput screening (HTS) for functional activity (using methods such as the FLUOROMETRIC IMAGING PLATE READER (FLIPR) and SECRETED ALKALINE PHOSPHATASE (SEAP) ASSAYS) has been useful for deorphanizing receptors, screening for mutant receptors (to understand structure/function relationships)[1] and screening ligand libraries (to identify receptor-selective ligands)[2]. In addition, X-ray crystallography and modelling-based analyses of GPCRs have provided information on the ligand–receptor interaction interface[3,4]. Refining these techniques will help in structure-based rational drug design. In addition, proteomics is being used to identify and quantitate proteins that associate with GPCRs, thereby delineating the signal-transducing complexes[5]. Finally, a variation of knockout technology (that is, targeted inducible deletion of individual GPCRs) is increasingly being used to define the spatio-temporal relationships of receptor activities[6,7].

Tamas Bartfai. The steady introduction of non-peptide ligands to neuropeptide GPCR receptors has enhanced research, and proven that these GPCRs are valuable drug targets in many important diseases. Such new ligands have arisen from industrial research, using binding, FLIPR and other cell-based assays in ultra-HTS formats, and chemical libraries enhanced with β-turn mimics, which often turned out to be recognized by GPCRs and were therefore good starting points for medicinal chemistry. Transgenic techniques have been important for validating the involvement of GPCRs in physiological and pathophysiological processes. No pharma project to generate GPCR ligands proceeds today without high-quality hits from screening and access to null-mutant animals and, if possible, access to animals with a mutation generating a constitutively active receptor. Academic GPCR research has benefited most from embracing more spectroscopic techniques, often using FLUOROPHORE-labelled ligands and/or receptors to follow interactions in real time, and to follow the trafficking of receptors during DESENSITIZATION and the development of TOLERANCE.

Philip G. Strange. Industry has been quick to use fluorescence-based technologies, in some cases in a single-molecule-detection mode — for example, fluorescence polarization, fluorescence intensity distribution analysis, FLUORESCENCE CORRELATION SPECTROSCOPY and FRET — for drug screening at GPCRs. This has been stimulated by the need for high-throughput systems and less reliance on radioactivity. Reporter-gene assays linked to fluorescence or luminescence readouts, and cell-based assay systems for examining the movement of proteins, have also been used. These technologies have had a big impact on industrial research into GPCRs.

The academic community has been rather slow to pick up on these techniques, perhaps owing to financial constraints, and much academic GPCR research remains rooted in older technologies. Nevertheless, some of these techniques are likely to be very important in pushing forward research on GPCRs into new areas. A few academic labs are using some of these technologies to study GPCRs, and I would single out the work from Brian Kobilka's lab on the use of fluorescence to probe conformational changes in GPCRs[8], and the use by one or two labs of cyan fluorescent protein (CFP)/yellow fluorescent protein (YFP) FRET for studying protein–protein interactions[9–11]. This latter technique may be of general use for examining the internal dynamics of receptors, and receptor–G-PROTEIN dynamics.

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Arthur Christopoulos. There are at least two important advances that I feel are having the greatest impact on GPCR research at the present time. The first is the application of genomics and proteomics to drug discovery. Based on recent estimates, there are approximately 300 non-sensory GPCRs in the human genome, which equates to a very (theoretically) target-rich environment (see BOX 1 (page 581) for a description of GPCR families) Accordingly, a shift from ligand-based to sequence-based drug discovery has led to the identification of a number of novel GPCRs, especially ORPHAN RECEPTORS. The second advance is the development/improvement of technologies that utilize fluorescence spectroscopy or related biophysical methods to

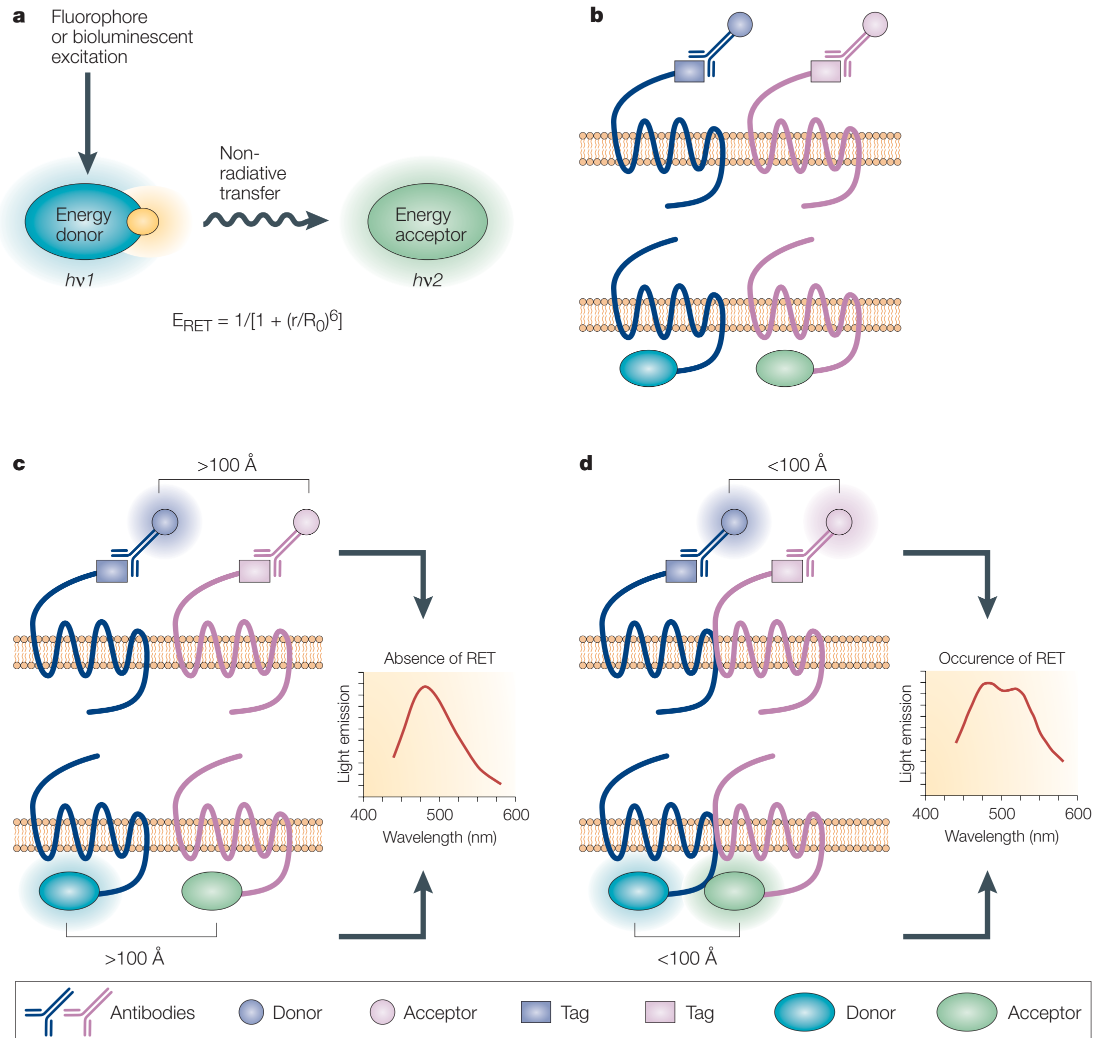


Figure 1 | **Use of RET to assess GPCR oligomerizaton.** Resonance energy transfer (RET) approaches are based on the non-radiative transfer of energy between a donor and an acceptor. **a** | The energy-donor moiety can either be a fluorophore that can be excited by an external source of light (fluorescence resonance energy transfer (FRET)) or a bioluminescent enzyme that emits light upon oxidation of a substrate (bioluminescent resonance energy transfer (BRET)). In both cases, the energy acceptor is a fluorophore. Because the efficacy of RET (E_{RET}) varies inversely with the sixth power of the distance (r) between the energy donor and acceptor, transfer of energy from the donor will result in the emission of light by the acceptor only if the two molecules are in close proximity (generally within 100 Å). R_0 is defined as the separation distance that results in 50% maximal RET efficacy. The exquisite sensitivity of RET to the distance separating the energy donor and acceptor can be used to assess protein–protein interactions. ($h\nu$ is the energy in a photon of electromagnetic radiation, where h is Planck's constant and ν is the frequency of radiation.) **b** | For G-protein-coupled receptors (GPCRs), two distinct strategies have generally been used. As shown in the top half of the panel, antibodies recognizing specific epitopes on the GPCRs ('tags'; denoted by coloured rectangles), generally at their amino terminus, can be coupled to the energy donor (blue circle) or acceptor (pink circle) fluorophores. Alternatively, as shown in the bottom half of the panel, the GPCRs can be genetically fused (generally at their carboxyl terminus) to fluorescent or bioluminescent proteins (denoted by coloured ovals), such as green fluorescent protein or luciferase. **c** | In the absence of dimerization, excitation of the donor will lead to the emission of light with a spectra characteristic of the energy donor itself (as in part **b**, both labelling strategies are shown). **d** | If dimerization occurs, bringing the energy donor and acceptor within 100 Å of each another, the energy transfer from the donor to the acceptor will lead to the appearance of an additional fluorescence signal that can be detected by the presence of a second light emission peak on the spectra (as in part **b**, both labelling strategies are shown). Figure prepared by Michel Bouvier.

study the molecular mechanisms underlying GPCR conformational changes. The GPCR field lags well behind the ion-channel field in terms of having a 'read-out' that reflects receptor activation/conformational events within a biologically relevant time frame (that is, micro- to milliseconds), but some of the latest developments in the area promise to address this shortcoming. For example, Jean-Pierre Vilardaga and colleagues[10] recently generated parathyroid hormone (PTH) receptor and α_2-adrenoceptor mutants into which the cyan- and yellow-emitting variants of GREEN FLUORESCENT PROTEIN (GFP) had been inserted in the third intracellular loop and carboxy-terminal (C-terminal) tail of each receptor, respectively. They then used FRET to monitor the rearrangement of the transmembrane helices of the receptors on binding agonists, partial agonists or antagonists, and were able to demonstrate real-time fluorescence changes that corresponded to millisecond receptor activation/deactivation cycles.

Susan R. George. The greatest impact on GPCR research has been generated by the use of genomics and bioinformatics approaches to search for novel members of this family, which are all potential targets for drug development. The discovery and identification of the entire complement of GPCRs in the human genome will have enormous implications for understanding normal physiology and pathophysiological mechanisms of disease, and for the advancement of pharmacology. The second technological advance that has had a significant impact is the development of fluorescence-based technologies to visualize and analyse receptor trafficking, as well as receptor–receptor and receptor–protein interactions, in living cells in real time. These methodologies have greatly aided our understanding of GPCRs and their function in a cellular milieu.

Thomas P. Sakmar. New technologies have provided the means for whole-genome DNA sequencing, and large GPCR sequence databases have been established. These databases have been interrogated using various bioinformatics approaches, in parallel with homology modelling and other computational methods. The identification of a large number of orphan receptor sequences stimulated significant technology development, mainly in the area of cell-based fluorescence detection systems, which could be automated to provide platforms for HTS of small-molecule libraries. Many of these libraries were constructed using combinatorial methods. Much of this recent technological development has been interdisciplinary. For example, classical molecular biology experiments provided the basis for many of the reporter-gene assays that have been adapted for high-throughput applications.

Brian Kobilka. Although not necessarily a new technology, the sequencing of genomes from yeast to humans has provided insight into the evolution of GPCRs and has facilitated the identification of orphan GPCRs and ligands. An extension of the Human Genome Project is the identification of GPCR polymorphisms by Steve Liggett and others[12], and the demonstration that specific alleles might be associated with an increased incidence of disease or altered responsiveness to specific drugs. Gene modification in mice has been very useful in characterizing the physiology of GPCRs, particularly those for which pharmacological tools are limited or don't exist. For example, the mouse orphan receptor PUMA-G (human HM74) was recently found to be responsible for the antilipolytic and lipid-lowering effects of nicotinic acid[13]. Fluorescence tools (fluorescent and bioluminescent proteins) have been used creatively to provide new insights into receptor oligomerization, receptor–G-protein interactions, receptor–arrestin interactions and the localization of second messengers, such as Ca^{2+} and cAMP.

Jean-Philippe Pin. One important issue is the identification of all protein partners that probably constitute what can be called the 'transductosome', in which GPCRs are involved. Such transductosomes might not be identical for a given GPCR in every cell expressing this receptor. As such, it will be important to pull down these protein complexes in specific cells and analyse their constituents using a proteomic (mass spectrometry) approach. Protein complexes other than those that control receptor targeting and trafficking within cells can be characterized using a similar approach.

The identification of a large number of putative GPCRs through sequencing programmes now enables the examination of the expression of all these genes in a single experiment using either arrays or high-throughput quantitative PCR. Such genomic technologies will enable a rapid examination of all GPCRs expressed in a specific organ under different pathological conditions and after treatment with various drugs.

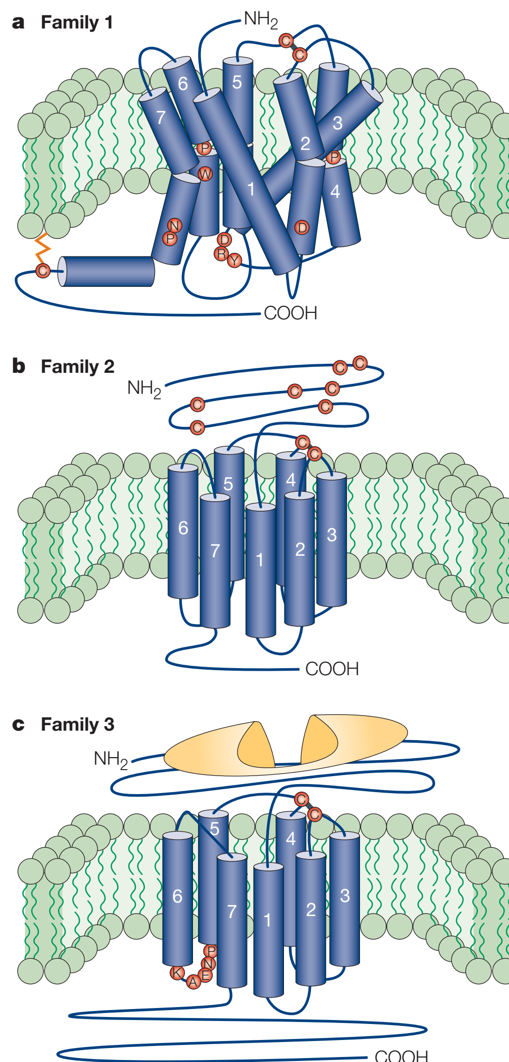
A number of biophysical techniques, such as single-fluorescent-molecule technologies, will also bring much information to the field. The knowledge gained is likely to include the stoichiometry of the different components of the macromolecular complexes in which GPCRs are involved, the precise mechanisms involved in their trafficking/targeting within cells, and the possible dynamics of association/dissociation of the interacting proteins. Conformational changes resulting from the binding of various types of ligand (agonists, antagonists, inverse agonists or allosteric modulators (BOX 2; page 586) could also be analysed using such technologies.

Although HTS of chemical libraries using functional tests helps in the identification of new leads for drug development, such technologies are also very useful for basic research, by generating highly specific tools, as well as identifying new compounds with specific properties, thereby providing much information on how GPCRs work. Such technologies have been widely developed in pharmaceutical companies within the past ten years, but not all identified leads are being made available for the scientific community, and not all GPCRs are being targeted. Therefore, such HTS technologies should also be developed for basic research purposes.

Bernard P. Roques. GPCR gene knockouts have allowed, in many cases, the determination of the main effects resulting from the TONIC or PHASIC stimulation of the target. A typical example is the knockout of the μ-opioid receptor[14], which demonstrated definitively the major role of this receptor in analgesia and opioid addiction, and the putative clinical interest of selective δ-opioid receptor stimulation[15–17].

In addition, the development of HTS methods by expressing GPCRs (including orphan receptors) in different types of mammalian cell has been useful. Using these preparations, a great number of pharmacologically useful probes have been created (including their radiolabelled counterparts when necessary), which has allowed the determination of receptor distribution, their principal functions and agonist versus antagonist activity. Expression in cells of both human GPCRs and their corresponding rodent proteins is an important step towards a rapid physiopharmacological, and putatively clinical, study of the target. At this time, there are

Box 1 | G-protein-coupled-receptor families



G-protein-coupled receptors (GPCRs) are the largest family of cell-surface receptors, and transduce the signals mediated by a diverse range of signalling molecules, including ions, biogenic amines, peptides and lipids, as well as photons, to mediate alterations of intracellular function. GPCRs can be divided into different families on the basis of their structural and genetic characteristics (see GPCR Database online). GPCRs in the different families do not share significant sequence similarity, although they all have the characteristic seven transmembrane (TM) domains. The figure shows schematic representations of receptor monomers (GPCRs have been shown to exist as dimers or oligomers), and illustrates some key structural aspects of the three main GPCR families known at present. Family 1 (panel **a**; also referred to as family A or the rhodopsin-like family) is by far the largest subgroup and contains receptors for odorants, important neurotransmitters, such as dopamine and serotonin, as well as neuropeptides and glycoprotein hormones. Receptors of family 1 are characterized by several highly conserved amino acids (some of which are indicated in the diagram by red circles) and a disulphide bridge that connects the first and second extracellular loops (ECLs). Most of these receptors also have a PALMITOYLATED cysteine in the carboxy-terminal tail, which serves as an anchor to the membrane (orange zig-zag). The recent determination of the crystal structure of rhodopsin[59] has indicated that the transmembrane domains of family 1 receptors are 'tilted' and 'kinked' as shown, due to the presence of amino acids such as proline that distort the helical transmembrane domain. Family 2 or family B GPCRs (panel **b**) are characterized by a relatively long amino terminus, which contains several cysteines that form a network of disulphide bridges. Their morphology is similar to some family 1 receptors, but the palmitoylation site is missing and the conserved residues and motifs are different from the conserved residues in the family 1 receptors. Little is known about the orientation of the TM domains, but given the divergence in amino-acid sequence, they are likely to be different from family 1 receptors. Ligands for family 2 GPCRs include hormones, such as glucagon, secretin and parathyroid hormone. Family 3 (panel **c**) contains the metabotropic glutamate, the Ca^{2+}-sensing and the $GABA_B$ (γ-aminobutyric acid, type B) receptors. These receptors are characterized by a long amino terminus and carboxyl tail. The ligand-binding domain (shown in yellow) is located in the amino terminus, which has been shown by the crystal structure of the metabotropic glutamate receptor to form a disulphide-linked dimer[103]. It is thought to resemble a Venus fly trap, which can open and close with the agonist bound inside. Except for two cysteines in ECL1 and ECL2 that form a putative disulphide bridge, the family 3 receptors do not have any of the features that characterize family 1 and 2 receptors. A unique characteristic of these receptors is that the third intracellular loop is short and highly conserved. At present, little is known about the orientation of the TM domains. Box adapted with permission from REF. 40 © 2002 Macmillan Magazines Limited. *Susan R. George*

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few GPCRs for which a non-peptide probe has not been rapidly found by screening, as successfully shown by both Merck and Sanofi-Synthelabo.

Moreover, the discovery that GPCRs can be stimulated (or blocked) by agonists (or antagonists) belonging to completely different chemical classes is of great clinical importance (for a review, see REF. 18). This amplifies the diversity of drugs available, thus allowing the replacement of an inappropriate molecule (such as one with high toxicity or low bioavailability) with another.

Finally, human genome analysis and genetic studies have been invaluable for their power to discover unknown GPCRs and defects that might be related to a given disease.

Joël Bockaert. HTS based on second-messenger production (for example, Ca^{2+} or cAMP) by GPCRs expressed in heterologous cell lines, as well as HTS based on the use of ubiquitous (G_{15}/G_{16}) or chimeric G proteins, is having the greatest impact (FIG. 2; page 589). Indeed, in heterologous systems, most GPCRs are able to activate G_{15}/G_{16} and thus the phospholipase C (PLC)/Ca^{2+} pathway. Chimeric G proteins can be obtained, allowing the coupling of non-PLC/Ca^{2+}-coupled receptors to this pathway. These technologies have enabled the discovery of a large series of antagonists (particularly non-peptidic antagonists of peptide-activated receptors). Some of the first examples of such non-peptidic antagonists of peptide-activated receptors were the ancestors of losartan (a series of imidazole-5-acetic acid derivatives patented by Takeda Pharmaceuticals). Losartan, an angiotensin-receptor antagonist, is actually largely used, with other similar antagonists, in the treatment of hypertension. In addition, fluorescence-based technologies, such as FRET, BRET and GFP-tagged receptors, have enabled the study of receptor internalization and desensitization, as well as the dynamic association of receptors with receptor kinases and arrestins, and receptor dimerization. They have also allowed direct structural changes to be monitored after agonist binding[8,10].

Proteomics techniques and the yeast two-hybrid system have been useful for studying protein networks associated with GPCRs. However, the association of these proteins with GPCRs does not implicate the involvement of G proteins, but rather indicates the involvement of domains such as PDZ BINDING MOTIFS or homer binding motifs. These proteins are involved in the trafficking, targeting and fine-tuning of signalling pathways[19]. For example, β_2-adrenoceptors activate both G_s and G_i proteins. Suppression of their binding to associated proteins, such as the Na^+/H^+ exchanger regulatory factor (NHERF) or *N*-ethylmaleimide-sensitive factor (NSF), which occurs via a PDZ binding domain, suppresses their coupling to G_i. It is of therapeutic importance that such coupling confers a protective effect on cardiac cells[20].

Rob Leurs. Expression profiling of diseased human tissues is having a major impact on identifying new GPCR targets. It will potentially lead to the prioritization of GPCR targets for given disorders. A good example is the recent observation of increased chemokine receptor CXCR4 expression in breast-cancer tumours and metastasis[21]. In addition, developments in the area of X-ray crystallography and related biophysical techniques (such as nuclear magnetic resonance (NMR) spectroscopy) are of huge importance to support the 'structure-based drug design' approach in the GPCR field. Using such techniques should eventually enable us to obtain direct structural information about ligand-binding events. With the knowledge of the structural features of both the receptor protein and the bound ligands, effective virtual screening technologies should become a reality.

Roland Seifert. The GPCR–Gα fusion protein technique[22] (FIG. 3; page 593) has had an important impact, because it allows for the comparison of closely related GPCR variants/isoforms/subtypes under precisely defined experimental conditions and with high sensitivity. Fusion proteins are sensitive models for the analysis of ternary complex formation (the complex consisting of agonist, receptor and nucleotide-free G protein displaying high agonist affinity and receptor-regulated guanosine-5′-*O*-(3-thiotriphosphate) (GTPγS) binding and steady-state GTP hydrolysis. Thus, receptors can be analysed directly at the G-protein level; that is, at a proximal level of signal transduction, without relying on effector system activity. In addition, the use of the Sf9 insect cell/baculovirus system greatly facilitated the molecular analysis of G_i-coupled GPCRs[23], G_s-coupled GPCRs[24] and G_q-coupled GPCRs[25] (see TABLES 1 (page 596), 2 (page 600) and 3 (page 604) for a summary of the different G-protein families).

I would like to emphasize that the fusion protein technique has greater importance for the analysis of G_s-coupled GPCRs than for the analysis of G_i- and G_q-coupled GPCRs, at least when using Sf9 cells as the expression system. With regard to the analysis of G_i-coupled receptors in Sf9 cells, it does not really matter whether we use a fusion protein or a co-expression system in terms of sensitive analysis of a GPCR[23,26].

Unexpectedly, for the analysis of G_q-coupled GPCRs in Sf9 cells, the co-expression of GPCRs with mammalian regulator of G-protein signalling (RGS) proteins — that is, just looking at the insect-cell G_q proteins 'talking' to mammalian RGS proteins — provides a much more sensitive system for receptor analysis than the fusion protein approach or the co-expression of GPCRs with mammalian G_q[25,27].

It should also be mentioned, however, that there is still a need for methodological advances in the field. Specifically, the analysis of GPCR–G-protein coupling would be greatly facilitated by the availability of a sensitive fluorescence assay that measured receptor-stimulated guanine-nucleotide exchange. Such an assay would substantially reduce the need for experiments with radiolabelled guanine nucleotides and offer novel opportunities in terms of kinetic analysis and HTS. We aimed at establishing such an assay using various GPCR–Gα fusion proteins in Sf9 membranes as targets, and 2′(3′)-*O*-(*N*-methylanthraniloyl (MANT)- and 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY)-labelled GTP analogues as fluorescent probes. Our efforts at establishing a fluorescence assay were compromised by the fact that the fluorescent nucleotides show a substantially reduced affinity for G proteins relative to their non-modified parent nucleotides, rendering the assessment of fluorescence changes by nucleotide binding difficult[28,29]. However, those disappointing results were more than compensated for by the serendipitous finding that MANT nucleotides constitute a novel class of potent and isoform-selective adenylyl cyclase inhibitors[28,30], which provides an interesting alternative approach for interfering with GPCR-mediated signalling at a more distal point at which signals from various GPCRs converge.

2

What tools and technologies for GPCR research would you most like to be available in the future, and why?

Roland Seifert. It would be nice to finally get some good GPCR crystal structures. This would allow us to compare the structures of GPCRs bound to various ligands. In addition to solving fundamental problems of receptor activation (for example, simple binding and activation versus the cubic ternary complex model of GPCR activation (BOX 3; page 606))[31], crystal structures may help us to identify specific drugs; for example, drugs that activate only one G protein. As I stated in my response to question 1 (page 582), it would be very important to have in hand a GPCR–G-protein coupling assay that takes advantage of fluorescent nucleotides, but the problem is to accommodate the fluorophore in the rather tight nucleotide-binding pocket of Gα. Moreover, I would like to see an increased availability of fluorescent ligands for GPCRs. We are currently working on fluorescent histamine-receptor ligands, but it is very laborious to find out where and how to attach which fluorophore in the carrier ligand.

Rob Leurs. For a medicinal chemistry group like mine, it would be fabulous to have more X-ray crystal structures available for the implementation of structure-based drug design for this family of proteins. To this end, 'standard' techniques for the production of milligram quantities of GPCRs, as well as their purification and functional reconstitution, would be useful, and progress is currently being made by several groups. Recombinant expression and purification of, for example, the muscarinic M_2 receptor[32] and the neurotensin receptor NTSR1 (REF. 33), have allowed the determination of the conformation of bound agonists to the respective GPCR by NMR techniques. Moreover, important progress with crystallization of light-activated rhodopsin was announced by Krzysztof Palczewski's lab at a recent Keystone Symposium (G-Protein-Coupled Receptors: Evolving Concepts and Drug Discovery, 17–22 February 2004, Taos, New Mexico).

Of course, an understanding of the experimental conditions required to facilitate crystallization for X-ray crystallography is also an extremely important issue. In addition to future insights into GPCR structure, the *in silico* (virtual) screening of GPCR ligands will hopefully soon be better suited to helping medicinal chemists discover new scaffolds for GPCRs.

Joël Bockaert. It is evident that there is an urgent need for the mass production of GPCRs and, even more importantly, for technologies that allow refolding of GPCRs. Indeed, some technologies, such as the production of GPCRs in *Escherichia coli*, lead to large amounts of protein production, but in a denatured conformation in detergent. Refolding is therefore needed to obtain a native conformation of the receptor. Attempts to do this are underway in several systems, including *E. coli*[34]. In addition, GPCR profiling using DNA chips or quantitative PCR in healthy and pathological cells or tissues, after drug treatment or during cell differentiation, will allow the discovery and identification of new GPCRs as druggable targets. Furthermore, molecular modelling of GPCRs is just in its infancy, but with the availability of new GPCR structures and modelling programs, the future of molecular modelling for finding new drugs has great potential.

Brian Kobilka. I would like to see the development of tools, reagents and methodologies to facilitate the use of NMR to study GPCR structure. This includes instrumentation and affordable media for labelling GPCRs generated in eukaryotic expression systems with ^{15}N- and ^{13}C-amino acids. Like crystallography, NMR has the potential for high-resolution structural determination, but can also address structural dynamics and potentially determine structures of receptors in a lipid bilayer.

Tamas Bartfai. I would like to see real-time NMR spectroscopy or other spectroscopic techniques for studying ligand, G-protein and GPCR interactions and movements, and other spectroscopic methods, such as FRET, for studying not only ligand–receptor interactions, but also receptor–receptor and receptor–modifying-protein interactions. The large-scale production of receptors for crystallization and as antigens would also be desirable.

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Joël Bockaert obtained his Ph.D. at the Pierre and Marie Curie University in Paris, France, and subsequently did postdoctoral work at Northwestern University, Chicago, Illinois, USA. He returned to France in 1975 as Assistant Professor at the Collège de France, Paris, and became Director of Research at Centre National de la Recherche Scientifique in Montpellier in 1982. At present, Bockaert is Professor of Neuroscience at the University of Montpellier. He is recognized for his work on signal transduction of GPCRs, and was one of the first researchers to use binding studies to detect receptors (oxytocin and vasopressin). During his postdoctoral training, Bockaert showed that homologous desensitization requires a phosphorylation step. On returning to France, he developed a miniaturized assay to measure the capacity of some GPCRs to stimulate cAMP production in discrete brain areas, and discovered a number of receptors, including adenosine A_2 receptors in basal ganglia and dopamine D_1 receptors in frontal cortex. His research in Montpellier has led to the discovery of the metabotropic glutamate receptors and 5-HT_4 receptors. His group also cloned the PACAP receptor and provided the first evidence that the NMDA glutamate receptors, which have been implicated in ischaemia, produce oxygen radicals. At present, Bockaert is using a proteomic approach to analyse protein networks associated with GPCRs.

Philip G. Strange. I think we need to move towards examining the detailed conformational changes that occur in GPCRs when ligands bind, so that we can understand the molecular basis of efficacy, and this will require new technologies. It would also be good to be able to measure several activities in one assay tube/well, such as ligand binding, G-protein activation and so on, so that the relative kinetics of the different processes could be examined. This kind of work could, in principle, lead to the development of techniques for studying numerous processes in the cell, eventually in three/four dimensions, although this is more in the realm of cell biology than straight GPCR research.

Thomas P. Sakmar. I am particularly interested in the molecular mechanisms of GPCR activation, so I would welcome any tools that would aid the evaluation of receptor dynamics, especially in native systems. These tools would presumably include specific time-resolved laser and optical spectroscopic and imaging technologies. Further developments and refinements of existing technologies, such as X-ray crystallography and NMR spectroscopy, are also required to advance structural and biophysical studies of GPCR function.

Lakshmi A. Devi. I would like to see the development of specific probes for the quantitative measurement of the dynamic spatio-temporal changes in the activity of key signalling proteins in live cells upon GPCR activation. It is conceivable that fluorescent small-molecule probes that accumulate at the site of activation could be developed, and specialized software to analyse the images of accumulated probes in specified locations within the cell could be used to extract quantitative information. This information could then be used to develop simulation programs to explore GPCR-mediated signalling networks and their interactions with networks activated by other receptor systems.

Susan R. George. I would like to see the development of sensitive, small-molecule fluorescent probes to analyse the structural topology of GPCRs and to track the intramolecular changes that result in the formation of their active and inactive conformations, together with their interactions with neighbouring proteins on the surface of living cells.

Rick Neubig. Tools that would be of great use in understanding both therapeutics targeted directly at GPCRs, and those that modulate GPCRs but do not necessarily interact directly with them (such as anti-depressants), would be simple and affordable methods for examining receptor expression and/or localization *in vivo*. Currently used POSITRON EMISSION TOMOGRAPHY (PET) methods are too expensive or cumbersome for routine research purposes. It is possible that fluorescence or luminescence technologies could move these types of study into more routine use. Another useful tool would be real-time measurements of the activity state of individual G proteins in intact cells.

Bernard P. Roques. I would like to see the development of fluorescent probes that can bind GPCRs covalently, to enable studies of agonist-induced conformational changes of GPCRs. This has been reported recently for the β_2-adrenoceptor[35], supporting the notion that, in GPCRs, there are different conformational states in the allosteric equilibrium, and allowing the kinetics of changes following binding of an agonist to be analysed. The rate of activation is slower for the β_2-adrenoceptor than for rhodopsin. It would be of interest to compare the rates of activation of such aminergic receptors with those of peptide-activated GPCRs. Indeed, the diffusion of a peptide is expected to be slower, possibly accounting for the notion of neuromodulation (the delayed response of a neuron bearing peptide-activated GPCRs, with the release of classical neurotransmitters being the final recorded step).

Fluorescent probes acting *in vivo* could also be designed by using existing irreversible ligands as templates (for example, β-funaltrexamine and its derivatives for the opioid receptors). These probes could allow an examination of the trafficking of GPCRs after stimulation, and the time-dependent interruption of the process by killing experimental animal models. They could also be used to investigate the fate *in vivo* of heterodimeric GPCRs with physio-pharmacological roles that have not yet been clarified.

Probes for PET scans of peptide-activated GPCRs have already been developed, particularly for the μ-opioid receptor. In addition, an ^{11}C-labelled naltrindole probe (a δ-opioid-receptor antagonist) has recently been designed.

The main purpose of these molecules is to investigate not only the *in vivo* labelling of GPCRs but, more interestingly, the changes in binding levels under various conditions (including stress, pain, inflammation, food intake and learning). This gives a direct picture in

different situations of the phasic release of the endogenous peptide effector, which competes with the PET-scan probe for GPCR binding.

It would also be useful to develop methods for differential analysis of the proteome in given structures (using methods similar to subtractive gene banks) following treatment with drugs that target GPCRs (compared with a saline control). Such methods could be used to analyse the effects of treatments for diseases such as depression and inflammatory pain in animal models, the aim being to determine whether the proteins modified on administration of a particular GPCR-targeting drug are also found in post-mortem tissues of patients with these diseases.

Rémi Quirion. NMR technologies applied to the structural studies of ligand–GPCR interactions will probably be available over the coming decade. In addition, nanotechnology and biophotonic approaches, such as QUANTUM DOTS and ATOMIC FORCE MICROSCOPY (AFM), should prove useful in the study of GPCR signalling in complex tissues and cell systems.

3

What are the main pitfalls and advantages of currently used cell-based assay systems for GPCRs?

Olivier Civelli. The cell-based assay systems allow GPCRs to be expressed in large quantities in any type of cellular environment. This has had a liberating effect on pharmacological analyses. But the transfected cells are artificial systems, and data obtained using them should be interpreted with caution.

Roland Seifert. Cell-based assays are often convenient to perform, but many researchers do not even bother to measure receptor expression levels in terms of moles of receptor per cell or milligram of membrane protein. Quite often, you only read about 'cpm bound' or 'amount of cDNA added to dish'. However, receptor expression levels may have a huge impact on the efficacies of partial agonists and inverse agonists[36]. Also, cell-based assays monitor signalling at very distal points, but this does not tell us anything about the events going on more proximally; that is, at the G-protein level. In fact, ligand efficacies and potencies measured at the G-protein level and effector level in a given signalling cascade can be quite different[37]. If we then look at activation of a reporter gene distal to some second messenger-activated kinase, things get even more complex. Thus, the question arises as to what the true efficacy and potency of a given ligand are. The answer may, in fact, be that there are multiple efficacies and not a 'true' single efficacy.

Bernard P. Roques. The advantages are that these are easy tests, and it is possible to find drugs that act on both human and animal GPCRs, thereby facilitating pharmacological investigations. They are also very useful for HTS. The main pitfall is the lack of reproducibility *in vivo*, in particular regarding signalling from cells generally transfected with high levels of GPCRs.

Jean-Philippe Pin. The pitfalls include false positives, and the fact that these techniques do not take into account the possible heterodimerization of the studied GPCR. Advantages include the compatibility with HTS, and the possibility of discovering new compounds that act on new sites, with unexpected properties.

Lakshmi A. Devi. Cell-based assays carried out in heterologous systems have been widely used for characterizing agonists, antagonists or allosteric ligands, as well as for defining the signalling system involved. The advantages are that these systems are simple and easily adapted to HTS. The pitfalls are that these systems differ in the repertoire of intracellular signalling components they contain compared with the endogenous system. Also, many of the peptide-activated GPCRs function by intra- as well as intercellular signalling. This complexity cannot be replicated in a cell-based assay. In addition, the modulation of receptor function by specific interacting proteins, including GPCRs of related families (that is, heterodimerization), cannot be explored.

Brian Kobilka. Cell-based assays for drug screening have been designed for sensitivity, reproducibility and versatility. They enable the detection of signalling by virtually any GPCR, including orphan receptors, for which nothing is known about G-protein-coupling specificity. The disadvantage of current cell-based assays is that they do not account for cellular context. There is a growing body of evidence that GPCR signalling *in vivo* is influenced by its location within signalling complexes that are cell-type specific[38,39]. The proximity of a GPCR to specific signalling molecules will probably influence the efficacy of a drug *in vivo*. Thus, the efficacy determined in a high-throughput assay might not be predictive of the efficacy *in vivo*.

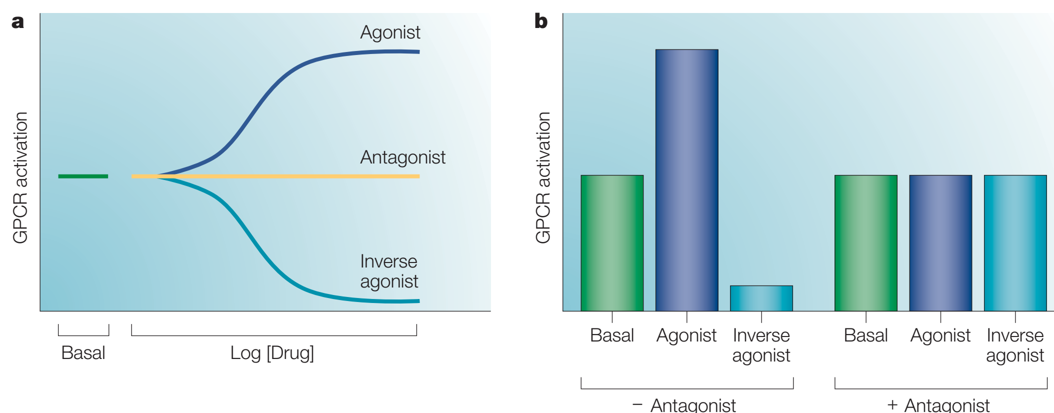
Michel Bouvier. Several pitfalls associated with currently used cell-based assays are linked to the expression levels of the heterologously expressed target proteins. On the one hand, overexpression can lead to aberrant responses that result from the inappropriate stoichiometry of the proteins involved in the measured response. This can be reasonably well controlled by selecting expression conditions that closely mimic physiological expression levels and/or by co-expressing signalling partners to restore appropriate stoichiometries. On the other hand, an increasing number of GPCRs are being found to be difficult to express at sufficient levels. Although many explanations could account for this, the lack of an appropriate molecular chaperone(s), escort protein(s) or co-receptor(s) are likely possibilities. However, identifying the appropriate partner(s) that would need to be co-expressed remains a difficult challenge. Another potential pitfall is related to the recent recognition that GPCRs can function as hetero-oligomers, which have different pharmacological and regulatory properties from each of the PROTOMERS (reviewed in REFS 40,41). This suggests that expression of individual receptors might not be sufficient

Box 2 | **Classification of GPCR ligands**

G-protein-coupled receptor (GPCR) ligands can be divided into three classes (panel **a**): agonists, which increase the proportion of active receptor states to cause a biological response; inverse agonists, which decrease the proportion of active receptor states, and thereby reduce constitutive (basal) receptor activity; and antagonists, which inhibit the action of other ligands (panel **b**)[188]. In systems that lack or display little constitutive receptor activity, an inverse agonist will seem to have a minimal effect on basal responsiveness, while retaining the ability to inhibit the actions of agonists. However, the same drug would be readily unmasked as an inverse agonist in a system with high constitutive receptor activity. It is likely that the majority of drugs classified as antagonists are actually inverse agonists[189]: only if a ligand inhibits drug action, while having no net effect on the distribution of receptor states, is it more correctly referred to as a 'neutral antagonist'. The ability of a compound to behave as an antagonist in one setting and an inverse agonist in another requires that screening systems be optimized to detect both behaviours. The increasing prevalence of inverse agonists has also affected views on therapeutics. For example, diseases characterized by inappropriate constitutive receptor activity would be better treated by inverse agonists rather than antagonists[189]. Long-term drug therapy with inverse agonists can also lead to different adaptive responses than those observed with agonists or antagonists[190].

GPCR ligands can also be classified on the basis of where they bind on the receptor. The majority of known GPCR ligands bind to the same domain recognized by the endogenous agonist for their target GPCR; that is, the orthosteric site[188]. However, many GPCRs have topographically distinct allosteric sites. This has led to the identification of allosteric modulators, which can indirectly regulate the activity of orthosteric ligands and/or directly mediate agonist/inverse agonist effects in their own right[191]. The presence of allosteric binding sites therefore offers additional avenues for attaining drug selectivity[191].

Arthur Christopoulos



to take full advantage of the pharmacological diversity of GPCRs. Combinatorial expression systems may therefore offer interesting alternatives.

Susan R. George. There are significant advantages of some cell-based assay systems, such as those using BRET and FRET, which have greatly enhanced the analysis of adjacent proteins *in situ* within a living cell. However, the interpretations made from some of these studies have to be viewed with caution, as a lot of assumptions are made that do not take into consideration the limitations of the methodologies. A positive energy-transfer signal detected by these methodologies indicates a very close association between the test proteins, in the order of 50–100 Å or less. Therefore, as expected, proteins that are constitutively associated with each other will have a positive 'basal' signal, and any increase or decrease in the signal by an experimental perturbation, such as agonist activation, could have several interpretations. For instance, an increased FRET signal following agonist administration might indicate a conformational change that decreases the distance between the attached fluorophores on adjacent proteins or, alternatively, a *de novo* increase in the association of the proteins. The opposite could be true for a decreased signal. Alternatively, if the signal is near maximal already, it is possible that no further increase would be measurable. The additional considerations to be borne in mind are the relatively large sizes of the fluorophores that are commonly used, and the choice of site on the protein at which the fluorophore is fused, as various domains might be expected to shift differentially, as shown with intramolecular FRET. Therefore, definitive conclusions regarding the dynamics of association or dissociation of interacting proteins might require additional supporting experimental evidence. Ultimately, the problem with most of the investigations we conduct in cell-based systems is the need to ascertain that the observations accurately reflect events in native systems.

Tamas Bartfai. The main pitfall is that many GPCR-dependent functions other than ligand–receptor binding might give a signal that could be picked up in currently used cell-based assays; for example, signals due to compounds that act on G proteins, such as regulator of G-protein signalling-4 (RGS4); interactive accessory proteins, such as receptor-activity-modifying proteins (RAMPs); or receptor dimerization. However, this may also be a benefit, because it allows us to analyse more than just the extracellular interactions between receptor

and ligand, which were preferred in the past because of the ease with which drugs can access these receptors without the need to cross a membrane. For this reason, many GPCR ligands have been put into clinical use, but we have relatively under-utilized intracellular signalling pathways initiated by agonist occupancy of these GPCRs. For example, the first protein-kinase and phosphatase inhibitors are only just reaching Phase III clinical trials. Secondary assays of ligand binding and GTPγS binding carried out on membrane preparations (that is, in a cell-free milieu) act to confirm whether the activity of a compound in a cell-based assay comes from affecting receptor–ligand interactions or from acting on downstream intracellular events.

Akio Inui. Cell-based assay systems are convenient for screening many prototypic compounds with agonist or antagonist activity for GPCRs. However, because of differences in the cell machinery between cell systems and tissues, data obtained from a single-cell-based assay system might not represent the *in vivo* behaviour of the compounds. A new level of complexity in GPCRs includes homo-/heterodimerization, post-transcriptional/post-translational modifications and interactions with proteins other than G proteins, which may be important for fine-tuning GPCR function[40,42]. Thus, although the information from cell-based assay systems is very valuable, caution is called for in interpreting its relevance to *in vivo* physiological signalling.

Rick Neubig. Currently used cell-based assay systems typically overexpress the human GPCR in a heterologous transformed cell line, and often engineer a chimeric G protein to permit Ca^{2+} signalling as a readout, whether or not that is the natural signal from the receptor. This approach works well for simply finding generic agonist/antagonist ligands, and is probably a good start for a novel receptor for which no such compounds exist. These assays are not, however, as suitable for the more subtle questions that I think have the greatest potential to generate novel GPCR therapeutics at existing receptors. Questions regarding up- or downregulation of receptors, the disruption of intracellular protein–protein interactions that contribute to localization and/or effector specificity and distinct effector outputs from a single receptor will not be answered well in such engineered systems, and will require more differentiated model systems that more closely mimic *in vivo* physiological settings.

Arthur Christopoulos. The main advantages of currently used cell-based assay systems are their scalability, their ability to provide a functional readout of receptor activity, their ability to identify a wider spectrum of biologically active molecules (such as allosteric modulators) than classical binding-based screens and their ability to provide ligand-independent screening platforms for orphan GPCRs. A major pitfall, however, is a reduced ability to validate the specificity of a given drug's site of action compared with the specific chemical probes that can be used in binding assays. Another disadvantage is that some functional cell-based assays do not always achieve the same level of reproducibility as do binding assays in terms of yielding quantitative parameters that can be incorporated into structure–activity programmes.

Joël Bockaert. The advantage is that they are easy and rapid. However, the pitfalls are, first, that heterologous expression in cell lines indicates only that the signalling pathway observed is 'possible', but the situation could be quite different in native tissue; and second, that the classification of a drug as an antagonist, inverse agonist, partial agonist or agonist in these systems can be quite different from the *in vivo* situation. A recent paper by Florence Gbahou and colleagues[43] clearly shows that a histamine H_3 receptor ligand (proxyfan) is a partial agonist for the inhibition of cAMP in heterologous cells, but a full agonist or a full inverse agonist for the sleep–wake cycles in cat and mouse, respectively.

Thomas P. Sakmar. Most of the high-throughput assays that are currently used are relatively straightforward in allowing for the rapid screening of hundreds of thousands of compounds. However, they are not necessarily very sophisticated with regard to monitoring the specific pharmacological effect of a compound on the conformation of a receptor. For example, a binding-competition assay will not differentiate between agonists, antagonists, partial agonists or inverse agonists. However, some cell-based assays can be designed to probe cellular responses to receptor activation or inactivation, which is a great advantage. Methods are currently available in cell-based systems for detecting receptor-induced changes in cellular GTP, cAMP, intracellular Ca^{2+} concentration, mitogen-activated protein kinase (MAPK) activity, and phosphorylation states of tyrosine and serine/threonine kinase substrates. In theory, these assays can be combined in high-throughput formats to provide detailed information about a specific receptor's control of the physiology of a particular cell type in culture. The most obvious shortcoming of even the best cell-based assays is that they neglect bioavailability issues, pharmacodynamics and toxicity concerns.

Philip G. Strange. If I understand the question correctly, the cell-based assays that are currently used have been enormously useful in defining the basic properties of GPCR systems. They also have their drawbacks, however. For example, my guess is that every clonal line, even in the same cell host with the same receptor, is different, and these properties continue to change with time. People have worried about the high expression levels achieved in recombinant systems, and this may be an issue, but it should be remembered that in a tissue such as the brain there are locally high concentrations of receptors. Perhaps more important is the complement of interacting proteins in a cell-based system. It is probably the case that a GPCR sees different proteins in a Chinese hamster ovary (CHO) cell compared with its native tissue, and we need to grapple with that issue. Having said all that, the properties of GPCRs expressed in recombinant systems are rather similar to those in native tissue if one sticks to ligand binding and possibly G-protein activation assays.

4

How will changing views of agonist and antagonist behaviour at GPCRs change the way we view these receptors as therapeutic targets?

Arthur Christopoulos. It is good to see that more attention is now being focused on the various manifestations of agonist/antagonist behaviour beyond the classic activation/antagonism paradigms. I think that there is not much doubt in the drug discovery field about the relevance of GPCRs as therapeutic targets, so, if anything, the identification of 'novel' GPCR behaviours (such as oligomerization), or additional GPCR drug-binding sites (such as allosteric sites), would further support this view.

Jean-Philippe Pin. The changing views of agonist and antagonist behaviour at GPCRs provide more possibilities for modulating their activities — for example, using positive or negative allosteric modulators, inverse agonists or PROTEAN LIGANDS[43,44] — but make it more difficult to predict the usefulness of a lead compound.

Rob Leurs. I feel that some quite interesting new opportunities are being explored. However, the increasing complexity that we continue to discover in this area (including non-G-protein signalling, dimerization and protean ligands) could make the GPCR family (in some eyes) less attractive. Despite the historical success of GPCR targeting, the wealth of new information could at some point trouble industry decision makers about, for example, the chance of achieving selective activation or inhibition.

Rick Neubig. Clearly, the concept of agonists and antagonists today is dramatically different from 15 or 20 years ago. We now have the distinction between pure antagonists and inverse agonists (antagonists that downregulate receptors), and emerging data that agonists can selectively modulate different functional outputs. So far, this richness of functional effects has not been used very effectively, but I think that more therapeutic examples using these concepts will emerge. The notion of enhanced agonist specificity due to selective activation of different G proteins has been discussed extensively in the literature (agonist-dependent trafficking of receptor stimuli) and a few well-documented examples of this phenomenon exist in *in vitro* models[45]. To date, there are no good clinical examples, but this remains a possibility — although perhaps a remote one. In some cases, it may be useful to downregulate a receptor (for example chemokine receptor-5 (CCR5) as the HIV entry co-receptor). Interestingly, several recent studies show that antagonists as well as agonists can lead to receptor downregulation, so it might be possible to eliminate receptors without ever turning them on. Unfortunately, I think that they are unlikely to totally revolutionize GPCR therapeutics, but they should provide some additional specificity to this class of drugs.

Tamas Bartfai. The changing views of agonist and antagonist behaviour are a greater surprise for academia than for industry, which has for a long time used very large numbers of synthetic ligands that have often turned out to be partial agonists or inverse agonists. By contrast, academics have not had access to such compounds and have instead used the endogenous ligands, which are not partial agonists. I therefore believe that the changing views of agonist and antagonist behaviour will not have a great influence on the way we develop drugs that target GPCRs. Most people involved in clinical trials have always favoured partial agonists as safer drugs, because the acute risk in the event of overdose is lower. Furthermore, in the chronic setting, they are less likely to cause desensitization and the development of tolerance than full agonists. Thus, they are more useful clinically.

Joël Bockaert. The most recent change is the observation that some antagonists are actually inverse agonists, which inhibit the constitutive activity of GPCRs. The therapeutic difference between an antagonist and an inverse agonist is not yet very clear. However, the observation that some natural inverse agonists do exist (such as the Agouti-related peptide (AgRP)), and that some receptors have natural constitutive activity (such as the melanocortin-4 receptor (MC4R)), indicates that these new pharmacological concepts are important. Note that MC4R is the target of α-melanocyte-stimulating hormone (α-MSH), a peptide that decreases the intake of food, and also of AgRP[46].

Philip G. Strange. One of the key changes over the past ten years has been the discovery that many of the drugs previously considered to be antagonists are actually inverse agonists. Indeed, it is rare to find a drug that is actually a neutral antagonist. I do not believe that the significance of this has been fully understood. One of the problems has been that inverse agonism is more apparent in recombinant cells and has been less easy to demonstrate in *in vivo* systems. One issue here is that people have been very keen to demonstrate agonist-independent activity in *in vivo* systems and its suppression by inverse agonists, but that may be difficult. Rather, another facet of inverse agonists may be the ability of the compounds to cause increases in receptor number in the relevant systems, and this may be apparent even in the absence of constitutive activity. Hence, in a native system, in which receptor expression levels may only be moderate and constitutive activity therefore low, inverse agonists may nevertheless increase receptor expression levels. These ideas have not yet been taken on board in drug design as far as I know.

For agonists, there is the question of how the agonist achieves its effects, including precisely how it activates the G protein, whether there are non-G-protein-linked responses for GPCRs, such as direct activation of Janus kinase (JAK), and whether one GPCR can direct responses to different G proteins (agonist-directed trafficking).

Another issue concerns the possible existence of multiple sites on receptors for ligand action. For several GPCRs, there is evidence that allosteric sites exist in addition to the primary ligand-binding site. This might be expected for a small molecule that affects the binding

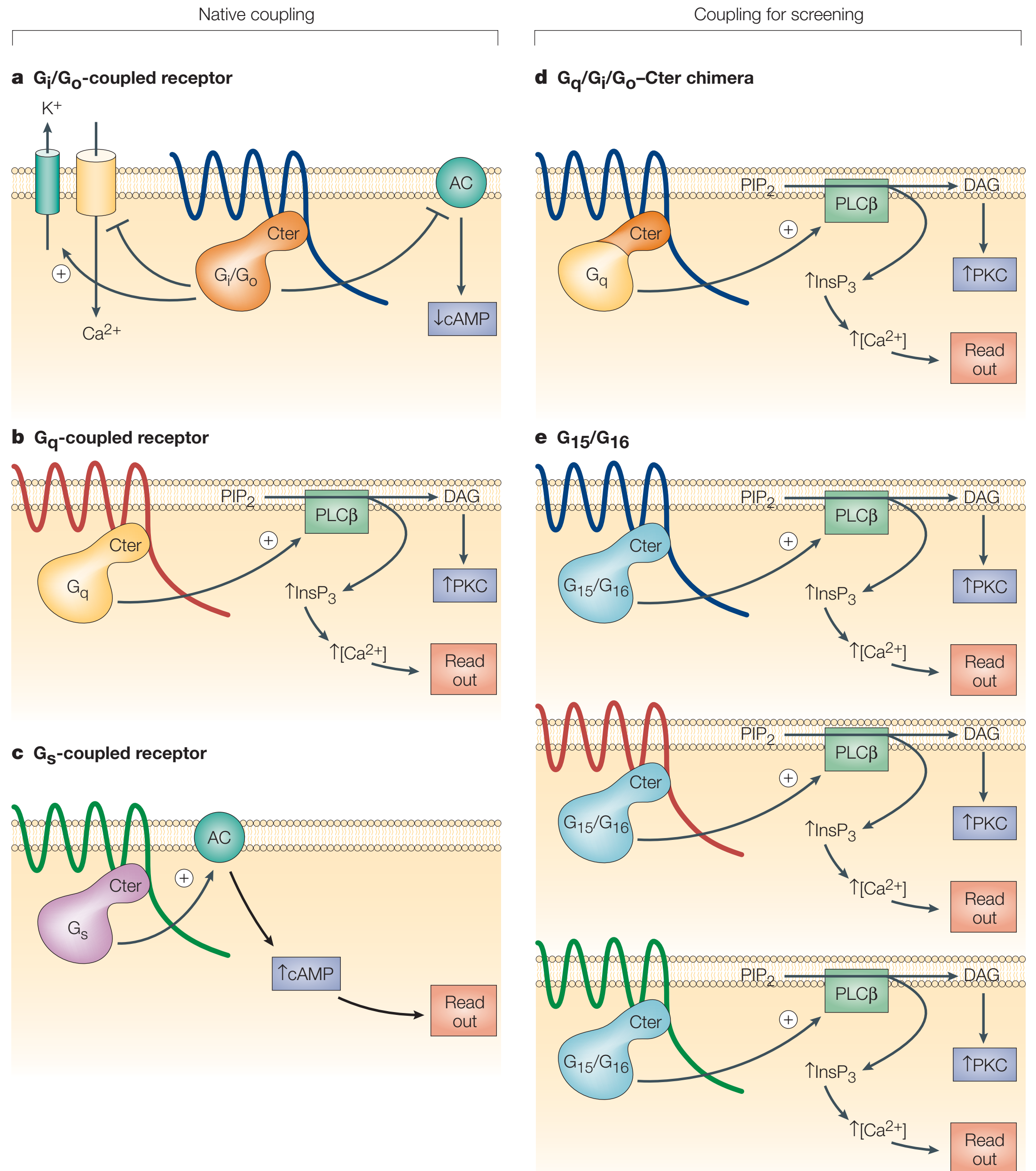


Figure 2 | **High-throughput screening based on second-messenger production has enabled the discovery of several GPCR antagonists.** Most G-protein-coupled receptors (GPCRs) are coupled to one or two members of the three main families of G proteins: G_i/G_o (orange), G_q (yellow) and G_s (pink). **a** | G_i/G_o-coupled receptors inhibit adenylyl cyclase (AC), activate K^+ channels or inhibit Ca^{2+} channels. **b** | G_q-coupled receptors activate phospholipase Cβ (PLCβ). This results in the intramembrane hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to inositol-1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). DAG increases the activity of protein kinase C (PKC) and $InsP_3$ increases intracellular Ca^{2+} concentration ($[Ca^{2+}]$). **c** | G_s-coupled receptors activate AC. Functional screening of GPCR ligands is generally done in cell lines expressing the GPCR of interest. High-throughput screening (HTS) readouts can be based on the detection of Ca^{2+} (by monitoring the increase in fluorescence of a Ca^{2+} indicator loaded into cells, such as Fluo-3). G_q ligands acting on G_q-coupled receptors can be directly screened in this way, as shown in part **b**. Ligands acting on G_s-coupled receptors (part **c**) can be directly screened by measuring cAMP production with appropriate kits; however, recording the increase in cAMP production is generally a more complex measurement than recording intracellular $[Ca^{2+}]$. The decrease in cAMP production by G_i/G_o-coupled receptors (part **a**) is generally not used in HTS. As measurement of $[Ca^{2+}]$ is generally more convenient, methods have been developed for coupling G_i/G_o or G_s to PLCβ. **d** | G_i/G_o coupling to PLCβ can be achieved by transfecting the GPCR of interest into cells, together with a chimeric $G_q/G_i/G_o$ protein. This chimeric G protein contains most of the sequence of G_q (yellow) fused with the carboxy-terminal (Cter) amino-acid residues of G_i/G_o (orange). **e** | In addition, most G_i/G_o-, G_q- and G_s-coupled receptors (shown schematically in the diagram in dark blue, red and green, respectively) can stimulate G_{15}/G_{16} (turquoise) when expressed together in the same cell. As G_{15}/G_{16} is coupled to PLCβ (REF. 197), Ca^{2+} readout can be used to monitor receptor activity. For more information on the use of chimeric G proteins in drug discovery, see REF. 198. Figure prepared by Joël Bockaert.

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Richard A. Bond earned a B.Sc. and a B.Pharm. at St Thomas University, Miami, Florida, USA, and the University of Houston, Houston, Texas, respectively. He received his Ph.D. in Pharmacology under the supervision of David E. Clarke, and did his postdoctoral training with Paul M. Vanhoutte at Baylor College of Medicine in Houston, Texas. His early work provided functional evidence for a β-adrenoceptor that was eventually found to be the β_3-adrenoceptor. Then, in collaboration with Robert Lefkowitz and others, he undertook studies on the spontaneous activity of GPCRs and compounds functioning as inverse agonists. Most recently, he became interested in the paradigm shift that has occurred with regard to the use of β-adrenoceptor agonists and antagonists/inverse agonists in the treatment of congestive heart failure. He is now attempting to determine whether the unexpected reversal in heart failure after administration of beta-blockers is a one-off event, or indicative of a more general pattern that is applicable to other diseases, such as asthma. At present, he is Associate Professor of Pharmacology at the University of Houston in Texas.

of a large molecule, such as a chemokine to its receptor, but seems to be more widespread. The wider acceptance of this concept should provide more sites for drug design.

Roland Seifert. I think that we will soon recognize that there are only very few truly neutral antagonists; that is, the majority of 'antagonists' are actually weak partial agonists or partial inverse agonists[36]. A major challenge will be to determine the therapeutic differences between a neutral antagonist and a partial agonist/inverse agonist; therapeutic areas that are responding in this regard are the areas of heart failure and schizophrenia. Metoprolol (Lopressor/Toprol) is an example of an inverse agonist that acts at the β_1-adrenoceptor, which is used for the treatment of chronic heart failure. Clozapine (Clozaril; Novartis), which is used for the treatment of schizophrenia, is an example of an inverse agonist that acts on several receptors; for example, the 5-hydroxytryptamine $(5\text{-HT})_{2A}$ receptor and dopamine D_1 receptor. Evidently, carrying out well-controlled and well-designed clinical studies that compare placebo versus inverse agonist versus neutral antagonist will be very time-consuming and expensive, as the differences between the drug groups may be small or only seen in patient subgroups, which mandates the examination of large cohorts. However, my undergraduate pharmacy students keep asking me about the clinical importance of inverse agonists, and I can only hope that we will ultimately fill the tremendous gap between our excellent basic pharmacology knowledge and our poor clinical pharmacology knowledge.

Lakshmi A. Devi. Recent studies show that a large number of classic antagonists have inverse-agonist properties. Pure antagonists block receptor activity and, as drugs, are thought to block the action of the endogenous ligand. Inverse agonists, on the other hand, also block the constitutive activity of the receptor. Thus, as drugs, inverse agonists would function in cases in which the basal signalling by the receptor is controlled mainly by its intrinsic activity rather than by the endogenous ligand.

Susan R. George. The realization that GPCRs can assume multiple conformations, instead of functioning as simple switches in 'on' or 'off' positions only, is very important conceptually. This helps us to understand the intrinsic activity or the 'unliganded' activity of GPCRs, and also the differential ability of agonists to induce different levels of activation and different effector coupling, thus generating different outputs. The differential ability of antagonists not only to block agonist action, but also to block the intrinsic activity of the unliganded receptor (as occurs with inverse agonists), has further reinforced the importance of recognizing multiple GPCR conformations. This suggests that the different receptor conformations could potentially be targeted selectively by drugs, and provides a mechanism through which a wider range of fine-tuning the control of GPCR function might be possible. This aspect has not been actively utilized in drug discovery, but provides a novel means of regulating, with drugs, the degree to which a GPCR is activated or inactivated. GPCRs seem to vary greatly in terms of the degree of intrinsic activity they exhibit, so the extent to which such modulations are possible will be distinct and individual to each receptor.

Bernard P. Roques. In the case of peptide-activated GPCRs (for example, angiotensin, bradykinin, tachykinin receptors and so on), antagonists rather than agonists have been developed in the clinic. The pharmacological activity and putative clinical interest of these compounds is based on the existence of a large tonic or phasic release of the endogenous effector.

For example, the great clinical success of angiotensin-converting enzyme (ACE) inhibitors as antihypertensives is due to an important basal release of angiotensin I, which is transformed into the vasoconstrictive peptide angiotensin II by ACE. In this case, the pharmacological responses that result either from inhibition of angiotensin II production, or blockade of its stimulation of the angiotensin receptor, are very similar.

Conversely, the basal (tonic) release of several neuropeptides is low. Thus, the opioid-receptor antagonist naloxone is unable to give a clear pharmacological response when administered in humans or animals in the absence of stressful conditions. This is one of the difficulties in the search for therapeutics in the field of neuropeptide GPCRs, which has no doubt limited the interest in peptide-activated receptors as therapeutic targets.

A large number of GPCRs induce signal transduction in the absence of agonist stimulation. In this constitutive state, the receptor is active, and inverse agonists are able to reduce this intrinsic activity. This is the case for compounds such as cimetidine, which acts as an inverse agonist of the constitutive histamine H_2 receptor[47]. It is now possible to create constitutive GPCRs by selective mutation of some amino acids in the internal loops[48]. The modified receptors could be used to screen for

inverse agonists[49], and these ligands may be used to investigate the *in vivo* pharmacology of the GPCR. This is useful when the basal release of endogenous effectors is very low, which prevents a clear response with competitive antagonists, and is complementary to GPCR knockouts. The only requirement is obviously the occurrence of a naturally constitutive state of the GPCR.

Thomas P. Sakmar. A more sophisticated and complete understanding of GPCR function, particularly with regard to the different conformational signalling states of a given GPCR, might allow for the design of improved drugs that could modulate GPCR function in very specific ways. GPCRs interact with many cell-signalling adaptor and transducer proteins, such as heterotrimeric G proteins, small G proteins and kinases. The current working hypothesis is that discrete receptor conformational states permit the receptor to recognize different binding partners[50,51]. These conformational states are stabilized by receptor ligands. Partial agonists, for example, might be used to selectively modulate only one of several signalling pathways, thereby avoiding some potential side effects of a full-agonist drug. Classical molecular pharmacology is based on a mathematical description of a drug binding to its receptor, but downstream signalling effects are obviously extremely important in drug action and pharmacology as well.

Jeffrey L. Benovic. Current theory suggests that GPCRs can exist in multiple conformational states[52]. Agonism is currently defined on the basis of the ability of a compound to activate or inhibit signalling, and thus largely reflects a readout of GPCR interaction with heterotrimeric G proteins. However, as GPCRs interact with additional proteins, and many of these interactions are ligand dependent (for example, interactions with GPCR kinases (GRKs) and arrestins), it is also important to consider such interactions in the characterization of ligand agonism. An interesting example of this is the μ-opioid receptor, for which some compounds — such as morphine — that serve as agonists in signalling (via G-protein coupling) do not promote receptor desensitization and endocytosis (via a GRK/arrestin-dependent pathway)[53]. Interestingly, the inability of an agonist to mediate μ-opioid-receptor desensitization seems to correlate with the agonist's propensity to promote physiological tolerance. Another interesting 'disconnect' between signalling and endocytosis is observed with the 5-HT_{2A} receptor, for which a number of compounds that serve as antagonists in signalling can function as agonists to promote receptor endocytosis[54]. On the basis of such studies, one can envision the identification of ligands that stimulate signalling without affecting regulatory processes such as desensitization, endocytosis and/or degradation, as well as ligands that selectively stimulate regulatory processes without affecting signalling.

Michel Bouvier. Although it is occurring only very slowly, there is no doubt that the evolving notion of drug efficacy will need to be taken into consideration in the development of new therapeutic agents. This includes the recognition that ligands can have negative intrinsic activity (inverse agonists), which inhibits the spontaneous/constitutive activity of receptors (reviewed in REF. 55). Such negative intrinsic activity can be beneficial when targeting diseases that result from constitutive receptor activity, but detrimental in other cases, such as the histamine H_2 receptor, for which inverse agonists promote more receptor upregulation than neutral antagonists[47]. The fact that drugs may have different 'apparent' efficacies, depending on the signalling pathway being considered (as recently discovered for β_2-adrenoceptor ligands, which act as inverse agonists inhibiting basal adenylyl cyclase activity, but partial agonists activating the MAPK pathway[56]), may also need to be considered in drug discovery efforts. Finally, several compounds act as pharmacological chaperones in assisting the folding and trafficking of nascently synthesized receptors. This was shown to be the case for vasopressin-receptor antagonists, which can rescue cell-surface expression and function of mutant forms of the vasopressin V_2 receptor that lead to nephrogenic diabetes insipidus[57], and which therefore have potential therapeutic applications. Clearly, the presence or absence of one or many of these pharmacological attributes may confer unique therapeutic properties on some compounds. Perhaps more importantly, these features have led to the realization that receptors exist in multiple conformational states that can be differentially recognized and stabilized by different ligands. Thus, different drugs may modulate receptor activity by promoting or stabilizing distinct conformational states that might be preferred in particular clinical indications. In other words, the simple binary classification of ligands into 'agonists' or 'antagonists' has become

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Michel Bouvier completed a B.Sc. in Biochemistry at the Université de Montréal, Quebec, Canada. Following his Ph.D. in Neurological Sciences at the same university in 1985, he spent four years as a postdoctoral fellow in the laboratory of Robert Lefkowitz at Duke University, Durham, North Carolina, USA. In 1989, he returned to Montréal as Professor of Biochemistry and Scholar of the Medical Research Council of Canada. He is now full Professor and Chairman of the Department of Biochemistry at the Université de Montréal. He holds the Hans-Selye Chair in Cell Biology and the Canada Research Chair in Signal Transduction and Molecular Pharmacology. Over the years, his research programme has focused on the processes involved in signal transduction across biological membranes. In particular, he has studied the processes controlling the efficacy of the G-protein-linked signalling pathways. In addition to studying the role of phosphorylation and palmitoylation in the control of GPCR signalling efficacy, his work has greatly contributed to the emergence of the concept of spontaneous activity and inverse agonism at GPCRs. More recently, his work provided the first direct biochemical and biophysical evidence that GPCRs exist as dimers in living cells. He also pioneered the use of bioluminescence resonance energy transfer (BRET) to explore the role of protein–protein interactions in signal transduction. Bouvier is the author of more than 140 scientific papers and has delivered more than 200 invited talks at conferences.

insufficient to explain drug efficacy fully. Establishing new classification systems that reflect the newly uncovered complexities, and incorporating them into drug design and screening campaigns, should increase the chances of developing compounds with unique therapeutic properties.

Richard A. Bond. I assume this question could mean: 'changing views of agonist, antagonist and inverse agonist behaviour'. Having said that, for the moment, I no longer believe in three classes of ligands. I believe that in a given system there are only agonists and the opposite of agonists (these could be termed either antagonists or inverse agonists). However, I am also against deleting either of the terms 'antagonist' or 'inverse agonist'. This is because they may serve a useful purpose in distinguishing between those compounds that are near zero efficacy (that is, antagonists that are actually weak partial agonists or weak partial inverse agonists), and those with clearly agonist or inverse agonist properties in a given system. Several years ago, when we were first generating data on inverse agonists at the β_2-adrenoceptor, Terry Kenakin told me, "it could be that zero efficacy is like a knife edge; most compounds will fall to one side or the other". It seems he was right.

I think, for the moment, that the message for the development of therapeutic targets is this: if one is clear about the ligand property needed in a particular disease state (agonist or inverse agonist), then 'do not get too close to the 'knife edge' (zero efficacy)' in designing a ligand. I say this primarily because of what has occurred in the field of heart failure, in which the compound bucindolol — which was apparently close to the knife edge, but probably on the agonist side — failed to be of benefit, whereas two other compounds more clearly on the inverse agonist side of the knife edge — carvedilol and metoprolol — were beneficial.

If we were to decide someday to delete either antagonists or inverse agonists as a class, I would probably vote for deletion of antagonists. I say this because to me the notion of 'antagonist' is one of a rather passive molecule whose only effect is seen in the presence of an agonist. I think this is erroneous. I believe compounds with negative efficacy in a given system produce an entire set of changes on their own, irrespective of whether there is agonist present or not. Therefore, to me, 'inverse agonists' convey that notion more appropriately.

As far as I can tell, industry's response to the discovery that compounds with negative efficacy produce differential effects from those with close to zero efficacy has been to design systems (usually via receptor overexpression or mutation) to amplify the efficacy of the ligand. However, this might be exactly the opposite of what is needed. It may be that the least sensitive systems will provide more appropriate answers for transferring results from the laboratory to the clinic. That is, compounds identified as agonists or inverse agonists in a less responsive system may retain that property more reliably as the signal transduction components are altered in the changing systems.

5

Many predictions for heptahelical receptors have been based on using the rhodopsin crystal structure as a template. How successful have these been?

Rob Leurs. Despite the fact that the overall homology of other GPCRs with rhodopsin is only very limited, the GPCR models that have been generated so far have, in my opinion, been very useful for the generation of new hypotheses and the rationalization of a variety of mutagenesis data. It should be noted, however, that most models generated on the template of rhodopsin have to be 'fine-tuned' on the basis of already known mutagenesis data.

Roland Seifert. I have worked on several GPCRs using the rhodopsin model. I would say that the rhodopsin model is very useful for some, but not all, GPCRs. For example, it was really useful for our molecular work on agonist–histamine-H_2-receptor interactions[58]. However, with respect to related work on the histamine H_1 receptor, the rhodopsin model did not give us the clues we hoped to obtain[25]. A major drawback of the rhodopsin model is that there is only an inverse agonist (11-*cis*-retinal), but no agonist[59].

Susan R. George. The predictions based on the rhodopsin crystal as a template have been enormously successful in many ways for the rhodopsin-like family 1 GPCRs, especially in furthering our understanding of the arrangements of the transmembrane helices relative to each other within a single monomer in the 'inactive' conformation. The spectacular analysis of rhodopsin in retinal disc membranes using AFM by Krzysztof Palczewski and colleagues[60,61], in combination with the analysis of its crystal structure, have enabled a glimpse into the arrangements of receptor oligomers at the cell surface. Although we are a long way from understanding the active conformation or the physiological arrangements of GPCRs on the surfaces of cells expressing a normal complement of multiple proteins, such as neurons, these studies represent very important initial landmarks.

Ronald E. Stenkamp. As of a year ago, when I looked at this issue in detail, most of the GPCR models based on the rhodopsin crystal structure had proven useful. They were used largely to provide a molecular model for a general understanding of mutagenesis and ligand-binding experiments. In a few cases, predictions were based on the models, which were subsequently confirmed by experiment. For example, in the case of the dopamine D_2 receptor[62], a model based on the crystal structure of rhodopsin was used to identify amino acids involved in cation binding. An earlier model based on another template had indicated that other residues were involved. Mutagenesis experiments were not consistent with the earlier predictions, but they were with those based on the rhodopsin crystal structure. The rhodopsin structure therefore does seem to be representative of other GPCRs (FIG. 4; page 613).

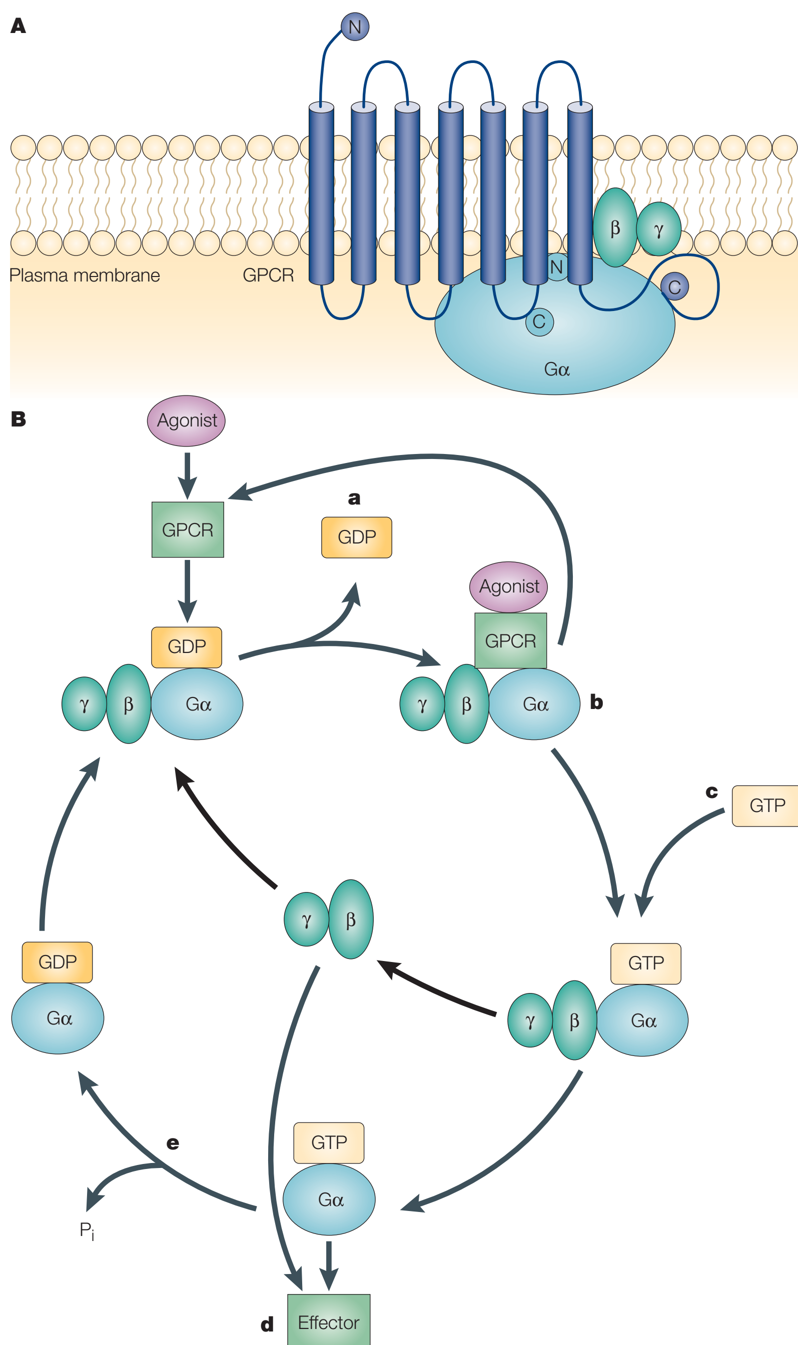

Figure 3 | **GPCR–Gα fusion proteins as a model system for the analysis of receptor–G-protein coupling. A** | Schematic of a G-protein-coupled receptor (GPCR)–Gα fusion protein. The GPCR carboxyl terminus (C) is fused to the amino terminus (N) of Gα[22], ensuring close proximity and defined stoichiometry of the two coupling partners. GPCRs can activate G proteins linearly (that is, one GPCR molecule activates one G protein) rather than catalytically (that is, one GPCR molecule activates several G proteins), which substantiates the relevance of the fusion protein technique[26,85]. Fusion proteins also enable the study of coupling between GPCR species isoforms[58] or intra-species polymorphic forms[185] and a particular Gα isoform. Correspondingly, the coupling of a particular receptor to various Gα isoforms can also be analysed[26,85]. Crosstalk between fusion proteins to non-fused Gα and between different fusion protein molecules[199,200] must be taken into consideration. **B** | G-protein cycling. Rate-limiting receptor-promoted GDP dissociation (**a**) is followed by ternary complex formation (**b**). The GPCR then catalyses the binding of GTP to Gα (**c**), which disrupts the ternary complex, causing dissociation of the G-protein heterotrimer into Gα and βγ. Both entities regulate the activity of effector systems (**d**; see also TABLES 1, 2 and 3). G-protein activation is terminated by hydrolysis of the Gα-bound GTP to GDP and P_i (**e**). GPCR–Gα fusions are useful for studying steps **b**, **c**, **d** and **e** of the G-protein cycle (to our knowledge, GDP dissociation (**a**) has not been directly studied with this system). Figure prepared by Roland Seifert.

Tamas Bartfai. Both inside and outside the pharmaceutical industry, the rhodopsin model has been combined with what we know about PHARMACOPHORES — which is a knowledge-rich area for several monoamine receptors — to cobble together models. However, such models have often been of *post factum* value; explaining rather than predicting results.

Joël Bockaert. The predictions have generally been 'correct', but the rhodopsin crystal was obtained in the presence of retinal, which is an inverse agonist. So far, the structure of an 'active' rhodopsin molecule is still lacking.

Arthur Christopoulos. The determination of the crystal structure of bovine rhodopsin at high resolution[59] has certainly been a boon to the GPCR field. One must bear in mind, however, that rhodopsin has low sequence similarity to most other GPCRs, has an inverse agonist incorporated into its structure, and the crystals obtained were of the receptor in its inactive state, so we should not necessarily expect to find high degrees of concordance between this structure and other GPCRs. Nevertheless, a good starting point is better than none, and there have been very successful predictions for the structures of some of the receptors for bioamines and related small molecules based on the rhodopsin model; for example, muscarinic M_1, dopamine D_2, α_1-adrenoceptor, histamine H_3 and adenosine A_1 receptors[4,63,64]. Of course, this only refers to the transmembrane domains of the GPCRs; we are still some way off determining the intra- and extracellular loop structures.

Jean-Philippe Pin. The structural predictions have been good enough for the general analysis of homologous protein structures, even for distantly related GPCRs, such as the heptahelical domain of class III (family 3/C) GPCRs, which include metabotropic glutamate, $GABA_B$ (γ-amino butyric acid, type B), Ca^{2+} and some taste and pheromone receptors (see, for example, REF. 65). However, one should take into consideration that the rhodopsin structure corresponds to a fully inactive state of a GPCR (stabilized by an inverse agonist), such that the various active states cannot yet be predicted with accuracy. Accordingly, such models are more useful for the characterization of the binding sites of inverse agonists. I am still not convinced that such models are accurate enough for a detailed analysis of ligand-binding sites and drug design. Recently, Didier Rognan and collaborators have used such a rhodopsin-based model of the vasopressin V_2 receptor for *in silico* screening of possible new agonists and antagonists[66]. This approach allowed them to identify known agonists and antagonists hidden in the chemical database, which suggests that it is a promising technique. However, whether new leads can be identified in this way remains to be shown.

Bernard P. Roques. Few breakthroughs in the field of GPCR structural determination have emerged from molecular modelling using the rhodopsin structure, except some data about the intra-cytoplasmic loops involved in

G-protein binding, although details on the specific amino acids involved and their spatial arrangements are still lacking. However, the domains of GPCRs that are involved in agonist or antagonist binding have sometimes been characterized using selective or systematic mutagenesis of rhodopsin-derived GPCR models. The existence of interconverting conformations of a given GPCR — which has led to the idea that there are discrete states of the protein with different affinities for agonist and different G-protein couplings — have also resulted in part from rhodopsin structural studies. However, other applications, such as the development of antibodies or characterization of regions of G-protein coupling, have not benefited greatly from the rhodopsin structure.

Thomas P. Sakmar. The rhodopsin crystal structure has been used as a template to create homology models for several GPCRs. For example, satisfactory homology models for the dopamine D_2 receptor, muscarinic acetylcholine M_1 receptor, CCR5 and a few others have been reported[4,63,67]. These select cases demonstrate that GPCR homology models are useful for understanding how a given drug interacts with its receptor. However, the rhodopsin structure has limited value for making predictions about the structures of the vast majority of GPCRs. For example, Elodie Archer and colleagues[68] tried without much success to model the cholecystokinin $(CCK)_1$ receptor using rhodopsin as the template. So even the CCK_1 receptor — a typical family 1b member — has apparently diverged far enough from rhodopsin to prevent successful homology modelling. Other more distant relatives of rhodopsin, by definition, resist even primary structural alignment, let alone homology modelling. For example, the rhodopsin structure provides little insight into the glucagon receptor, which is a prototypical member of GPCR family 2. In addition, even successful homology models contain only the transmembrane segments — loops are generally not homologous. Finally, the rhodopsin structure represents the inactive state of the receptor. Therefore, it is unlikely that existing GPCR models, either based on rhodopsin or designed *ab initio*, will be sufficient to be used for *in silico* screening of compound libraries.

6

How close are we to having further GPCR structures, and what are the main barriers to obtaining these?

Philip G. Strange. So near, yet so far, I believe. For receptors for which there is enough pure protein available, it seems that crystallization is the hold-up.

Tamas Bartfai. Obtaining large quantities of good quality crystals has been the bottleneck — much larger membrane protein complexes have been solved much more easily; for example, photosynthetic reaction centres and, more recently, multimeric ion channels. There are no known theoretical reasons for these difficulties with GPCRs.

Joël Bockaert. The main barrier is certainly obtaining a correctly folded, stable GPCR protein in a suitable detergent.

Arthur Christopoulos. I think that the biggest barrier to determining further GPCR structures remains our general inability to express these proteins so that they remain biologically active and in sufficient quantities to facilitate structural studies. Novel expression systems are still needed for complex integral membrane proteins such as GPCRs.

Rémi Quirion. We are making good progress, and I am optimistic that we will determine many structures over the next five years. The key, however, is progress in crystallography, and ensuring that crystallography experts will focus their attention on solving the structures of GPCRs. This is far from guaranteed, as the recent explosion in the field of proteomics has led to a great demand for crystallographers to work with GPCR experts on the determination of three-dimensional structures.

Lakshmi A. Devi. We are fairly close to having further GPCR structures because of recent advances in a number of areas. Techniques for generating large quantities of recombinant membrane proteins, optimization of membrane-protein purification techniques and the development of selective affinity probes have helped in GPCR isolation. The main barriers are that large amounts of homogeneously pure GPCRs (that is, with the same post-translational modifications) cannot be easily generated, mainly because GPCRs undergo multiple modifications and become increasingly unstable during purification. AFM is increasingly being used to probe the nature of GPCRs under native conditions. AFM measurements have been employed to determine intramolecular and intermolecular forces[69]. Individual SINGLE-MOLECULE FORCE EXTENSION measurements can provide information on the movement of individual domains during resting compared with ligand-bound states. Furthermore, intermolecular measurements provide information on the forces and energy barriers traversed upon dissociation of the ligand, G proteins and/or other interacting proteins from GPCRs. Improving the sensitivity of this and related imaging techniques would facilitate investigations on the nature of GPCRs in an endogenous system.

Ronald E. Stenkamp. It is impossible to predict when a particular set of structures will become available. It could take only a single breakthrough or a lot of hard work to suddenly produce many more structures for this class of protein. Unless you poll every researcher in the field, it will be impossible to say how close we are to having more structures.

So far, the main barrier to additional structures has been obtaining large quantities of purified GPCRs. Crystallization issues cannot be addressed without that. I am not experienced in the generation of GPCR samples, so I do not understand all the difficulties there. I expect that crystallization and X-ray structure

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Arthur Christopoulos gained a B.Pharm. before completing a Ph.D. in Pharmacology at the Victorian College of Pharmacy (Monash University), Melbourne, Australia, in 1997. His doctoral research focused on mechanisms of allosteric modulation of muscarinic acetylcholine receptors under the supervision of Fred Mitchelson. Christopoulos then joined the Department of Psychiatry, University of Minnesota, USA, as a postdoctoral associate in the laboratory of Esam E. El-Fakahany, where he investigated novel modes of regulating cholinergic signalling pathways. In 1999, he returned to Australia as Head of the Amrad Drug Discovery Laboratory in the Department of Pharmacology at the University of Melbourne, and was subsequently appointed a C. R. Roper Senior Research Fellow in 2000, and a National Health and Medical Research Council (NHMRC) of Australia Senior Research Fellow in 2003. Christopoulos now heads the Molecular Pharmacology Laboratory in the Department of Pharmacology, and is investigating novel modes of regulating GPCRs, especially allosteric ligand–receptor and receptor–protein interactions. In addition, he has strong interests in analytical pharmacology, and has published numerous articles and two monographs on the application of computer models to biological data analysis.

analysis of the samples will follow the same course of problems, hassles and successes as found for other macromolecules. The fundamental breakthrough will come in the protein preparation stage.

Jeffrey L. Benovic. At present, there are significant academic and industrial research efforts aimed at elucidating GPCR structure. Many GPCRs can now be expressed and purified in milligram quantities, so quantity no longer seems to be a major impediment to determining structure. A major barrier in crystallography involves stabilizing a particular conformation of the protein that enables the growth of diffractable crystals. For example, a key feature in the crystallization of rhodopsin was the presence of 11-*cis*-retinal in the ligand-binding site, which stabilized the inactive conformation of the receptor. Light activation destroyed the crystal[59]. Similar strategies need to be used for other GPCRs. In addition, co-crystallization of GPCRs with additional proteins such as G proteins, GRKs, arrestins and antibodies holds significant promise in helping to stabilize receptor conformation. However, although these efforts should eventually prove successful, it is difficult to predict a time frame for such success.

Susan R. George. The limitations of receptor crystallography are well known, such as the difficulty of obtaining large quantities of receptor and so on. However, the bigger limitation is deciphering whether the structure visualized in the crystal has maintained a physiological conformation and/or whether any relationship to neighbouring receptors has been maintained. There will be enormous interest in analysing a GPCR structure in the various stages of activation; that is, in the conformations stabilized with neutral antagonist, inverse agonist and full agonist.

Brian Kobilka. It is likely that high-resolution crystal structures of one or a few of the more tractable GPCRs will be available within the next one to two years; however, a general solution for generating high-quality GPCR crystals will probably take more time. Recent success with ion channels is encouraging[70–72]; however, GPCRs might prove more difficult targets. To date, ion-channel structures have been obtained from prokaryotic proteins that have remarkable thermal stability. By contrast, there are no prokaryotic homologues of GPCRs. So, what are the major problems to be solved? Obtaining sufficient quantities of protein is no longer the major impediment. Although production and purification remain technically challenging and expensive, it is now possible to prepare milligram quantities of several GPCRs using different expression systems[73–76]. Moreover, the use of robotic systems that are capable of setting up trials with 20–100 nanolitres of protein solution greatly extends the number of parameters that can be examined with a limited supply of protein.

I believe that the most significant obstacles to obtaining high-quality crystals are protein heterogeneity, protein stability and the dynamic nature of GPCRs, which are flexible molecules that are dependent on a lipid environment for structural integrity and stability. Moreover, it is possible that the structure and function of some GPCRs might be different in different lipid environments. Most GPCRs retain function in only a limited number of detergents, which typically have a relatively low critical micelle concentration and a relatively large micelle size. These properties are not ideal for crystal formation.

Some of these problems might be resolved by the development of new detergents, the addition of lipids to detergent micelles, or possibly by the use of lipid matrices for crystal formation. Heterogeneity of GPCRs, which can be attributed to incomplete post-translational modifications such as glycosylation, fatty acylation and phosphorylation, will interfere with crystal formation or limit crystal quality. This heterogeneity might be an artefact of overexpression. Viral overexpression of GPCRs might overwhelm the enzymes responsible for post-translational processing, as well as the protein-folding and editing machinery. Consequently, the expressed protein is a mixture of functional and non-functional protein, and even the functional protein might be heterogeneous with respect to glycosylation, fatty acylation and phosphorylation.

Despite the apparent unsuitability of GPCRs for crystallographic analysis, not all GPCRs are created equal, and it is likely that screening a large number of GPCRs from different species will enable the detection of those that are expressed at high levels, are more efficiently processed and are biochemically stable. Other approaches include generating fusion proteins to enhance expression and stability, and to increase the amount of hydrophilic surface area. Functional fusions with G proteins, arrestins and other proteins have been described[73,77,78]. Complexes of GPCRs with G proteins, arrestins or conformationally specific antibodies might also increase the likelihood of crystal formation.

Table 1 | **Classification and functional properties of Gα subunits***

Family‡	Isoform	Gene	Expression	Effectors	PTX/CTX sensitivity
G_s	$G\alpha_{s(s),s(l)}$§	*GNAS*	Ubiquitous	AC ↑	CTX
	$G\alpha_{s(xl),s(xxl)}$§	*GNAS*	Neuronal, neuroendocrine	AC ↑	CTX
	$G\alpha_{olf}$	*GNAL*	Olfactory epithelium, brain, testes, pancreas	AC ↑	CTX
G_i	$G\alpha_{t(r)}$	*GNAT1*	Retinal rods, taste cells	cGMP-PDE ↑	CTX, PTX
	$G\alpha_{t(c)}$	*GNAT2*	Retinal cones	cGMP-PDE ↑	CTX, PTX
	$G\alpha_{gust}$	*GNAT3*	Taste cells, GI brush cells	PDE ↑?	CTX, PTX
	$G\alpha_{i1}$	*GNAI1*	Widely, preferentially neuronal	ACI,V,VI ↓, (GIRK ↑, PLCβ ↑?)‖	PTX, (CTX)
	$G\alpha_{i2}$	*GNAI2*	Ubiquitous	ACI,V,VI ↓, (GIRK ↑, PLCβ ↑, PI3K ↑)‖	PTX, (CTX)
	$G\alpha_{i3}$	*GNAI3*	Widely, preferentially non-neuronal	ACI,V,VI ↓, (GIRK ↑, PLCβ ↑, PI3K ↑?)‖	PTX, (CTX)
	$G\alpha_{o1,o2,o3}$¶	*GNAO*	Neuronal, neuroendocrine, cardiac myocytes?	AC ↓?, VDCC ↓‖, GIRK ↑‖, PLCβ ↑?‖	PTX, (CTX)
	$G\alpha_z$	*GNAZ*	Neuronal, endocrine, platelets	ACI,V ↓, (GIRK ↑, VDCC ↓)‖	
G_q	$G\alpha_q$	*GNAQ*	Ubiquitous	PLCβ ↑, Rho-GEF	
	$G\alpha_{11}$	*GNA11*	Widely, not platelets	PLCβ ↑, Rho-GEF	
	$G\alpha_{14}$	*GNA14*	Testes, haematopoetic cells and tissues	PLCβ ↑	
	$G\alpha_{15/16}$#	*GNA15*	Haematopoietic cells and tissues	PLCβ ↑	
G_{12}	$G\alpha_{12}$	*GNA12*	Ubiquitous	Rho-GEF ↑, Btk ↑, Gap1m ↑, cadherin	
	$G\alpha_{13}$	*GNA13*	Ubiquitous	Rho-GEF ↑, radixin	

*For selected reviews, see REFS 204–224. ‡Heterotrimeric G proteins are composed of three different subunits, and are classified into four subfamilies on the basis of their Gα amino-acid-sequence similarity. §(s) and (l) indicate short and long splice variants of $G\alpha_s$; (xl) and (xxl) indicate additional (extra)-long splice variants of $G\alpha_s$. Receptor coupling of $G\alpha_{s(xl),s(xxl)}$ has not been demonstrated so far. ‖Regulation of the effector presumably depends on direct interaction with Gβγ dimers released from a PTX-sensitive heterotrimeric G protein. For additional effectors, see TABLE 3. ¶$G\alpha_{o3}$ corresponds to deamidated $G\alpha_{o1}$ (^{346}Asn→Asp), representing 30% of total G_o in brain. #$G\alpha_{15}$ and $G\alpha_{16}$ are the mouse and human homologues of the *GNA15* gene product, respectively. ↑ = stimulation; ↓ = inhibition; AC, adenylyl cyclase; Btk, Bruton's tyrosine kinase; cGMP-PDE, cGMP-dependent phosphodiesterase; CTX, cholera-toxin-sensitive; (CTX), CTX-sensitive only in the presence of agonist-activated receptors; Gap1m, Ras GTPase-activating protein; GI, gastrointestinal; GIRK, G-protein-regulated inward rectifier K^+ channel; PI3K, phosphatidylinositol 3-kinase; PLCβ, phospholipase Cβ; PTX, pertussis-toxin-sensitive; Rho-GEF, guanine-nucleotide-exchange factor of the Rho GTPase; VDCC, voltage-dependent Ca^{2+} channel.
Table prepared by Bernd Nürnberg, Institut für Biochemie und Molekularbiologie II, Klinikum der Heinrich-Heine-Universität Düsseldorf, Germany. e-mail: bernd.nuernberg@uni-duesseldorf.de.

Jean-Philippe Pin. Some new structures should come out in the next few years. The main problem is the production of sufficient quantities of pure and correctly folded GPCRs. Clearly, the solution of the structure of rhodopsin[59] demonstrated that this is possible and, as such, stimulated many labs to move in this direction. This increases the chance of success. Although a crystal structure usually constitutes a breakthrough, other biophysical methods will also provide structural information about GPCRs. For example, recent developments in NMR technology[79] suggest that the determination of small membrane proteins should be feasible using this technique, and possibly provide information complementary to X-ray crystallography. Small-angle neutron scattering can provide important structural information on protein complexes in which GPCRs are involved, such as the mean radius and the molecular mass of the complex. This recently allowed the demonstration that a dimeric GPCR is associated with a single heterotrimeric G protein[34].

Roland Seifert. I think that we still have a long way to go to get novel GPCR structures. I have discussed several approaches with researchers working in the area — for example, using GPCR–Gα fusion proteins and non-glycosylated receptors — but it just seems to be very hard to grow the crystals. There may be many reasons for the failure. Perhaps one has to use the right ligand, a specific G-protein heterotrimer, a specific detergent, a specific salt and so on. Also, low constitutive activity may be important to prevent denaturation of the receptor during procedures (we know that some GPCRs with high constitutive activity also exhibit high structural instability; however, this does not seem to be a general rule)[36]. Unfortunately, many popular GPCRs used for structural studies do have appreciable constitutive activity[36].

Bernard P. Roques. We are probably not that close! The main barriers to obtaining the structure of a GPCR at high resolution are, first, difficulties in purifying the protein in large quantities in an active state; and second, difficulties in crystallizing such flexible and unstable molecules, even in the presence of lipids. A solution could come from the development of an appropriate lipophilic, somewhat rigid, 'crown', which could stabilize the GPCR in a monomeric or dimeric form.

The circumference of the packaged seven transmembrane domains of many GPCRs is known. The amino-terminal (N-terminal) domains of several of these GPCRs can be deleted without adversely changing their properties. Therefore, two semi-circles of identical, semi-rigid fatty-acid chains corresponding to the length

of the membrane bilayer could be designed by linking at each extremity of the chain an oligomeric peptide sequence of appropriate size, consisting of cysteine or serine residues and reduced amide bonds. The two semi-circles could enclose the seven transmembrane domain of the GPCR preferentially bound with a ligand to reduce the mobility. The two semi-circles could be equipped with fluorescent probes for FRET between these two species or between these molecules and the receptor labelled by a cysteine-selective fluorescent probe, such as IANBD[80]. Reactive groups that allow the two semi-circles to close could also be introduced into the reduced polypeptide chain.

Thomas P. Sakmar. I hesitate to make a prediction about when we will have any additional structures. Some of the barriers are technical, but most are intrinsic to the biology of the receptors themselves. There is no true prokaryotic homologue of a eukaryotic GPCR, and heterologous high-level expression of GPCRs has, in general, not provided enough homogeneous material to allow crystallization trials. Even in the case of one recent example of a membrane protein crystal structure — bacterial lac permease[81] — literally hundreds of grams of protein were prepared over many years before crystals were obtained, and even then only a mutant locked into a particular conformation, not the native transporter, formed crystals. Rhodopsin is unique among GPCRs in that it can be isolated from retinae in large quantities and is locked into its inactive state by a covalently bound inverse agonist, 11-*cis*-retinal[59].

7

What big questions remain for GPCRs at the structure/function interface?

Tamas Bartfai. We do not have a detailed dynamic picture of agonist binding, of dimerization or of G-protein interactions. In short, most of the field remains open.

Thomas P. Sakmar. What does the active conformation of a GPCR look like? Is there more than one active conformation for a given receptor? How is G-protein specificity/selectivity achieved? How does the receptor catalyse nucleotide exchange in a heterotrimeric G protein? What is the structural basis for allosteric regulation of receptor function?

Joël Bockaert. The big challenges are to obtain structures of active conformations of GPCRs, and to obtain a crystal of an active GPCR in the presence of a G protein. Ideas about the correct interaction between GPCRs and G proteins, and the mechanism of activation of the latter, are still largely speculative.

Brian Kobilka. A crystal structure will provide a high-resolution snapshot of a single receptor conformation, most likely in an inactive state. Of equal interest and importance for understanding how these proteins work are the dynamic aspects of the receptor. GPCRs are conformationally complex molecules. Evidence for ligand-specific conformational states comes from observations that agonist potency for a given GPCR can be influenced by the G protein[45] or other effector system[56] to which it is coupled. Moreover, biophysical studies provide direct evidence for ligand-specific conformational states[8], as well as the existence of intermediate conformational states for receptors activated by a single agonist[8,52,82]. There are a number of important questions to address, including what conformational changes are needed for activation? How do ligands change the conformation of the receptor? What is the role of the lipid environment on receptor structure and dynamics? How do cytosolic signalling molecules influence the structure and dynamics of GPCRs?

Michel Bouvier. We need to determine the molecular correlates of drug efficacy; that is, establish the links between specific conformational changes and efficacy profiles. We also need to understand the dynamics of formation of the protein complexes ('signalosomes') that control signalling selectivity and efficacy. Other challenges include determining the role of GPCR dimerization in receptor functions, establishing whether or not GPCR heterodimerization has general physiological implications, and unravelling the rules dictating heterodimerization specificity.

Philip G. Strange. One of the big questions to me is the organization of GPCR systems. Are they in the form of signalling complexes? What is the composition of such complexes and how does this affect selectivity? A simple but fundamental point concerns the ratio of receptor to G protein *in situ*. Gross measurements of receptor and G protein suggest an excess of G protein, but many experimental studies and models suggest an excess of receptor. Does this imply functional microdomains and, if so, how much restriction is there on GPCR activity?

Jean-Philippe Pin. A number of questions remain. First, we need to determine the structures corresponding to the various states of GPCRs, especially in the active state. Second, we need to understand how GPCRs interact with and activate heterotrimeric G proteins and, in that respect, the precise role of GPCR dimerization in G-protein coupling will have to be solved. Third, we need to gain a molecular understanding of the mechanism of action of allosteric modulators, as such compounds constitute promising leads for drug development (owing to higher subtype selectivity and maintenance of the kinetics of the response to natural ligands).

Lakshmi A. Devi. What are the conformations of the receptor in an agonist-occupied activated state, and how do they differ from the antagonist-occupied state (especially those antagonists that are structurally similar to agonists)? Is the conformation of the receptor the same when activated by a small molecule compared

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Olivier Civelli earned his undergraduate degree and Ph.D. from the Swiss Federal Institute of Technology, Zurich, Switzerland. His thesis research on the mechanism of translation was carried out at the Jacques Monod Institute, University of Paris VII, Paris, France. As a postdoctoral fellow, he joined Edward Herbert's laboratory at the University of Oregon, Eugene, Oregon, USA. There, he became interested in neuropeptide expression and cloned the rat prodynorphin precursor. He began his studies on the cloning of GPCRs as an assistant professor and founding member of the Vollum Institute in Portland, Oregon. He was the first to clone and describe the structure of a dopamine receptor, the D_2 receptor, and used that clone to unravel the diversity of the dopamine receptor system by discovering the D_4 and D_5 receptors. In 1992, he joined F. Hoffmann-La Roche, Basel, Switzerland, as Head of the Department of Biology, where he designed and applied a strategy that uses orphan GPCRs as targets to discover new neurotransmitters and neuropeptides. This allowed him to identify orphanin FQ/nociceptin as the first novel transmitter discovered using this strategy. In 1996, he returned to academia in the Department of Pharmacology at the University of California, Irvine, California, USA, where he holds the Eric L. and Lila D. Nelson Chair of Neuropharmacology. In this position, Civelli has continued his search for new transmitters and their receptors and discovered, in particular, the MCH1 and urotensin II receptors. His current interests are focused in two directions: the use of orphan GPCRs as tools to identify novel transmitters; and the search for the functional significance of the novel transmitters discovered using this approach.

with a large peptide (a 10–30-amino-acid peptide)? What is the mechanism of receptor activation? Is there a generalized mechanism for all GPCRs or mechanisms specific to families of GPCRs? What is the dimerization interface? Does this differ between homodimers and heterodimers? Is there a common 'dimerization' motif for different GPCRs?

Rob Leurs. Although we are starting to have some idea about the mechanism of activation of GPCRs, we still lack detailed information on a variety of structurally diverse GPCR members. For example, are the structural changes for all agonists similar on binding? (Probably not.) And what structural changes are induced by partial agonists, inverse agonists or allosteric modulators? Allosteric modulators are of particular interest, as many small-molecule inhibitors of peptide-activated GPCRs (especially those targeted by larger peptides) tend to act as allosteric modulators. In summary, what determines (positive or negative) efficacy? Of specific interest in this respect is also the issue of agonist-directed signalling; how do different agonists induce distinct conformational changes that lead to the differential activation of various signal transduction pathways? What is the structural basis of these phenomena? Which distinct conformational changes at the GPCR–G-protein interface are associated with the activation of different G-proteins?

For me, it is not yet clear how GPCR pharmacology (in general) is determined by GPCR (hetero)dimerization. It is clear that (hetero)dimerization occurs in many cases, but there are not many examples of a major impact on GPCR pharmacology, although some good papers on $GABA_B$ and opioid receptors[83,84] indicate that the changes can be very significant. In this area, it would be of interest to learn how specificity in GPCR heterodimerization is determined, and if ligands regulate the extent of dimerization.

Rémi Quirion. (Please also see my answer to question 16, page 617.) The big questions remaining are what leads to an agonist-like effect versus an antagonist-like effect, and how to promote receptor–G-protein coupling. We do not understand enough about the three-dimensional and active conformations of neuropeptides and chemokines. Truly useful structural information is still scarce, when we consider, for example, the gaps in our knowledge of most peptide-activated receptors. The complexity of interactions when dealing with a peptide as a ligand compared with a small-molecule ligand is the major issue, and this has a major impact on GPCR-based drug development. Can we expect a 'one model fits all' situation (as for β-adrenoceptors or rhodopsin), or will we have to painstakingly work our way through the various subfamilies of GPCRs? I fear that the second outcome is more likely.

Roland Seifert. We have no idea about the specific conformational changes going on in the intracellular domains of receptors following agonist-dependent or -independent activation and how this affects the G protein. We find complexity at any given structure/function interface. First, there is evidence to support the notion that receptors are not only simple on/off switches for G proteins, but rather can modulate their activity in an agonist-dependent manner[31,37]. Second, the pharmacological profile of specific GPCRs may depend on the identity of the G protein to which the receptor is coupled[85]. Third, there is evidence for multiple active conformations of a given G protein, which depend on the purine or pyrimidine nucleotide to which it is bound[37,86].

Will we ever be able to generate all possible crystal permutations of a given receptor bound to various ligands, and coupled to various G proteins bound to different nucleotides, to understand this amazing complexity? I guess that nature will keep its secret of how to fine-tune signal transduction for many more years. However, I believe that elucidating these delicate details is not only an academic exercise but will help us understand the pathophysiology of many diseases and develop GPCR ligands with greater therapeutic efficacy and fewer side effects.

Bernard P. Roques. Is the function(s) of a given GPCR modified by its membrane environment? For example, does the activation of a dopamine D_2 receptor in the cortex lead to identical intracellular signalling to its activation in the ventral tegmental area? Are the completely different pharmacological responses produced by two ligands endowed with similar high affinity and selectivity for a given receptor due to various types of oligomerization of the target, or a result of the chemical

composition of the plasma membrane in a particular structure? Is the heterodimerization of μ-opioid receptors and CCK_2 receptors responsible for the high potentiation of analgesia produced by endogenous or exogenous opioid-receptor agonists and CCK_2-receptor antagonists? The investigation of such a hypothesis could open the way for specific stimulation by appropriate ligands (for example, CCK_{2A} or CCK_{2B})[87]. It is highly likely that other proteins interact with the intracellular domains of GPCRs. How could they be characterized? Could multiple hybridization techniques be used? What could be the roles of such proteins?

Ronald E. Stenkamp. The basic questions remain the same as they have been for some time. How do ligands bind to the receptor? How is a signal transmitted across the membrane? How do G proteins bind to the receptor? The rhodopsin crystal structure is a useful molecular model[59], but it has not provided the answers. The structure tells us little about the binding of non-retinal ligands, so this remains a crucial question, especially for drug designers. The rhodopsin structure places some limits on what the activation process is, but as it is only one of the relevant conformational states, it tells us little about the detailed structural changes connected with signal transmission. And finally, the intracellular surface of the rhodopsin structure is ill-defined, and is not even the conformation that binds G proteins, so it tells us little about the intermolecular interactions involved there. Thus, we are facing the same question that existed before the structure came out; that is, how do GPCRs work?

8

How widespread do you think GPCR heteromerization will turn out to be, and how great a functional significance will it have?

Joël Bockaert. I personally think that all GPCRs will turn out to be dimers. Current evidence certainly indicates that a GPCR dimer interacts with only one trimeric G protein, and that in some cases (such as for members of GPCR family 3), only one subunit of the dimer binds the ligand.

Jeffrey L. Benovic. GPCR heterodimerization seems to be widespread, and the potential functional significance is tremendous (FIG. 5; page 616). There are now examples of GPCR heterodimerization resulting in changes in ligand-binding specificity, trafficking and signalling. Although this provides another level of complexity in understanding the biology of GPCRs, it also provides another potential means of regulating GPCR function.

Ronald E. Stenkamp. I suspect heteromerization will be a major issue for most biological systems involving GPCRs. Molecular models of GPCRs will be essential for thinking about the chemistry of these systems, but the control, feedback and interlocked systems found in biology will be based on complexities such as the formation of heteromers.

Lakshmi A. Devi. From the studies carried out so far (which have mainly been in heterologous systems), it seems that GPCR dimerization is a fairly universal phenomenon. There is accumulating evidence that a GPCR dimer couples to a single heterotrimeric G protein, and that dimerization is a prerequisite for G-protein activation, at least in the case of the leukotriene B_4 receptor[76]. By contrast, a limited amount of information is available on the extent of heterodimerization in endogenous tissue. It seems that in the majority of cases there is a subtle change in response as a result of heterodimerization. Thus, the functional consequence will be limited to only those heterodimers that show robust changes in receptor function. We need a better understanding of novel pharmacology due to dimerization, including information regarding the selectivity of interaction and co-localization of two interacting GPCRs, and the extent of heterodimerization *in vivo*.

Michel Bouvier. The clear physiological significance of heterodimerization has now been obtained for family 3 GPCRs — for example, $GABA_B$[88] and taste receptors[89] — and family 2 GPCRs — for example, calcitonin-receptor-like receptor (CRLR) heterodimerization with RAMPs[90]. In these cases, the functional importance of heterodimerization was relatively easy to establish, as it was found to be obligatory for receptor ONTOGENY and function.

For the rhodopsin-like family 1 receptors, most evidence comes from heterologous expression studies, which show that, on co-transfection, receptor heterodimers can form and change the functional properties of the co-expressed receptors[40,41]. Depending on the receptor pairs considered, changes in the pharmacological, signalling and/or regulatory properties have been reported. One of the most striking examples is the change in the pharmacological properties of the δ- and μ-opioid receptors upon their heterodimerization. Indeed, co-expression of these two receptor subtypes led to a loss of binding of both δ- and μ-opioid-receptor-specific ligands when tested individually, although binding could be observed on simultaneous addition of the two classes of ligands, indicating a strong positive cooperativity[83]. Whether or not such phenomena mimic situations in native tissues naturally co-expressing different receptor subtypes remains to be determined.

Arguments in favour of a functionally important role for heterodimerization include the observation, in well-controlled studies, that heterodimerization is a selective process that allows the formation of only certain heterodimeric pairs at physiological expression levels.

For instance, heterodimerization between the closely related δ- and κ-opioid receptors was found to occur at lower expression levels than heterodimerization between either of the two opioid-receptor subtypes and the more distantly related β_2-adrenoceptors[91]. Quantitative studies also indicate that certain heterodimers can form with the same propensity as homodimers, as recently shown for the β_1- and β_2-adrenoceptors[92], as well as for the

Table 2 | **Diversity and expression of Gβ and Gγ subunits***

Isoform	Gene	Expression
Gβ subunits		
$G\beta_1$	*GNB1*	Ubiquitous
$G\beta_2$[‡]	*GNB2*	Ubiquitous
$G\beta_3$[§]	*GNB3*	Widely, cone cells, taste cells
$G\beta_4$	*GNB4*	Widely, brain, lung, placenta
$G\beta_5$[§‖]	*GNB5*	Neuronal, lung, germ cells, lymph, ovary
Gγ subunits		
$G\gamma_1$ ($G\gamma_{rod}$)[‡]	*GNGT1*	Retinal rods, neuronal
$G\gamma_2$ ($G\gamma_6$)	*GNG2*	Widely, neuronal
$G\gamma_3$	*GNG3*	Widely, neuronal, haematopoietic cells
$G\gamma_4$	*GNG4*	Widely
$G\gamma_5$	*GNG5*	Widely, placenta, liver
$G\gamma_7$	*GNG7*	Widely, brain, thymus, eye
$G\gamma_8$ ($G\gamma_{olf}$)	*GNG8*	Neuronal, olfactory epithelia
$G\gamma_9$ ($G\gamma_{cone}$,$G\gamma_{14}$)	*GNGT2*	Retinal cones, neuronal
$G\gamma_{10}$	*GNG10*	Widely, brain, placenta
$G\gamma_{11}$	*GNG11*	Widely, non-neuronal
$G\gamma_{12}$	*GNG12*	Ubiquitous
$G\gamma_{13}$	*GNG13*	Widely, lingual and olfactory epithelium, neuronal

*For selected reviews, see REFS 204–224. For Gβγ-regulated effectors, see TABLE 3. [‡]$G\beta_2$ cannot form dimers with $G\gamma_1$. [§]Splice variants exist. [‖]$G\beta_5$ forms dimers with certain regulators of G-protein signalling (RGS6,7,9,11); dimerization of $G\beta_5$ with Gγ has been observed following recombinant expression of $G\beta_5$ and Gγ.
Table prepared by Bernd Nürnberg, Institut für Biochemie und Molekularbiologie II, Klinikum der Heinrich-Heine-Universität Düsseldorf, Germany. e-mail: bernd.nuernberg@uni-duesseldorf.de.

vasopressin V_{1A} and V_2 receptors and the oxytocin receptors[93], suggesting that heterodimerization of some family 1 receptors could occur as readily as homodimerization in native tissues. Contrary to the often held view that heterodimerization is observed only in systems in which receptors are expressed at high, non-physiological levels, an increasing number of studies, including those cited above, are carried out at expression levels that resemble physiological conditions. Recently, heterodimerization between α_{1D}- and α_{1B}-adrenoceptors has been shown to play an important role in the cell-surface expression of the α_{1D} subtype[94], indicating that a role for heterodimerization in receptor ontogeny and cell-surface trafficking may not be limited to family 2 and 3, but could also apply to family 1 GPCRs.

Despite the increasing evidence suggesting the physiological significance of heterodimerization in cellular systems, data confirming its importance in living animals are still largely missing. Although difficult to do, such *in vivo* studies are of primary importance, as establishing the functional importance of heterodimerization would completely change the way we envisage pharmacological selectivity, and hence change our approach to drug development and screening.

Arthur Christopoulos. In receptor biology, protein oligomerization is generally the rule rather than the exception, so it is perhaps not surprising that GPCRs have been shown to oligomerize. From the work done so far, it seems that most GPCRs, especially those that are structurally related, have the potential to form dimers or higher-order oligomers, so the phenomenon is likely to be widespread. For the majority of cases, however, and in contrast to other receptor superfamilies, I am not convinced that GPCR oligomerization is ligand regulated and/or a requisite property for the physiological function of these receptors. There are important exceptions, such as the $GABA_B$ heterodimer[95–97], but these serve to highlight the work that still needs to be done to elucidate fully the role of oligomerization in GPCR pharmacology. Overall, I think that the greatest potential for drug discovery in this area will not be through the development of ligands that regulate the GPCR oligomerization process *per se*, but via targeting (constitutive) GPCR (hetero)oligomers that exhibit novel pharmacology compared with either receptor expressed alone.

Susan R. George. It seems that GPCRs homodimerize and form higher-order oligomeric structures. I believe that heteromerization is probably widespread, and has very important functional significance in specific instances, quite distinct from homodimerization[40]. However, we are at a very rudimentary stage of understanding these processes in native tissues. Some of the published studies in this area do not seem to have a firm rationale for throwing two GPCRs together. If two proteins never see each other in reality, they may not have evolved to stay apart when expressed together in a heterologous system. For instance, many receptor subtypes within a subfamily of the family 1 GPCRs might have high sequence similarity to each other, especially within the transmembrane domains, which contain the important sites that mediate the homodimerization of these receptors. Therefore, if subtypes that are never co-expressed in native cells are expressed together in heterologous systems, they may interact physically in a completely non-physiological manner, but the significance of this would be questionable. Thus, it is very important to demonstrate a functional consequence resulting from the GPCR heteromerization, and also a physiological basis for a presumed interaction, including an anatomic cellular co-localization. Moreover, even if two GPCRs do interact, it does not mean that the entire population of the two receptors in any cell that expresses both receptors are in a heteromeric form *in vivo*. We have some interesting preliminary data showing homogeneous and heteromeric populations of receptors within separate microdomains of the same neuronal cell[98]. However, we have insufficient knowledge at the present time regarding the kinetics and dynamics of these heteromeric interactions within the cell and on the cell surface, and whether they are constitutive, reversible, ligand-induced or cell-type specific.

Philip G. Strange. My current guess would be that all GPCRs will turn out to be oligomers, although the functional significance of this is very difficult to assess. There is increasing, but still limited, evidence that GPCRs are exhibiting the sort of behaviour seen in oligomeric enzymes, such as cooperativity in ligand binding. There has been, however, a paucity of evidence in functional

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Lakshmi A. Devi earned her B.Sc. and M.Sc. degrees from the University of Mysore, Karnataka, India, and her Ph.D. degree from the University of Windsor, Ontario, Canada. She did her postdoctoral training with Avram Goldstein at the Addiction Research Foundation, Palo Alto, California, USA, and with Edward Herbert at the Vollum Institute, Portland, Oregon. Devi joined the faculty of Pharmacology at New York University School of Medicine in 1987, and then the faculty of Mount Sinai School of Medicine, New York, in 2002 as Professor of Pharmacology and Biological Chemistry. With Bryen Jordan and Ivone Gomes, Devi discovered the role of opioid receptor dimerization in the modulation of function and showed that this molecular mechanism could, at least in part, account for the enhancement of morphine analgesia by δ-opioid receptor antagonists. Her group also showed that opioid receptors dimerize with other GPCRs, leading to changes in their signalling and trafficking properties. Devi's current research efforts are focused on exploring the consequences of GPCR interactions, molecular mechanisms of narcotic addiction and the regulation of neuropeptide levels by post-translational processing.

assays that the oligomers are important, but perhaps this reflects the view that prevailed until recently that GPCRs were monomers.

Linked to these ideas is the question of whether the models we are using for GPCR function are correct. The dominant model is the ternary complex model and its extensions, in which the receptor exists in several conformational states and agonism is achieved by stabilization of an agonist–receptor–G-protein ternary complex[99]. Some of the data from the work on oligomers, notably from Jim Wells's lab, suggest that the model may need to be modified to take account of the existence of oligomeric species of receptors and potential allosteric effects of G proteins (see, for example, REF. 100).

Jean-Philippe Pin. I believe that GPCR dimers constitute the functional unit of the receptor: one heptahelical domain interacting with the α-subunit of the G protein, and the other with βγ. As such, all GPCRs should be dimers. Although some receptors have been shown not to form homodimers, it is still possible that they can form heterodimers with other GPCRs expressed in the cells used in these studies. Such an example is the *N*-formyl-peptide receptor, as evidenced by the absence of dominant properties of receptor mutants[101]. For example, mutants of this receptor that do not internalize are unable to prevent internalization of the co-expressed wild-type receptor. Although clear examples of heterodimers have been reported (for example, the $GABA_B$ receptors[88] and the TAS1 receptors[89,102]), and although a number of possible heterodimers have been observed (including some *in vivo*[103]), it is quite difficult to estimate how widespread this is. I guess that it is likely to be more widespread than we thought a few years ago. However, we need to consider what we actually mean by a 'heteromer'. If one accepts the idea that the functional unit is a dimer, then I would be tempted to define a heteromer as a functional unit composed of two distinct subunits (like IONOTROPIC RECEPTORS). However, many of the data gathered in recent years favour the existence of oligomers of dimers and, as such, some of the described 'heteromers' may simply correspond to the association of two distinct homodimeric functional units. To illustrate this point, I would not call the observed association between a GPCR and an ionotropic receptor a heteromer (for example, the association of dopamine receptors with $GABA_A$ or *N*-methyl-D-aspartate (NMDA) receptors). Similarly, the described association of the adenosine and metabotropic glutamate (mGlu) receptors is unlikely to correspond to a heteromeric functional unit, as, according to our view of the activation mechanism of mGlu receptors, a dimeric mGlu receptor is required for glutamate action[104]. Such receptor associations could still have functional consequences (such as synergy between the responses generated by the associated receptors), but I would not call these heteromers.

Rick Neubig. My guess, and it is a complete guess, is that heterodimerization does occur, but will not be of great functional or pharmacological importance. By contrast, homodimerization may well be crucial for the mechanism of G-protein activation — as illustrated by the $GABA_B$ receptor[105].

Thomas P. Sakmar. A growing body of evidence, and probably an equal volume of speculation, suggests that GPCRs might exist and be active as oligomeric complexes, similar to some other cell-surface receptors. Because GPCRs are major pharmacological targets, their existence as homo- or heterodimers could have very important implications for the development and screening of drugs. Ali Salahpour and colleagues[106] argue that "although much has to be done, it is clear that the concept of GPCR dimerization challenges the current models describing GPCR function. The notion of GPCR dimerization should also have impact on the development of new therapeutic agents that act through this class of receptor. For instance, compounds that selectively interfere with GPCR dimerization might represent a new class of pharmacological agents that could regulate receptor functions". So, the idea of receptor–receptor interactions has not only resulted in an important revision of the traditional models of GPCR structure and function, but has also created interesting new ones.

However, it should be pointed out that methods used to study GPCR homo- and heterodimerization are problematic and prone to producing artefacts. As such, it might well be that some of what has been reported will not withstand the test of time. Although much effort has been concentrated on this topic in recent years, there are very few examples in which GPCR dimerization has been implicated definitively in receptor function. It is possible that GPCR dimerization affects non-G-protein-mediated functions of GPCRs, such as protein folding and transport to the cell surface, rather than the canonical G-protein mediated pathways.

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Susan R. George earned her M.D. from the University of Toronto, Toronto, Canada, and completed her residency in Internal Medicine at Toronto General Hospital followed by sub-specialty training in Endocrinology. She was a Fellow of the Medical Research Council (MRC) of Canada during her postdoctoral training with Philip Seeman at the University of Toronto, and was subsequently appointed Assistant Professor and MRC Scholar, and then Professor, in the Faculty of Medicine and Pharmacology. George's interest in the molecular pharmacology of neurotransmitter receptors, and their role in drug addiction and other diseases of the central nervous system, led her to accept a Senior Scientist post at the Centre for Addiction and Mental Health in Toronto in 1996, where she was appointed Section Head in 2000. In 2002, she received the Novartis Senior Scientist Award, and was also awarded the Canada Research Chair in Molecular Neuroscience. George is interested in the biology of the GPCRs through which neurotransmitters such as dopamine, serotonin and opioids function. Her research is focused on the regulation of receptor function, receptor homo- and hetero-oligomerization, second-messenger coupling, structure–activity relationships and distribution in the brain of the receptors and the mRNAs encoding them. Her lab uses receptor-gene-deleted animal models to define the specific functions of the closely related receptor subtypes, particularly the receptor systems contributing to vulnerability to substance abuse and neuropsychiatric disease. The lab is also searching for novel human receptor genes that are expressed in the brain, and is using knowledge of structural conformations to devise screening strategies for identifying novel compounds to target these receptors pharmacologically.

In classical models of GPCR signal transduction, signal amplification is generally thought to occur only at the level of the G protein or the effector, and not at the receptor level. Oligomerization could provide a means of signal amplification through the activation of many receptors by a single ligand. Oligomerization may be an early event during GPCR transport, as shown by the observation that the intracellular retention of receptor complexes occurs when truncated mutants of GPCRs are co-expressed with the wild-type receptor[40,93]. GPCRs interact and associate with a large number and wide range of proteins, including other types of receptor, ion channel and chaperone protein. Hetero-oligomerization has been invoked to explain the crosstalk that has been observed between two receptor systems, and is usually associated with an increase in the diversity of receptor pharmacology and function.

Tamas Bartfai. So far, heteromerization does not seem to be that important for acute signalling, but it may turn out to be important in desensitization, tolerance and regulation of receptor biosynthesis. Of course, enzymology is full of examples of heterodimers in which one subunit is regulatory and the other is catalytic, and we may see a similar division of labour for GPCRs, which would contribute to even greater promiscuity in terms of which receptor activates which G protein.

Bernard P. Roques. Heterodimerization has been demonstrated, mainly using *in vitro* techniques, using different cells for different types and subtypes of receptor[107]. It seems that this phenomenon, which is probably essential for receptor trafficking and recycling, could have interesting pharmacological consequences. For example, in rats, tolerance to morphine, which is linked to the very low internalization of the occupied μ-opioid receptor, disappears after administration of a peptidic μ-opioid-receptor agonist[103]. This suggests that homodimerization could be induced when the same GPCR is occupied by two chemically different ligands. Nevertheless, the functional significance of heterodimerization could come from genomic and proteomic approaches demonstrating that, in a given tissue, the expression of GPCR receptors that are expected to heterodimerize occurs at the same level and at the same time.

Roland Seifert. From my personal experience with several combinations of chemoattractant and biogenic amine receptors expressed in Sf9 insect cells, I would say forget heterodimerization. Perhaps we have worked with the wrong receptors, the wrong expression system or inappropriate methodology. Cautiously, I would conclude that heterodimerization is not a general property of GPCRs and is not universally observed.

We have observed homodimerization of certain human formyl-peptide-receptor isoforms[108] and the human histamine H_2 receptor[27] expressed in Sf9 insect cells. In the formyl-peptide receptor, a single amino acid in the extreme C-terminus is crucial for homodimer formation, and dimer formation seems to reduce the constitutive activity of the receptor. By contrast, we have no information about what the homodimerization of the H_2 receptor could imply functionally.

Personally, I would be most interested to answer the question of whether GPCR homo- and/or heterodimerization has an impact on the pharmacological properties of a receptor in terms of changing the order of potency and efficacies of a series of ligands, but, so far, I have not been able to set up a system to address this important issue in an unambiguous manner.

Rémi Quirion. Heterodimerization is most probably a rather generalized phenomenon (after all, these receptors are anchored and interact with various plasma membrane receptors). However, I am much less convinced about its eventual relevance for disease states and drug development. Moreover, although very interesting academically, current studies on GPCR heteromerization are possibly artefacts produced in artificial models by overexpression of various GPCRs. Basically, the issue here is that most data on homo- and heterodimerization are derived from transfected cell models, in which the receptor(s) under study is markedly overexpressed compared with normal and even pathological conditions. The level of receptor expression can be thousands of times greater than that seen in normal cells, leading to artificial protein–protein interactions, signal transduction and so on. *In vivo* studies are thus urgently needed to truly establish the functional significance of receptor heterodimerization.

9

Almost two-thirds of drugs on the market are thought to interact with GPCRs, by far the largest family of targets, and yet new drugs targeting GPCRs are few and far between. Why?

Joël Bockaert. I believe this is because what is easy and obvious is generally done first. The screening for lead compounds is done on a cellular basis, and *in vivo* studies are generally done too late. Pharmaceutical companies seem to be looking at the same targets for the same applications.

Richard A. Bond. I think the main answer to this comes from radical changes to the way in which drug discovery is performed. If you change methods and get a different outcome, then OCCAM'S RAZOR says it was the change in methods that produced the different outcome. The heavy reliance on cell-based and reconstituted systems in identifying ligands has not led to increased numbers of compounds in the clinic. This has been the topic of numerous articles, one of the more recent being David Horrobin's article published in *Nature Reviews Drug Discovery*[109]. Although I am not nearly as negative as Horrobin about current drug discovery techniques being little more than an intellectual puzzle-solving exercise, I do think he has a point. The point is, I believe, that the timescale required for current strategies to yield results extends beyond the foreseeable future. It may well be that the reliance on faster, higher-throughput methods will one day be accurately translated into success in the clinic; likewise for all the money invested in genomics and proteomics. The 'experiment' of relying on molecular biology and engineered systems is now at least 20 years old and correlates with the drought in new drug discovery quite well. I believe there is nothing 'wrong' in continuing with this method, provided shareholders are alerted to the fact that the company is taking a much longer route to obtaining successful drugs.

The one reason often cited for the diminished output of new ligands that really irks me is "all the low-hanging fruit is gone". This is absolute rubbish in my opinion. We do not know how a single cell operates, and yet we have the arrogance to state that all the best answers have been discovered. This is sheer nonsense. I am convinced that some of the absolute best antihypertensives, antidepressants, hypoglycaemics and so on are still to be discovered. Maybe it seems that "all the low-hanging fruit is gone" because we have dug ourselves a rut as a result of many of us taking the same path; and from this lower level, the new fruit are now higher than our reach. How else do we wind up with dozens of selective serotonin-reuptake inhibitors, beta-blockers, angiotensin-receptor blockers and so on?

In summary, I think that if we want different results, then we simply need to do things differently — both from what we do now and from each other.

Michel Bouvier. This is obviously a complex question to which one can only offer tentative answers. First, it must be recognized that developing drugs is a difficult endeavour, for which the chances of failure greatly exceed those of success, even in the best of situations. That said, I think that the low success rate may result in part from a lack of innovative and original approaches to drug discovery. This may seem paradoxical at a time when most drug companies have integrated sophisticated and costly genomic and proteomic platforms into their drug discovery efforts. Unfortunately, however, most major drug companies do exactly the same things. Not only do they focus largely on the same targets, but they use the same validation and screening tools. Combined with the lack of chemical-scaffold diversity, this seriously limits the chances of success for the industry as a whole; and when success does occur, many come out with very similar molecules targeting the same receptors. Metaphorically speaking, it is as if everyone is looking under the same lamppost to find the key to the biological problems being considered, because they only feel comfortable in that well-lit environment.

A better integration between biology and drug discovery efforts would certainly lead to greater diversity in both target identification and screening methods. Contrary to what could be imagined, the choice of screening assays can have important consequences on the type of molecule that will be identified as a potential hit. Taking advantage of biological diversity, and taking into account the evolving concept of drug efficacy when selecting screening assays, would certainly increase the chances of identifying molecules with novel properties that might be easier to distinguish from existing therapeutic agents at the clinical-trial level. Introducing the complexity of biological systems into the drug discovery process would certainly go against the current efforts of many companies to streamline the process, and would represent a real challenge. Unfortunately (or maybe fortunately), drug discovery is not like manufacturing cars, and the assembly-line model may not be applicable.

Philip G. Strange. I do not believe that this is because GPCRs have not been looked at as targets. The orphan GPCRs that have been deorphanized have not yet been exhaustively examined. Perhaps this also reflects the sort of diseases that produce best-selling drugs. If you look at

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Akio Inui completed his M.D. and Ph.D. at Kobe University, Kobe, Japan. He was appointed Assistant Professor at the same university in 1984, and became Associate Professor in 1998. Between 1985 and 1989 he also served as Chief Physician of the Medical Ward at Kobe University Hospital. Since 2001, Inui has been Associate Professor in the Division of Diabetes, Digestive and Kidney Diseases in the Department of Clinical Molecular Medicine at Kobe University Graduate School of Medicine. He is recognized for his studies in elucidating the role of gut–brain peptides in the regulation of gastrointestinal motility, feeding and body weight, and the identification of the ghrelin–neuropeptide Y pathway from stomach to brain in the pathogenesis of obesity and cachexia. Inui received the Janssen Award of the American Gastrointestinal Association in 2004. He is Editor of *Peptides*, *Nutrition* and the *International Journal of Oncology*, and between 2000 and 2002 he also edited the *International Journal of Molecular Medicine*. His efforts are now focused on translational research on peptides, which bridges the gap between basic and clinical disciplines for better understanding and management of human body-weight regulation disorders, including obesity, cancer cachexia and eating disorders.

Table 3 | **Gβγ effectors***

Effector	Response to Gβγ
ACI	↓
ACII, IV‡, VII‡	↑
GIRK1–4	↑
N-, P/Q-, R-, T-type VDCC	↓
PLCβ1–3	↑
PLA_2	↑
PI3K-β§,-γ	↑
GRK2,3	↑
Raf-1	↑
$p140^{Ras\text{-}GEF}$	↑
P-Rex1‖	↑
Btk	↑
Tsk	↑
Phosducin and phosducin-like proteins	(–)¶

*For selected reviews, see REFS 204–224. For isoform diversity of Gβ and Gγ subtypes, see TABLE 2. ‡AC activity is superactivated by Gβγ only if coactivated by $G\alpha_s$. §Stimulation has been demonstrated under *in vitro* conditions only. ‖P-Rex1, a guanine-nucleotide-exchange factor of the Rac GTPase, is synergistically activated by phosphatidylinositol-3,4,5-trisphosphate and Gβγ. ¶Phosducin and phosphoducin-like proteins regulate G-protein-mediated signalling by binding to Gβγ and removing the dimer from cell membranes.
↑ = stimulation; ↓ = inhibition; AC, adenylyl cyclase; Btk, Bruton's tyrosine kinase; GIRK, G-protein-regulated inward rectifier K^+ channel; GRK, G-protein-coupled-receptor-kinase; $p140^{Ras\text{-}GEF}$, guanine-nucleotide exchange factor of the Ras GTPase; PI3K, phosphatidylinositol 3-kinase; PLA_2, phospholipase A_2; PLCβ, phospholipase Cβ; Raf-1, member of the raf/mil subfamily of serine–threonine protein kinases; Tsk, interleukin-2 (IL-2)-inducible tyrosine kinase (Itk); VDCC, voltage-dependent Ca^{2+} channel.
Table prepared by Bernd Nürnberg, Institut für Biochemie und Molekularbiologie II, Klinikum der Heinrich-Heine-Universität Düsseldorf, Germany. e-mail: bernd.nuernberg@uni-duesseldorf.de.

the top-selling drugs, these are for ulcers, cholesterol lowering, blood pressure and depression; for these indications, GPCRs do not currently seem to be useful targets.

Susan R. George. There may be limitations in the combinatorial libraries available and the screening assays used. Along with new chemistries and new screening technologies, another fundamental problem that needs to be addressed is that of identifying the GPCR subtypes that are involved in particular pathophysiological processes.

Lakshmi A. Devi. The majority of GPCR ligands that are therapeutics were developed on the basis of their functional activity in endogenous systems. Generally, plant or fungal preparations with activities that affected specific targets and modulated function were first identified. This led to the identification of active ingredients, and the subsequent development of drugs. The proteins activated by these compounds were later identified as GPCR targets. Thus, in this strategy, the target (disease) and compound (drug) were identified first. By contrast, current strategies are generally focused on the identification of selective ligands for GPCRs in a heterologous system, and are therefore further removed from the disease indication (and thus the target in an endogenous system).

Jeffrey L. Benovic. The difficulty in identifying new drugs that target GPCRs partially reflects the inherent complexity of GPCR signalling, and the difficulty of identifying a link between a particular GPCR and a disease. The disease aspect adds a particularly difficult dimension, as most diseases rarely involve dysfunction of a single gene product. We can also add to this the difficulty of identifying appropriate ligands to either activate or inactivate a target GPCR, and finally, the need to demonstrate that the identified ligands are beneficial in treating disease.

Roland Seifert. We need to learn more about the physiological and pathophysiological roles of receptors. Just remember how our concepts about targeting β-adrenoceptors in heart failure have changed during the past few years. Specifically, about two decades ago, we thought that β-adrenoceptor antagonists were deleterious in heart failure, and the suggestion of treating heart failure patients with these drugs would have failed you in a pharmacology exam. Then, the paradigm slowly changed. Not only was it shown that β-adrenoceptor agonists are deleterious in heart failure, but it also emerged that β-adrenoceptor antagonists actually increase life expectancy and life quality in heart failure patients[110]. In this specific case, the clinical pharmacology is actually supported nicely by studies with transgenic animals[111].

Olivier Civelli. The fact that many drugs target GPCRs can be rooted to the fact that GPCRs bind naturally small ligands that lend themselves to friendly chemistry. Serendipity most often led to the discovery of a particular drug that much later was found to be GPCR-related. However, due to their simple structures, these initial drugs permitted the synthesis of numerous chemical variants that, over a few decades, saturated the original indication or spread across to related indications. In the majority of cases, however, the molecular targets of these drugs were not characterized. Drug discovery took a new turn with the cloning of GPCRs, which only started at the end of the 1980s. As it takes more than a decade to bring a drug to the market, I believe that we have not yet seen the full impact that GPCR molecular biology will have on the development of novel drugs. Another outcome of the application of molecular biology to the GPCR field has been the discovery of numerous, often unexpected, receptor subtypes. This has delayed drug discovery programmes, as they faced questions of drug specificity — an issue that does not have such a strong effect on non-GPCR drug discovery programmes. GPCR-related drug discovery may look like it is lagging behind, but I believe this is only temporary.

Thomas P. Sakmar. Most of the existing GPCR drugs target a relatively small number of well-characterized GPCRs, mainly biogenic amine receptors, and these drugs are very often structurally related to physiological

ligands that essentially served as lead compounds for drug development. It is much more difficult to find a specific small-molecule drug for 'new' GPCR targets that have protein or peptide natural ligands. With a relatively small number of lead compounds from which to start, the even bigger problems of pharmacology, bioavailability and toxicology become enormous. Many of the remaining GPCR drug targets are peptide-activated receptors located in the central nervous system (CNS), and significant work has been concentrated on finding non-peptide mimics. But most non-peptide antagonists or agonists have been very toxic. Peptide analogues of the native ligands should make less toxic and more effective drugs. However, the bioavailability of potential peptide drugs, the cost-effectiveness of manufacturing and marketing peptides, and peptide drug delivery methods all have to be worked out. For example, the half-life of a peptide hormone in the gut is about 2–3 minutes. Therefore, protease-sensitive peptide bonds need to be modified to increase drug bioavailability. One successful peptide drug, octreotide (Sandostatin; Novartis), which is targeted to the somatostatin receptor, is used to treat malignant carcinoid syndrome and other endocrine conditions. Modifications to the native cyclic peptide structure increased the drug's half-life from 2 minutes to 3 hours[112].

Rob Leurs. In my opinion, it is important to note that many of the newer GPCRs that we are trying to target are peptide-activated receptors. Small ligands are less likely to act as competitive antagonists at these GPCRs, but rather will probably function as allosteric modulators. This has important consequences for screening strategies (for example, competition binding assays against radioactive peptide will not always readily yield high-affinity ligands). Often the physiology of the 'newer' (often deorphanized) GPCRs is not known in full detail, which might hinder the rapid development of selective ligands during later phases of the drug discovery process. Target validation using knockout mice and other techniques often gives hints of whether a drug is selective or not, but these are not fully predictive. Because of the lack of detailed structural information, structure-based drug design (rational and virtual) is not very well developed for GPCRs, in contrast to some other favourite drug classes, such as kinases, for which high-quality structural data are often available.

Tamas Bartfai. In most cases, it is the size of the endogenous ligand that causes problems; for example, large neuropeptides, such as calcitonin, PTH and glucagon-like peptide-1, or protein ligands, such as the follicle-stimulating hormone (FSH). These large ligands interact with their receptors at multiple sites to evoke agonist action and to reach the free energy of binding (9–12 kcal mol^{-1}, or K_d values that are nano- to picomolar). We have not been very successful in creating multiple interactive points in synthetic ligands, so it is hard to make synthetic-peptide receptor agonists that will cause the conformational changes in the receptor required for G-protein signalling. However, it is not an impossible feat — morphine and some other neuropeptide agonists do exist. The task becomes harder as the size of the endogenous ligand being mimicked increases. The difficulty in designing agonists is that we are not very good at mimicking protein–protein interactions. In the past, the focus was on antagonists, for which we have a good record. Site-directed mutagenesis and affinity labelling with antagonists show that they often bind to a small pocket between the transmembrane domains of the GPCR, thereby preventing the conformational change that agonist binding would cause. In other words, our GPCR antagonists are, for the most part, allosteric antagonists.

Akio Inui. It is noteworthy that the majority of GPCR-targeted drugs elicit their biological activity by selective agonism or antagonism of biogenic monoamine receptors, whereas the development status of compounds targeting peptide-activated GPCRs is still in its infancy. Automated combinatorial chemistry, multiple parallel synthesis and HTS have dramatically altered the process of finding leads, and are expected to facilitate the overall drug discovery process. The choice of a disease-relevant target is one of the most crucial, but also most difficult, steps in initiating a drug discovery project. To date, the REVERSE PHARMACOLOGY strategy has led to the identification of ~40 ligand–receptor pairings, with the pace of discovery accelerating in the past several years[113,114]. The identification of either its endogenous 'natural ligand' or a synthetic, surrogate ligand is a direct means of revealing the physiological function of an orphan GPCR. This ligand–receptor pairing can then be used to investigate the G-protein coupling and effector-regulating specificities of the receptor, and mutagenesis studies can be used to identify key amino-acid residues within the receptor that are involved in ligand binding. Such functional information and tissue-distribution data, coupled with knowledge of the physiological role of the endogenous ligand, can provide invaluable information regarding the potential role of a novel GPCR in disease. A major challenge for the pharmaceutical industry in identifying drugs of the future is to associate the many novel GPCRs with disease. Careful evaluation of the clinical relevance of lead compounds may be helpful for that.

Brian Kobilka. In addition to their interactions with receptors, compounds must have appropriate pharmacokinetic and toxicological properties, which limits the range of chemical structures that drugs can have. Another potential problem is that, owing to technological advances over the past two decades, the criteria for candidate drugs with respect to selectivity might have become too stringent. Lead compounds are typically screened against large panels of GPCRs to determine selectivity. Compounds found to interact with other GPCRs are often discarded before animal tests are done. In the past, companies did not have the full spectrum of receptors required to look for crossreactivity. In some cases, these older 'dirty drugs' might be effective because they act on more than one GPCR.

10

What are the barriers to achieving specificity using GPCR ligands?

Joël Bockaert. I do not see any theoretical barrier.

Bernard P. Roques. There is, theoretically, no barrier to achieving specificity for GPCR ligands, despite the similarities between GPCRs. For example, zinc metalloproteases, such as ACEs, endothelin-converting enzymes and matrix metalloproteases have very similar active sites, but this has not hindered the design of highly selective ligands[115]. The challenge with GPCRs is determining the clinically important state of the receptor on which to direct a ligand (for example, monomer, homodimer or heterodimer).

Rob Leurs. In general, there are no bigger hurdles for medicinal chemists in achieving ligand specificity for a given GPCR than for other multi-gene families. It has already been proven by currently marketed drugs that it is possible to find the right key for the right lock. The GPCR expression profile is an important factor for specificity of action of the ligand in the *in vivo* situation. With current techniques of expression profiling, one should be able to assess such risks early on in a drug development programme.

Tamas Bartfai. The barriers to achieving specificity and selectivity for GPCR-based drugs are no different to those for other drug-target classes. First, there is the issue of chemical specificity (interestingly, interactions with α-adrenoceptors and with human ether-a-go-go-related (hERG) channels have been much highlighted, but we do not understand why so many GPCR ligands interact with these channels, we just test for such interactions). Second, there is the problem of avoiding drug interactions at the level of cytochrome P450 isoenzymes and other metabolizing pathways.

Philip G. Strange. I do not work in industry, so it is easy for me to answer this question! The ingenuity of the medicinal chemist seems to be sufficient to overcome specificity problems if enough time is available. For example, when the D_2-like dopamine receptors were cloned (D_2, D_3 and D_4)[116], it proved fairly straightforward to find D_4-selective antagonists, although they have so far not been useful as drugs. D_3-selective antagonists were more difficult to synthesize, but they have come with time.

Thomas P. Sakmar. The majority of GPCRs belong to subfamilies of closely related receptors with similar small-molecule-binding pockets. Also, existing compound libraries consist of compounds that in many cases already interact with other targets. Furthermore, in most cases, small-molecule GPCR drugs are basic, lipophilic compounds, which tend to interact with ion channels — in particular, the hERG channel — leading to common cardiac side effects such as QT INTERVAL prolongation.

Susan R. George. One of the fundamental hurdles is achieving a greater understanding of receptor-subtype-specific functions and defining the structural and functional differences among the receptor subtypes.

Roland Seifert. If we knew the crystal structures of receptors, we could do much more *in silico* drug design rather than relying on brute-force HTS.

Jeffrey L. Benovic. GPCRs provide tremendous diversity in regulating many biological processes. However, most GPCRs are members of subfamilies (for example, there are nine adrenoceptors), which makes the task of identifying subtype-specific ligands very

Box 3 | Several models have been proposed for GPCR activation

In the simple binding and activation model of G-protein-coupled receptor (GPCR) activation (**a**), an agonist, A, binds to an inactive receptor, R_i, to form the complex AR_i, which then undergoes conformational change to the active state (AR_a; orange). However, several multi-state models have also been proposed[45,192], and an increasing amount of experimental evidence supports the relevance of these models.

In the simple ternary complex model (**b**), activation of the receptor is followed by topographically distinct binding of the active-state receptor to the G protein (G; light green); the resulting ternary AR_aG complex then mediates effector release. The extended version of this model[193] (**c**) includes a situation in which a receptor is activated independently of a ligand (R_a; turquoise), and can then interact with, and activate, a G protein (pink). Finally, the cubic ternary complex (CTC) model[194–196] (**d**) models the same concept but includes the formation of a non-signalling complex between the inactivate-state receptor and G protein (R_iG; red).

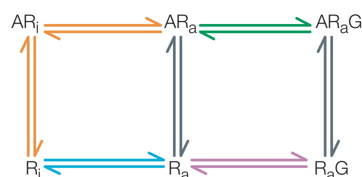
Numerous experimental data support the notion that model **d** is more appropriate than models **a–c**: non-signalling ternary complexes between GPCRs and G proteins have been observed[31]; for the β_2-adrenoceptor, dissociations between conformational changes observed at a molecular level and effector activation exist[80]; and the efficacies and potencies of ligands at a given GPCR vary depending on which G protein the GPCR is coupled to and which nucleotide is bound to a given G protein[37,85]. These experimental observations point to the existence of ligand-specific GPCR conformations that vary in their capacity to interact with, and activate, G proteins.

Although the experimental analysis of GPCRs becomes much more complex if one accepts model **d**, this model is very helpful in designing ligands that selectively activate defined signalling pathways instead of stimulating all signalling pathways that can be activated by a given GPCR. Box adapted, with permission, from REF. 192 © 2002 Macmillan Magazines Ltd, with contribution from Roland Seifert.

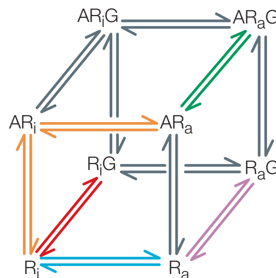
a Simple binding and activation

b Simple ternary complex model

c Extended ternary complex model



d Cubic ternary complex model



Ligand-mediated receptor activation
Receptor activation by allosteric modulation
Activation of G protein in absence of ligand
Activation of G protein in presence of ligand
Inactive receptor coupled with G protein

challenging. Once a target is identified, screening for target-specific ligands needs to include the target and target-related family members. Ultimately, ligand specificity will need to be further established *in vivo*.

Jean-Philippe Pin. The most promising highly selective drugs are those not interacting at the binding site for the natural ligand. Indeed, the natural binding site is under strong pressure during evolution to keep its ability to bind the natural ligand with the correct affinity. As such, closely related receptors have a similar ligand-binding site, making the identification of highly specific ligands difficult. However, recent studies have described the identification of allosteric ligands for GPCRs, which interact at a site distinct from the orthosteric site (where the natural ligand binds)[117,118]. Such allosteric sites have not been under specific selective pressure during evolution and, as such, are quite distinct between highly homologous receptors. They therefore constitute an excellent target for highly selective drugs. A good example is the recent description of highly selective subtype-specific allosteric regulators (positive or negative) for the class III (family 3) GPCRs, which can discriminate between $mGlu_1$ and $mGlu_5$ receptors[117], or between $mGlu_2$ and $mGlu_3$ receptors[119].

Akio Inui. A thorough examination of crossreactivity with other receptors and receptor subtypes is necessary to achieve specificity with GPCR ligands, but this may be difficult for some GPCRs, such as 5-HT receptors, of which there are approximately 20. GPCRs will have different functions in the body depending on the tissues in which they are expressed. For example, most of the feeding stimulatory peptides increase appetite when administered in the CNS, yet decrease appetite after peripheral administration. The drug delivery system is apparently a key component to achieving specificity, allowing the specific targeting of GPCRs in the CNS, periphery or target tissue.

11

How will our increasing knowledge of interacting protein partners for GPCRs change the way that we think about achieving selective GPCR modulation?

Olivier Civelli. The interacting proteins that link GPCRs to other signalling molecules inside the cytoplasm are mainly pleiotropic: they can interact with several GPCRs. As such, they will not offer an advantage for achieving selectivity, although they remain very useful in understanding the function of the system. Proteins that interact directly with GPCRs at the membrane, such as RAMPs, exhibit specificity. However, their use as targets to achieve specificity will prove challenging, as designing small molecules to facilitate or prevent topologically large protein–protein interactions (such as GPCR–RAMP interactions) is very difficult at present.

Roland Seifert. It will certainly render issues more complex. At the minimum, any reasonable drug development programme should take into consideration several G proteins to which a given GPCR can couple. Based on my experience, I do not believe that the efficacies of full agonists are that much affected by various G proteins, but the story is very different with partial agonists and inverse agonists[85]. It is really difficult to predict which specific receptor is affected by switching it from one G protein to another in terms of agonist potencies and efficacies. For example, the pharmacological properties of agonists of the β_2-adrenoceptor fused to $G\alpha_s$ splice variants differ considerably from each other[120], but this is not the case for the histamine H_2 receptor fused to $G\alpha_s$ splice variants[121]. Moreover, the formyl-peptide receptor fused to various G_i proteins exhibits very similar pharmacological properties[26]. To render issues even more complex, in contrast to the fusion protein system, $G\alpha_s$ splice variants do not have an impact on the constitutive activity of the β_2-adrenoceptor in a conventional co-expression system[122]. The latter differences highlight the importance of studying several experimental systems for a given receptor — but which system is the best?

With respect to regulatory proteins other than G proteins, I can say that we have no evidence for a specific effect of the RGS protein RGS4 and the $G\alpha$-interacting protein (GAIP, also known as RGS19) on the pharmacological properties of the histamine H_1 receptor[25]. Again, I would be cautious about extrapolating these observations to other GPCRs and RGS proteins.

Some years ago, we started a project to study the impact of various G-protein $\beta\gamma$-complexes ($\beta_x\gamma_y$) on the pharmacological properties of the β_2-adrenoceptor coupled to $G\alpha_s$ splice variants. However, we realized relatively quickly that the project would go beyond the capabilities of our group if we wanted to be systematic and comprehensive — that is, to study not only one full agonist but a complete series of partial agonists and inverse agonists — and we therefore abandoned it.

Thomas P. Sakmar. New therapeutic approaches will aim at selectively modulating the interaction of a GPCR with a particular subset of interaction partners. For example, a drug could induce receptor downregulation without stimulating receptor signalling. However, many downstream GPCR signal mediators, modulators and regulators, such as RGS proteins, are ubiquitous, so it might be difficult to target their activities for therapeutic effects in the absence of toxicity.

Brian Kobilka. These protein–protein interactions might determine the efficacy of a particular drug for a specific disease process. Developing high-throughput assays that account for these interactions could become a routine part of drug discovery.

Rémi Quirion. This is a fascinating research area that has yet to yield clear applications at the therapeutic level. Moreover, it is not clear whether proteins such as RAMPs and receptor protein components are uniquely associated with the calcitonin-gene-related peptide

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Brian Kobilka received his B.S. degree in Biology and Chemistry from the University of Minnesota, Deluth, USA, in 1977 and his M.D. degree from Yale University, New Haven, Connecticut, in 1981. He completed residency training in internal medicine at Barnes Hospital, Washington University School of Medicine, St Louis, Missouri, in 1984. From 1984–1989 he was a postdoctoral fellow in the laboratory of Robert Lefkowitz at Duke University, Durham, North Carolina. In 1990, he joined the Faculty of Medicine and Molecular and Cellular Physiology at Stanford University, California. Kobilka's research interest has focused on the structure and mechanism of action of GPCRs, with a focus on adrenoceptors as model systems. His lab uses a broad spectrum of approaches to study GPCRs, from using biochemical and biophysical tools to elucidate ligand-induced conformational changes, to using *in vitro* and *in vivo* systems to determine the structural basis of more complex functional properties that are observed only in differentiated cells. They have developed direct methods to monitor ligand-induced conformational changes in the purified β_2-adrenoceptor, and are attempting to obtain a high-resolution structure of this receptor. They have also developed strains of knockout mice for five adrenoceptor subtypes, which have provided insights into the physiological roles of these receptors *in vivo*.

(CGRP)/adrenomedullin receptor family, or whether they can also modulate biological responses to other GPCRs. My guess is that RAMP-like proteins may exist for other GPCRs, and not only peptide-activated GPCRs. It may be that drugs could be targeted to RAMPs instead of GPCRs *per se*. For example, in the case of the CGRP/adrenomedullin receptor family, the CRLR can function as either a CGRP receptor or an adrenomedullin receptor, depending on which members of the RAMP family are expressed (RAMP1 or RAMP2, respectively)[90]. This is a promising finding, but it is still early days in terms of leading to potential therapeutic opportunities.

Tamas Bartfai. So far, we have been able to synthesize selective, high-affinity GPCR ligands, such as antagonists to β-adrenoceptors, histamine H_1 and H_2 receptors, and D_2 dopamine receptors, which have been used to treat more than 100 million people for disorders such as hypertension, allergy, gastric-acid secretion and psychosis. We are far worse today at dealing with blocking protein–protein interactions that involve multiple interaction points distributed over a large surface, which we have not mimicked successfully synthetically, except for some rigid natural products with large surface areas, such as cyclosporine. Consequently, until we improve our ability to affect protein–protein interactions generally, we will prefer to deal with the ligand-binding aspects of GPCRs.

Another example is the function of the CRLR, which acts as a CGRP receptor in the presence of RAMP1, or as an adrenomedullin receptor in the presence of RAMP2 (REF. 123). These are heterodimeric receptors of two membrane-bound protein subunits. It is clear that blocking the interaction between the subunits would be one way to affect signalling through these receptors — it would provide a good alternative to using ligands that directly compete for the binding of CGRP. I do not expect these interactions, or the interactions between monomers of the homo- or heteromeric GPCRs, to provide easy or often-used pharmacological ways for acting on GPCRs in the near future. Of course, cell-based assays may pick up compounds that could target such protein–protein interactions, and therefore have the potential to be very instructive.

Jeffrey L. Benovic. Our increasing knowledge of interacting partners should have a tremendous impact on how we think about achieving selective modulation. A particular receptor may well interact with a dozen or so additional proteins, each of which may provide cell-type-specific physiological effects. For example, the prototypic β_2-adrenoceptor interacts with G proteins such as G_s and G_i; protein kinases such as protein kinase A, protein kinase C, GRKs and tyrosine kinases; scaffolding proteins such as A-kinase anchoring proteins, arrestins and NHERF; and additional proteins such as NSF and eukaryotic initiation factor-2Bα. Arrestins are particularly interesting as they interact with many GPCRs, their interaction is often driven by ligand binding, and they serve as scaffolds to mediate many processes, including receptor desensitization, trafficking and signalling. Thus, one could envision that identifying ways of regulating receptor–arrestin interactions might provide an additional avenue for modulating GPCR activity.

Joël Bockaert. It will allow us to fine-tune the action of GPCRs, particularly when splice variants do not differ in their binding sites. For example, there are two splice variants of the prostaglandin F2α receptor — FP2A and FP2B — that differ only in their C termini, but activate quite different signalling pathways[124]. To differentially modulate their action would therefore require modifying their interactions with intracellular partners.

Michel Bouvier. Protein–protein interactions have long been recognized as central determinants of cellular activity, as exemplified by the transcriptional and translational machineries. For GPCRs, however, the role of protein-complex formation involving more than just the receptor and its cognate G proteins was only recently uncovered (reviewed in REF. 125). We are still at the stage of identifying the various partners that can be engaged by the receptor, and we are only just getting the first glimpses into their potential roles in controlling signalling selectivity and efficacy. Much more work will be required to identify all the partners that can participate in the formation of specific signalling complexes (signalosomes) and, more importantly, to understand the dynamics that regulate their assembly/disassembly cycles. Undoubtedly, this will dramatically change the way that we approach signalling selectivity, and will hopefully provide new insights into how we can modulate GPCR signalling in a selective manner.

Arthur Christopoulos. This question is related to the GPCR oligomerization issue. Receptor-interacting proteins have the potential to define target phenotype. The best example is the family of RAMPs. The interaction of RAMP1 with CRLR yields the CGRP-receptor phenotype, and recently developed selective CGRP-receptor antagonists have been shown to act specifically at the RAMP–receptor interface[126]. In addition to forming complexes with GPCRs that help determine the physiological/pharmacological ligand-recognition characteristics of the receptor (receptor phenotype), another more widespread mode of GPCR modulation by interacting proteins is at the level of the signal transduction processes mediated by the GPCR[127]. In this latter instance, the interacting protein itself can become the therapeutic target, rather than its receptor partner. Overall, the area of interacting proteins has the potential to yield an enormous array of additional GPCR-related drug targets.

Rick Neubig. I think this will be a very important area for developing more subtle modulators of GPCR function. Interacting partners have several potential roles in GPCR signalling. They will directly mediate signalling outputs that may be independent of G proteins, enhance or inhibit the classic G-protein signals of a given receptor in particular tissues or subcellular locations, and modulate receptor expression. Unlike the G proteins themselves, these novel interacting partners will be tissue-specific and permit the development of GPCR modulators that have more selective actions than ligands that bind to the primary agonist/antagonist recognition site.

Jean-Philippe Pin. Recent findings indicate that such interacting proteins are not only involved in the desensitization process of GPCRs, but can have multiple effects, such as the determination of the precise subcellular localization of the receptor[128] and its specific association with effector proteins (enzymes producing second messengers, or ion channels)[129]. As such, interacting proteins can play a major role in the specific intracellular pathways activated by a receptor. Intracellular interacting proteins can even 'activate' GPCRs, as illustrated by the Homer1a protein, which unmasks constitutive activity of mGlu receptors[130]. Manipulating these interactions with drugs can therefore have several effects, depending on the cell type expressing a given receptor. However, interacting proteins are usually not specific for a given GPCR. This constitutes, in my opinion, a limitation for drug development in that area.

12

Could there be an approach to GPCR pharmacology based around monoclonal antibodies or proteins, rather than small molecules?

Roland Seifert. I doubt that this is going to be productive. Remember, most of the relevant receptor structure is buried in the membrane, so how are you going to target that? I am personally not too convinced about the quality of currently available GPCR antibodies. From a research perspective, tagging GPCRs N-terminally and C-terminally with epitopes that are detected by monoclonal antibodies is a great approach, but one always has to be concerned that tagging may somehow modify receptor function, at least slightly.

Rick Neubig. I think this is unlikely. Most GPCRs represent relatively good targets for small-molecule ligands. Given the huge advantages of small molecules in terms of ease of use, I would be surprised if there were many situations in which antibodies would be preferable.

Olivier Civelli. If we're thinking about therapies, then probably not. The GPCRs are naturally well suited to modulation by small-molecule surrogates. The difficulties in preparing and delivering large proteins mean that they might be useful only in very rare cases.

Bernard P. Roques. Small molecules are likely to remain the ligands of choice for the pharmacological study of GPCRs. They have all the properties required to move rapidly from the research laboratory to clinical trials, including the potential for large-scale synthesis, good bioavailability and low cost. A disadvantage of monoclonal antibodies is the uncertainty about issues such as the fate of internalized receptors, their pharmacological action, the dose–response, the duration of action and so on. However, they could be used in cases in which small ligands cannot be obtained, particularly for orphan GPCRs. Monoclonal antibodies could also be interesting as vectors to target drugs to tumours, which very often exhibit hyperexpression of GPCRs.

Joël Bockaert. I prefer small molecules. But in some cases, such as GPCR family 2, for which no companies have succeeded in finding ligands, antibodies may be a solution, albeit a difficult one.

Jeffrey L. Benovic. This is certainly a possibility. One could envision using antibodies or other proteins to target the extracellular surface of a GPCR and either regulate ligand binding, lock the receptor into an active or inactive conformation, or regulate receptor localization.

Tamas Bartfai. There have been observations of antibodies that bind to the extracellular domains of GPCRs and act as agonists, but the use of antibodies as therapeutic ligands for GPCRs will depend on the size of the ligand being replaced by the antibody. For example, if the PTH receptor (PTHR) could be targeted with a good monoclonal antibody that has PTH effects but ten times the half-life, then why not? However, as long as orally available small molecules can be developed as GPCR ligands, they will be preferred.

Thomas P. Sakmar. Yes. One recent example of a potential monoclonal antibody drug lead is based on the observation that certain monoclonal antibodies directed against CCR5 block HIV entry[131]. However,

monoclonal-antibody-derived drugs might not become first-line drugs because of their lack of oral bioavailability. In addition, from a theoretical point of view, there is no obvious advantage of monoclonal antibodies over small molecules, assuming that a small molecule with the correct properties can be found. Therefore, although it might be faster to find a monoclonal antibody to modulate the function of a specific GPCR in the short term, small molecules should prove superior in the long run.

Arthur Christopoulos. For many GPCRs, the extracellular loops tend to show less sequence similarity across subtypes than do the transmembrane domains, and autoantibodies directed against GPCRs in some disease states recognize specific epitopes within these loops. Thus, nature has already provided an additional means of achieving GPCR subtype selectivity, and I think there are now some good examples of how monoclonal antibodies can be used to target these receptor domains instead of small molecules. For example, Jean-Christophe Peter and colleagues[132] have recently shown how a functional monoclonal antibody against the human β_2-adrenoceptor was used to derive an scFv fragment that was specific both *in vitro* and *in vivo* for β_2-adrenoceptor GPCRs.

Lakshmi A. Devi. One could envision an approach using monoclonal antibodies or proteins rather than small molecules to study GPCR pharmacology, followed by their use as therapeutic agents. This would mainly involve the use of monoclonal antibodies that would stabilize the naive or agonist-mediated change in the conformation of the GPCR.

Mutagenesis and other related techniques are beginning to unravel the many conformational states of the peptide-activated receptors. However, relatively little is known about the change in conformation of the extracellular domain, and of the N-terminal tail in particular. Monoclonal antibodies provide a highly specific (GPCR-subtype selective) tool for the identification of agonist-mediated conformations of individual GPCRs. The development of monoclonal antibodies that bind to the extracellular region and stabilize the active conformation of the receptor would mimic a highly selective agonist; such an agent would serve as a receptor-subtype-selective agent. Monoclonal antibodies that stabilize the native conformation of the receptor have been found to function as inverse agonists[132].

Akio Inui. Monoclonal antibodies are already being used successfully in several fields, including cancer therapy, with much less toxic side effects than chemotherapeutic agents. For example, trastuzumab (Herceptin; Roche), which is a humanized monoclonal antibody specific for the extracellular domain of ErbB2, a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, is used to treat breast-cancer patients. With regard to GPCRs, an anti-PTH-related protein antibody is now being considered, not only for improving hypercalcaemia of malignancy, but also for treating cancer anorexia-cachexia syndrome and for preventing or treating bone metastasis of breast cancer[133,134]. Such antibodies may be an effective approach to targeting GPCRs that are outside the blood–brain barrier. The entire surface of a GPCR can be considered a potential binding site for biologically active molecules — both proteins and small molecules. Synthetic peptides or food-derived peptides, some of which are resistant to peptidases, may also be effective targeting molecules, which could be administered not only intravenously and intramuscularly, but also by oral and intranasal routes. An example is growth-hormone-releasing peptide-2 (GHRP2), which is in clinical trials for treating patients with short stature[135].

Jean-Philippe Pin. Sure, activating and inactivating antibodies have already been identified for GPCRs. For example, antibodies that activate β_2-adrenoceptors have been identified[136]. It is interesting that whereas these monoclonal antibodies are active, the Fab fragments are not, highlighting the probable importance of receptor dimers for G-protein activation. Inactivating antibodies have been found against the $mGlu_1$ receptor[137,138]. These could correspond to autoantibodies, which are probably responsible for the severe cerebellar ataxia observed in patients with Hodgkin's disease.

Rob Leurs. Yes. Many GPCRs are already activated by proteins (or peptides)! The action of antibodies against thyroid-stimulating hormone (TSH) receptor in Graves' disease also gives an indication that the action of antibodies can be quite effective on GPCRs[139]. Moreover, the use of antibodies against peptide or

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Rob Leurs earned his Master of Medicinal Chemistry and Ph.D. in Molecular Pharmacology at Vrije Universiteit in Amsterdam, the Netherlands. Leurs received his postdoctoral training with Jean-Charles Schwartz at the Institute National de la Sante et Recherche Medical (INSERM), Paris, France. With Martial Ruat and Jean-Michel Arrang, he was involved in cloning the histamine H_1 and H_2 receptors, and discovered the previously unknown serotonin 5-HT_6 and 5-HT_7 receptors. In 1993, Leurs received a five-year fellowship of the Royal Netherlands Academy of Arts and Sciences, and in the same year, he introduced site-directed mutagenesis approaches into medicinal chemistry efforts directed towards the design and synthesis of histaminergic ligands. In 2000, he was appointed Professor of Medicinal Chemistry at Vrije Universiteit in Amsterdam, and since 2003 he has been one of two scientific directors of the Leiden/Amsterdam Center for Drug Research. Leurs received the Galenus Research Prize in 1997, the Organon Prize in Pharmacology in 1999 and a Pfizer Academic Award in 2001. His current research efforts are focused on the molecular pharmacology of histamine and chemokine receptors; the design and synthesis of ligands acting at chemokine CXC3 receptors, viral chemokine receptors and histamine H_3 and H_4 receptors; and the high-resolution structures of GPCRs.

protein ligands could also be of interest. The recent use of 'degrakines' against chemokine receptors[140] is another interesting approach to modulating GPCR expression and thereby function. However, as with all targets, if one can use small ligands instead of 'biologicals', this will often still be preferred, as absorption, distribution, metabolism and excretion (ADME) properties tend to be easier to optimize.

Glossary

ANORECTIC
Suppressing or causing loss of appetite.

ATOMIC FORCE MICROSCOPE
(AFM.) A microscope that nondestructively measures the forces (at the atomic level) between a sharp probing tip (which is attached to a cantilever spring) and a sample surface. The microscope images structures at the resolution of individual atoms.

CHROMOPHORE
A light-absorbing molecule, such as pterin or retinal. Often physically associated with a protein partner to form a photoreceptor/phototransducer.

DESENSITIZATION
The mechanism by which a ligand becomes less effective on a receptor during a prolonged application.

DIPSOGENIC
Something that induces a strong need or desire to drink fluids; thirst-inducing.

FEAR CONDITIONING
A test to measure the ability of a rodent to learn and remember an association between an aversive experience and environmental cues. Learning and memory are assessed by scoring freezing behaviour in the presence of the cue or context.

FLUORESCENCE CORRELATION SPECTROSCOPY
(FCS.) A single-molecule technique that examines the diffusion of fluorescent molecules across a small confocal volume. It can be used to examine ligand binding based on the different diffusion speeds of a fluorescent molecule, when free and bound to a receptor.

FLUOROPHORE
A small molecule or a part of a larger molecule that can be excited by light to emit fluorescence.

FLUOROMETRIC IMAGING PLATE READER
A high-throughput screening device used to quantify real-time intracellular calcium fluctuations simultaneously in multi-well plates. In this cell-based assay, cells are loaded with a dye that can be excited once bound to calcium. Various GPCRs can be coupled to calcium pathways using G-protein chimeras or promiscuous coupling to $G\alpha_{15}/G\alpha_{16}$.

FLUORESCENCE LIFETIME IMAGING
An imaging technique that takes advantage of the change in the life-time of fluorescence of an energy donor when fluorescence resonance energy transfer occurs.

G PROTEIN
A heterotrimeric GTP-binding and -hydrolysing protein that interacts with cell-surface receptors, often stimulating or inhibiting the activity of a downstream enzyme. G proteins consist of three subunits: the α-subunit, which contains the guanine-nucleotide-binding site; and the β- and γ-subunits, which function as a heterodimer.

GREEN FLUORESCENT PROTEIN
An autofluorescent protein, originally isolated from the jellyfish *Aequorea victoria*, that can be genetically conjugated with proteins to make them fluorescent.

GTPγS
A hydrolysis-resistant analogue of GTP that binds to G proteins with high affinity. $[^{35}S]$GTPγS is frequently used to monitor GPCR-mediated guanine nucleotide exchange at G proteins.

INOTROPIC
Affecting the contraction of muscle, especially heart muscle.

INTERNAL FLUORESCENCE REFLECTION
An imaging technique that allows the detection of single-molecule fluorescence signals.

IONOTROPIC RECEPTOR
A term that describes a receptor that exerts its effects through the modulation of ion-channel activity. This term is now commonly used for receptors with intrinsic ion channels.

OCCAM'S RAZOR
A principle of 'postulate parsimony', articulated by William of Occam in the thirteenth century, which suggests that all else being equal, simpler explanations should be preferred over more complex ones.

ONTOGENY
In this context, the production and formation (in terms of adoption of three-dimensional structure) of receptor proteins.

ORPHAN RECEPTOR
A receptor for which no endogenous ligand has been identified.

PALMITOYLATION
A post-translational modification in which palmitic acid, a fatty carbon chain, is attached to a cysteine residue by a thio-ester bond.

PDZ BINDING MOTIF
Protein–protein interaction domain that often occurs in scaffolding proteins and is named after the founding members of this protein family (PSD-95, DLG and ZO-1).

PHARMACOPHORE
The ensemble of steric and electronic features that is necessary to ensure optimal interactions with a specific biological target structure and to trigger (or to block) its biological response.

PHASIC
Physiological events that occur only transiently with intervening periods of inactivity.

POSITRON EMISSION TOMOGRAPHY
A radioisotope method for localizing chemicals, such as receptor-bound ligands, *in vivo*.

PROTEAN LIGAND
A ligand that acts as an agonist on one specific pathway, and as an inverse agonist on another pathway.

PROTOMERS
Identical subunits in an oligomeric protein complex.

QUANTUM DOTS
Nanometre-scale particles of semiconductor materials. Optically, quantum dots are similar to fluorophores in that they absorb light at one wavelength (colour) and emit light at another. They could potentially replace fluorophores in many bioassays, as their emission efficiency does not fade over time as occurs with regular fluorophores. Similar to fluorophores, they can also be conjugated to biomolecules.

QT INTERVAL
On an electrocardiogram, the QT interval represents the time between the electrical activation and inactivation of the ventricles, the lower chambers of the heart.

REVERSE PHARMACOLOGY
The process that leads from an orphan receptor to the identification of its endogenous ligand.

SECRETED ALKALINE PHOSPHATASE (SEAP) ASSAY
A gene-reporter assay in which transcription of a thermostable secreted alkaline phosphatase is induced by the accumulation of an intracellular second messenger. The SEAP is quantitatively secreted in the supernatant, which simplifies the detection step of this assay.

SINGLE-MOLECULE FORCE EXTENSION
A measurement of mechanical properties between single molecules using atomic force microscopy.

SMOOTHENED RECEPTORS
Receptors involved in development first identified using *Drosophila* genetics. These receptors are constitutively active in the absence of their associated protein Patch, the receptor for Hedgehog. Hedgehog is supposed to prevent Patch from inhibiting constitutive activity of Smoothened, leading to Smoothened activation.

TOLERANCE
Reduced drug responsiveness with repeated exposure to a constant drug dose.

TONIC
Physiological events that occur in a sustained manner.

13

How close are we to being able to integrate understanding of how individual GPCRs function with their role in disease states?

Roland Seifert. We have a very long way to go, even for 'oldies', such as β-adrenoceptors. I think a major problem in this respect for us as researchers is the fact that the human body possesses an amazing capability to compensate for defects in a specific cell type and/or organ, and those adaptive changes often prevent us from detecting a disease process in the early stages. In addition, many organ functions are regulated in parallel by various GPCRs converging on the same G protein; for example, the heart, in which the β_1-adrenoceptor, histamine H_2 receptor and glucagon receptor all couple to G_s to mediate positive INOTROPIC effects. Thus, if one GPCR fails to function properly, another GPCR may readily fill the void, preventing us from observing a severe clinical phenotype.

Let me illustrate our dilemma using two examples. First, when I entered the field of receptor/G-protein research in the mid-1980s, most people expected the formyl-peptide receptor to play a crucial role in host defence against bacterial infections[141]. However, even the complete absence of function of the formyl-peptide receptor in humans induces only a very discrete phenotype; that is, a specific form of periodontopathy[142]. It is likely that other chemoattractant receptors, such as receptors for leukotriene B_4 and complement C5a, take over the job of the formyl-peptide receptor[143].

Second, every physiology and pharmacology textbook highlights the pre-eminent importance of the β_1-adrenoceptor for inducing positive inotropic and chronotropic effects in the heart. Nonetheless, knockout of the β_1-adrenoceptor in the mouse does not compromise heart function[144]. Thus, it is tempting to suggest that either the histamine H_2 receptor or glucagon receptor takes over the function of the β_1-adrenoceptor[145], but this is still pure speculation.

Theoretically, systematic gene arrays in tissue samples of patients during the course of a disease constitute an approach to monitoring changes in the expression of GPCRs and regulatory proteins. However, from an ethical and financial point of view, this approach is probably not realistic. In addition, we should not forget that gene arrays do not provide information about the actual protein expression level of a given gene.

Rémi Quirion. We are still a long way from being able to extrapolate from our understanding of individual GPCR functions to determining their roles in disease states, except for a few very rare disorders associated with mutations in specific GPCRs. More globally, this will take some time, as there is tremendous redundancy in the organism for most crucial survival functions (such as sex, eating, regulation of vegetative functions, stress responses and so on).

Thomas P. Sakmar. The answer to this question depends on what disease state is considered. There are some examples of abnormalities resulting from GPCR alterations for which the molecular pathophysiology is understood in great detail. For example, although it is not a target of pharmacological intervention, we certainly understand the genetic and molecular basis of red/green colour blindness. Many inherited endocrinopathies are also well understood. The roles of defects in receptor synthesis or transport, however, are more difficult to sort out. For example, how do mutations in the gene encoding rhodopsin lead to the activation of apoptotic pathways in autosomal-dominant retinitis pigmentosa? Subtle defects in GPCR-mediated signal transduction pathways that cause disease will also be more difficult to sort out. Specific molecular correlations may be difficult to establish in complex disorders such as hypertension, for example. In many cases, GPCRs act in the context of complex signalling networks, and the specific function of different GPCR subtypes, or even different members of a GPCR subfamily, is not well understood. In the case of the chemokine network, for example, there is a high degree of promiscuity among chemokines and their receptors. For the majority of the chemokine receptors, the consequences of malfunction or pharmacological intervention are not well understood. Furthermore, the issue of the physiological and pathophysiological relevance of GPCR polymorphisms is only just starting to be addressed.

Philip G. Strange. I think we understand quite a bit about the role of some GPCRs as drug targets, and this is sometimes taken as meaning that we understand the role of GPCRs in diseases. In fact, we do not know very much in terms of relating the function of particular GPCRs to disease. There are notable examples for which we do know quite a bit, such as the chemokine receptors CCR5 and CXCR4, whose roles in the entry of HIV-1 into cells are well described (see, for example, REF. 146). There is still, however, a lot to learn about the role of GPCRs in diseases, and the analysis of polymorphisms may yield some interesting data in this respect.

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Rick Neubig earned a B.S. in Chemistry at the University of Michigan, Ann Arbor, USA, and completed his M.D. and Ph.D. degrees at Harvard University, Cambridge, Massachusetts. He completed his residency in internal medicine at the University of Michigan Medical School, where he was appointed Assistant Professor in the Department of Pharmacology and Internal Medicine in 1984. In 1995, Neubig became Professor in the Department of Pharmacology. Neubig was the Founding Chair of the Molecular Pharmacology Division at the American Society for Pharmacology and Experimental Therapeutics (ASPET) from 1997–1999, and became a member of The International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) in 2001. He has served on several editorial boards, and now sits on the editorial boards for the ASPET journals *Molecular Pharmacology* and *Molecular Interventions*. Neubig is interested in the mechanisms of signal transduction through G proteins and the receptors that activate them. His research is focused on the structural and kinetic mechanisms of GPCR signals, and the development of drugs that target novel sites in signal transduction processes. Three areas of recent interest to Neubig are the novel family of RGS proteins; scaffolding proteins that contribute to specificity of receptor–G protein–effector signalling; and the structural basis of the receptor–G-protein contact site.

Akio Inui. We are still some way from a clear understanding of individual GPCR function. Receptor agonists/antagonists and knockout animal models are useful approaches for addressing this issue. However, it seems that somewhat different conclusions can be reached by using different types of GPCR antagonist. An example is the neuropeptide Y_5 receptor antagonists, which only sometimes show ANORECTIC properties[147]. The reason for this is not clear at present, although lack of specificity could be an explanation for some compounds; however, an inherent problem is that drug effects are usually evaluated by a limited number of research laboratories, making a direct comparison between drugs difficult. Redundancy in the system or compensation for the loss of a particular GPCR is also not unusual in conventional knockout models. This might modify the phenotype of the animals and mask the physiological function of the GPCR. With the exception of some rare congenital forms of the disorders[148], the polymorphisms in GPCRs that relate GPCR function to disease states in complex disorders, which are likely to be influenced by multiple alleles of multiple genes[149], largely remain to be determined.

Jean-Philippe Pin. In most cases, we have a long way to go, but in others we are very close. The case of the Ca^{2+}-sensing receptor is a good example. This receptor plays a major role in 'measuring' calcium concentration in the blood and inhibiting PTH release by the parathyroid gland[150]. In agreement with the major roles of this receptor subtype, loss-of-function mutations are responsible for familial hypocalciuric hypercalcaemia and neonatal severe hyperparathyroidism[151], whereas gain-of-function mutations are responsible for autosomal-dominant hypocalcaemia[152]. For some of these patients, positive or negative allosteric regulators may have beneficial effects. A second interesting example is that of the vasopressin V_2 receptor. Loss-of-function mutations of this receptor are responsible for 90% of cases of nephrogenic diabetes insipidus. Most of the identified mutations result from the misfolding of the receptor and its retention in the endoplasmic reticulum. Interestingly, ligands that enable the correct folding of the receptor and, as such, allow the receptor to reach the plasma membrane, have been identified (and called 'pharmacological chaperones')[57]. Such compounds are good candidates to treat these patients.

Joël Bockaert. In some cases, it is easy; for example, the involvement of TSH receptors in toxic thyroid hyperplasia, or luteinizing-hormone receptors (LHR) in male precocious puberty. By contrast, the roles of individual GPCRs in depression, anxiety, diabetes, cell metastasis and obesity are far from being understood.

Tamas Bartfai. In the case of the role of monoamine GPCRs in cardiovascular disease, and in some neurological, endocrinological and mental diseases, we are starting to understand the intricacies of chronically blocking or activating GPCRs. In most other areas, however, our knowledge is still in its infancy.

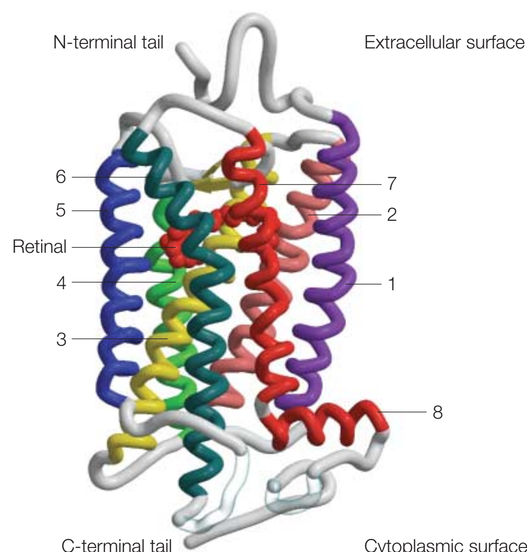

Figure 4 | **The crystal structure of rhodopsin.** (Protein Data Bank entry 1HZX.) The polypeptide sequence of the rhodopsin protein starts at the amino terminus, which is located on the extracellular surface of the membrane bilayer discs in the retina. The protein folds into seven transmembrane helices (labelled 1 through 7), and a short helix (8) that runs across the cytoplasmic surface of the membrane. The crystal structure was obtained in the presence of the retinal chromophore[59] (denoted by red spheres), which is important for photon absorption in this vision protein, and is buried within the protein. Two of the protein loops on the cytoplasmic surface were not located in the crystal structure, presumably due to their dynamic motion. They are denoted in the figure by transparent regions. The crystal structure of rhodopsin has served as a model for understanding G-protein-coupled receptor (GPCR) activation and signalling processes, and has also been the starting point for predicting the structures of other GPCRs. Figure drawn using Molscript[201] and Raster3d[202]. Figure prepared by Ronald E. Stenkamp.

Bernard P. Roques. Genomic and proteomic approaches have enabled the analysis of proteins that are up- or downregulated in a given disease in which a GPCR is involved. The question remains as to whether it is only a defect in receptor activation that is linked to a given disease, or whether it is a result of changes to signalling pathways. The discovery that GPCR activation could result in direct (for example, dimerization of tyrosine kinase receptors[153,154]) or indirect (for example, stimulation of the RAS cascade[155]) responses is of major importance in diseases such as cancer and neurodegenerative disorders.

Michel Bouvier. In some rare cases, this has already been achieved. Indeed, in the case of a few monogenic diseases, mutations leading to loss or gain of signalling activity have been identified as the direct causes. These include mutations leading to the misfolding of the vasopressin V_2 receptor[156], the gonadotropin-releasing-hormone receptor[157] and rhodopsin[158], which are associated with nephrogenic diabetes insipidus, hypogonadotropic hypogonadism and retinitis pigmentosa, respectively. Also, several mutations that lead to increased constitutive activity of receptors, such as the

thyrotropin receptor involved in thyroid toxic adenomas[159], have been reported[160]. Unfortunately, the small number of patients afflicted by these diseases means that there is a lack of incentive to transform this knowledge into new drug therapies. In the case of more complex diseases, dysfunctions are likely to affect more than one GPCR signalling system. Thus, a better understanding of the integration between various signalling pathways and of their regulatory processes will be required before we have a complete picture of the role of individual GPCRs in each of the disease states considered. Fortunately, having such an understanding is not always required to develop an effective treatment. Indeed, blockade or activation of a unique receptor (even if this receptor is not directly involved in the pathogenic process) is often sufficient to promote homeostatic changes that are beneficial and can be translated into therapeutic efficacy. Nevertheless, a better picture of the overall signalling abnormalities associated with specific disease states should offer new opportunities for the development of drugs targeting the integrative and/or regulatory processes instead of individual receptors.

Arthur Christopoulos. In some cases, we have a fairly good idea of how we can relate GPCR function to a given disease state, but these invariably relate to the clear identification of a mutation or polymorphism in the GPCR that gives rise/contributes to the disease state. In the majority of cases, however, we are simply not there yet. If we consider just one of the main therapeutic areas targeted by the pharmaceutical industry — the CNS, for example — a consistent message that filters through time and time again is that the suspected causes of most mental-health-related disorders are multifactorial, and probably involve more than one type of receptor family and other proteins.

14

How successful are the animal models that seek to recapitulate diseases linked to the aberrant function of peptide-activated GPCRs?

Bernard P. Roques. This is one of the most important problems in the field. The development of GPCR gene knockouts in a given tissue at a given time, and tissue regulation in animals other than mice that have sequenced genomes (for example, rats), is a priority. The validity of animal models that have mainly been developed to study classic neurotransmitters or hormones has to be questioned when examining the mode of action of peptides. Neuropeptides seem to play a crucial role in adaptation to different types of situation (such as stress, vigilance, fear and food or sexual rewards); monitoring such responses requires more subtle assays than those currently used to study the responses to aminergic GPCRs.

Rémi Quirion. For those of us interested in neuroscience and mental health, we are still a long way from the finishing line, as we have two major challenges: to find more appropriate animal models of human brain diseases, and then to determine the relevance of neuropeptides and their receptors in the aetiology of these diseases. We still have major hurdles to overcome for diseases such as schizophrenia and depression, although progress is being made in anxiety research. Most of our current animal models of mental-health disorders and addiction involve the analysis of classical neurotransmitter systems (such as knockout mice of various dopaminergic, 5-HT and noradrenergic receptors, and their re-uptake carrier proteins) and their aberrant functions after exposure to amphetamines or neurotoxins (for example, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4), 5,7-dihydroxytryptamine (5,7-DHT), 6-hydroxydopamine (6-OHDA)). It may not be so surprising that such models are not really informative about the role of neuropeptide GPCRs, when we consider that classical neurotransmitters are usually fast acting, whereas peptides are slower in their onset of action, but have a longer duration of effect. Accordingly, new models must consider, first, the difficulty of modelling many human brain disorders in animals and, second, the distinct mode of action of peptides versus classical, fast-acting neurotransmitters. This is a double challenge for us all.

Olivier Civelli. Mice devoid of a peptide-activated GPCR gene, or of the gene for the peptide precursor itself, are being established at an increasing pace. In general, these animal models have been used to confirm, rather than to discover, one or several aspects of the function of the peptide-activated GPCR system. Some of these have been evocative of a link to a particular disorder. One of the best examples is the orexin/hypocretin knockout mice, which exhibit a narcoleptic-like phenotype that parallels exactly the

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Jean-Philippe Pin is a former student of the Ecole Normale Supérieure, Paris, France. He earned his Ph.D. in Molecular Biology in 1987 at the University of Science and Technology in Montpellier, while working in a unit of the Centre Nationale de la Recherche Scientifique (CNRS). With Fritz Sladeczeck, Max Recasens, Samuel Weiss and Joël Bockaert he discovered the metabotropic glutamate (mGlu) receptors. He then participated in the demonstration of synergism between various glutamate receptor subtypes for the activation of phospholipase A_2. In 1990, he joined Steve Heinemann's laboratory at the Salk Institute, La Jolla, California, USA, as a postdoctoral fellow, being a recipient of both a NATO and a National Institutes of Health (NIH)–Fogarty fellowship. He identified new mGlu receptor splice variants and demonstrated differences in their transduction properties. In 1992, he returned to France to set up a research team working on the structure/function relationship of mGlu receptors within a CNRS laboratory headed by Joël Bockaert. He identified the molecular determinants responsible for mGlu–G-protein-coupling selectivity, and showed that mGlu receptor constitutive activity can be regulated by intracellular proteins. Since 2003, he has been Head of the Molecular Pharmacology Department within the Laboratory for Functional Genomics at CNRS. His research efforts are focused on the activation mechanism of class C GPCRs, mainly mGlu and $GABA_B$ receptors, and their regulation by intracellular proteins.

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one described for narcoleptic dogs that have mutated orexin-/hypocretin-2 receptors[161,162]. This is a research field that is still at an early stage.

Akio Inui. Functions of peptide-activated GPCRs are well depicted by recently developed peptide-overexpressed or peptide/receptor-deficient animal models. For example, the deletion or mutation of leptin, melanocortin or their receptor genes led to obesity in mice and humans, with essentially similar phenotypic characteristics[163,164]. Abnormalities in the orexin system caused narcolepsy-related symptoms in orexin and orexin-receptor knockout mice, as well as in humans and dogs with naturally occurring genetic mutations in orexin and orexin receptors[165]. Observations in mutant mice thus shed new light on the role of peptide-activated GPCRs, not only in energy homeostasis, but also in other integrative functions. Advanced gene-targeting strategies, in which genes can be turned on or off in desired tissues at desired times, are beginning to produce a better understanding of GPCRs and animal models linked to their aberrant functions.

Tamas Bartfai. We need more imaginative animal models that enable the observation of more behavioural, endocrine and immune parameters, and, most importantly, that employ a greater number of stimuli. We so often passively look for a phenotype that would need to be part of an evoked response to be recognized; that is, an endocrine, immune or sensory challenge is needed to discover that a peptide-activated GPCR is part of a response, which, in the naive animal, would not be seen. At present, our phenotyping techniques are grossly inadequate for monitoring responses and changes in response to stimuli. I believe that we are way ahead of ourselves in generating new transgenic strains, and are spending too little time developing better phenotyping techniques. At present we use too few challenges to enable a full understanding of the role of peptide-activated GPCRs; even in endocrinology, stress and hormonal challenge are not used often enough when looking for a phenotype. Similarly, our analysis of behavioural effects is inadequate, mainly because we tend to use high-throughput tests such as FEAR CONDITIONING to monitor both anxiety and cognition, when we know that both cognition and anxiety are in fact much more complex, and we have many better but slower tests for them. The problem is that when high-throughput is required, the fastest, most robust tests are often chosen at the risk of missing the sometimes more subtle neuropeptide-GPCR-mediated effects.

Roland Seifert. It is a mixed bag. For example, the endothelin-receptor knockout in the mouse is a good model for Hirschsprung's disease[166], but the formyl-peptide-receptor knockout in mouse does not recapitulate the localized juvenile periodontitis observed in humans[167].

15

In your opinion, which diseases are most strongly associated with aberrant function of peptide-activated GPCRs?

Bernard P. Roques. Physiological over- or under-stimulation of opioid receptors in limbic structures could be involved in depression (δ-opioid receptor)[16], risk for drug abuse (μ-opioid receptor) and so on. The brain cannabinoid CB_1 receptor could play a major role in the memory process and the coordination of neuronal networks, such as those joining the cerebellum to the thalamus and then the frontal cortex. A few schizophrenic syndromes could be related to a defect in this pathway that is related to or caused by a hypofunctioning of the CB_1 receptor. Glaucoma could be related, at least partially, to local under-stimulation of the atrial natriuretic peptide receptor. Obesity is very probably the result of a defect in the balance between the activation of various types of peptide GPCR in different central and peripheral structures. In addition, it could be important to investigate the role of overexpressed peptide-activated GPCRs (such as gastrin, CCK and neurotensin receptors) in various types of cancer.

More generally, it could be interesting to create animal models of diseases (such as food deprivation or the reverse, hypertension, repetitive stress, atherosclerosis or arthritis) and to investigate the possible changes in expression of GPCRs in various tissues using microarray technology. Could such studies open the way to preventing a given disease by activating or blocking one or more GPCRs that are as yet unknown? Microarray studies have identified polymorphisms of GPCRs in various diseases of the CNS, such as schizophrenia, major depression, anxiety and so on. Nevertheless, the relationship between these diseases and dysfunction of GPCR mutants remains to be definitely confirmed. This

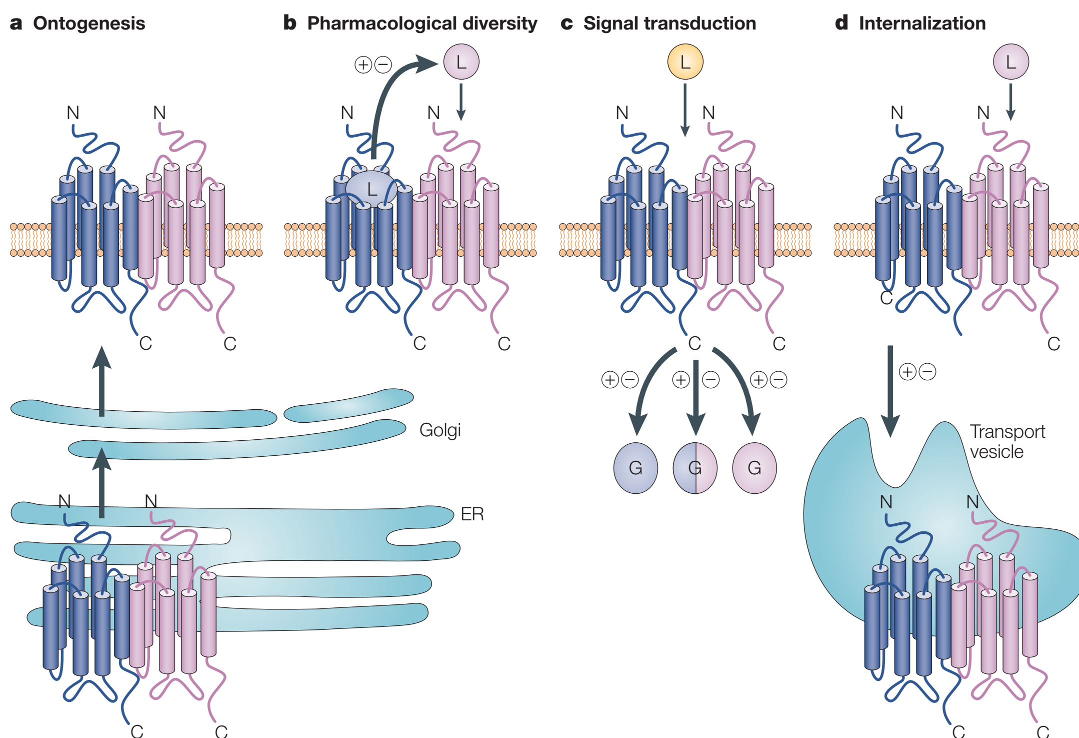

Figure 5 | **Proposed roles for GPCR heterodimerization.** Although in most cases the physiological relevance of G-protein-coupled receptor (GPCR) heterodimerization still remains to be demonstrated, several studies carried out in heterologous expression systems have suggested many distinct roles for heterodimerization in GPCR function. Similar roles have been proposed for heterodimerization between distinct GPCRs, and between GPCRs and the receptor-activity-modifying proteins (RAMPs). In this figure, the heterodimerization of distinct GPCRs is used to illustrate these potential roles (see REF. 203 for an illustration of the interaction between RAMPs and GPCRs). **a** | Ontogenesis. Heterodimerization can be involved in the quality control of folding and membrane targeting of newly synthesized receptors. **b** | Pharmacological diversity. The ligand-binding selectivity of the receptors can be affected by heterodimerization, which can promote either positive or negative binding cooperativity. In this schematic representation, heterodimerization enables the ligand (L) for one receptor (blue), to influence the binding of another ligand to the second receptor within the dimer (pink). **c** | Signal transduction. Heterodimerization can influence the signalling properties of a given ligand (yellow) by affecting the selectivity of interaction between the target GPCR and its cognate G protein, resulting in different G-protein coupling. **d** | Internalization. The endocytic pattern of a given receptor can be modified by heterodimerization. For instance, heterodimerization between endocytosis-prone and endocytosis-resistant receptors has been found to lead to receptor heterodimers that adopt the endocytic pattern of one of the protomers (shown here as the 'pink' receptor). ER, endoplasmic reticulum; G, G protein; L, ligand. Figure prepared by Michel Bouvier.

indicates that the physiological ligand(s) of normal receptors are unable to induce the expected responses issued from stimulation of unmodified GPCRs. This could open the way to expressing recombinant GPCR mutants in cells, followed by HTS of agonists or antagonists selectively directed towards these altered GPCRs. Among diseases in which alterations of neuropeptide GPCRs play a role, severe depression, schizophrenia, some tumours, glaucoma, infertility, inflammatory diseases, cardiovascular diseases, obesity, pain and atherosclerosis are just a few examples.

Joël Bockaert. Pain, cancer (metastasis), stem-cell differentiation, depression, schizophrenia and diabetes spring to mind.

Tamas Bartfai. Pain, neurological diseases, endocrine diseases (fertility-related diseases in particular) and mental diseases other than anxiety are all more or equally important GPCR-related disorders.

Akio Inui. Obesity, anxiety and irritable bowel syndrome are linked strongly with aberrant function of peptide-activated receptors, although these are multifactorial in causation. Other diseases to which peptide-targeted compounds are being applied or are under consideration include cancer cachexia, eating disorders, pain, hypertension and heart failure.

Olivier Civelli. First are diseases that are transmitted by viruses that use cytokine GPCRs as docking targets. These GPCRs hold great promise for antiviral therapies. Next are the diseases that will be associated with the malfunctioning of the ~200 GPCRs that are (or will be found to be) peptide-activated. On the basis of this diversity alone, the peptide-activated GPCRs can be expected to be involved in numerous disorders, in particular those involved in brain function. What we have learned about neuropeptide GPCRs suggests that it is likely they will be involved in most psychiatric disorders and, indeed, carry the best hope for new therapies in this medical field.

Rémi Quirion. It is hard to tell at this stage, but most certainly pain on the basis of the well-known effects of opioids, neurokinins and CGRP. Migraine is probably another one (CGRP), as well as various cardiovascular diseases, obesity and mental illnesses, although these diseases are all multifactorial. One idea that is gaining credence is that neuropeptides and their receptors are involved in depression and associated illnesses (for example, REF. 168). Although current drugs are based on monoamines, the next generation of clinically effective molecules could be related to peptides such as substance P and other neurokinins, including corticotropin-releasing hormone and neuropeptide Y, and/or certain trophic factors. In spite of the relative weakness of all animal models related to depression, it is clear that all current drugs are slow acting, requiring many weeks of treatment before clinical improvements are seen. Could this be because monoaminergic transmission is one of the final steps in the disease process, and that more slowly acting transmitters, such as neuropeptides, are in fact key, which would explain the clinical lag phase? This idea is certainly worth exploring further, and could potentially have a great clinical impact. Of course, transgenic and knockout animal models of various peptide-activated receptors could turn out to be most informative in that context.

Rob Leurs. I would like to bring up the family of chemokine receptors, which are likely to be involved in a variety of important disorders, including cancer and inflammatory conditions, such as rheumatoid arthritis and multiple sclerosis.

Roland Seifert. Aberrant peptide-receptor function presumably plays a role in many disease states, including all kinds of inflammatory diseases, pain states, hypertension, obesity, endocrine diseases and carcinogenesis. For example, in some endocrine diseases, such as renal diabetes insipidus, we have a clearly defined defect of a receptor: the vasopressin V_2 receptor[151]. The aforementioned defect of the formyl-peptide receptor, which causes a defined type of periodontopathy, is another example of a peptide-receptor defect that causes a clearly defined phenotype[142]. By contrast, in other cases, such as Kaposi sarcoma, we have a constitutively active and virally pirated chemokine receptor that causes a disease[169]. Pathologically increased constitutive activity of the CCK_2 receptor, thyrotropin receptor, luteinizing-hormone receptor and PTH receptor also results in defined disease states[36]. However, in most inflammatory conditions, in which numerous peptide mediators play a role, it will be much more difficult to define the exact role of a given peptide receptor in pathogenesis, simply because there is significant overlap in function.

16

What are the main hurdles that will need to be overcome to create further successful therapeutic approaches based around targeting GPCRs?

Brian Kobilka. I believe that there is great potential for new GPCR-based therapies for neuropsychiatric disorders. However, target identification and validation is still a major limitation. At present, evidence in support of a particular target comes from expression profiling and, when available, knockout studies. Animal models (particularly mouse models) are limited in their ability to reproduce complex human neuropsychiatric disorders; however, with the growing number of mutant strains of mice and the development of technology for phenotyping, the predictive value of these animal models will probably improve[170]. Another possible problem is our desire for highly selective drugs. Older drugs that are on the market now are not completely selective. It is possible that the efficacy of drugs might in part be attributable to their effect on more than one receptor. Drugs might have to be used in combination. Thus, target identification may require looking at two or more GPCRs at a time.

Bernard P. Roques. A major hurdle is the development of appropriate animal models and adapted pharmacological tests directed towards a particular disease in which a GPCR is thought to play a role (for example, from studies with knockout mice).

Thomas P. Sakmar. I believe that target validation is a major hurdle. A drug might be found that blocks or activates a specific GPCR, but does this intervention have the desired effect *in vivo* on complex physiological problems such as obesity or autoimmune and inflammatory diseases? Animal models might be misleading, and until one has a drug candidate that is approved for clinical trials, the final answer cannot be known for sure. Assuming that there is good evidence that a GPCR target is valid for pharmacological intervention, finding a specific drug can be extremely laborious and time consuming. However, this process is often straightforward compared with the subsequent stages of optimization of the pharmacological and toxicological profile of a compound series in animal models and in human trials.

Rémi Quirion. The key to success is our understanding of what it takes at the structural and molecular levels to generate an agonist or an antagonist, and the various permutations in between (partial agonists, inverse agonists and so on). We still know very little in that respect, and therefore cannot provide truly useful information to help rational drug design. Another key issue is G-protein–receptor coupling. We still know little about how this occurs at the molecular level, and it is hard to extrapolate from one GPCR to another. I see these two issues as major drawbacks.

Tamas Bartfai. The validation of new GPCRs as drug targets in disease models is the number one problem. This is a big problem, as there is a growing feeling that if the knockout of a GPCR does not have a striking phenotype (and knockouts often do not, but this may be because of our poor phenotyping techniques, as mentioned in my response to question 14 (page 615)), then

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Bernard P. Roques earned degrees in pharmacy and pharmacology at René Descartes University, Paris, France, and a Ph.D. in Physical Chemistry at the Ecole Polytechnique, Paris, where he was Assistant Professor from 1970–1976. He was then Professor of Pharmacochemistry at René Descartes University and Director of the Department de Pharmacochimie Moleculaire et Structurale associated with the Centre Nationale de la Recherche Scientifique (CNRS) and Institut National de la Santé et de la Recherche Médicale (INSERM) from 1976–2001. His laboratory designed the first DNA polyintercalators, in addition to discovering potent and selective agonists of the δ-opioid receptor and subsequently demonstrating their potential interest as antidepressants. In 1980, he developed selective inhibitors of enkephalin-inactivating enzymes, one of them being marketed as an antidiarrheal agent, and proposed the concept of dual inhibitors for the complete protection of enkephalins, and their extension to metallopeptidases involved in cardiovascular diseases. At present, his laboratory specializes in the development of inhibitors of the zinc metallopeptidases involved in peptide metabolism, which are of potential interest in the cardiovascular field, neurodegenerative processes, glaucoma and cancer. Among international awards, Roques has received the Charles Mentzer Prize from the Medicinal Chemistry Society (1994), the INSERM Academy of Sciences Award (1994), the G. Wittig/V. Grigard Prize from the Gesellschaft Deutscher Chemiker (1996), the Galien Prize of Pharmaceutical Research (1977) and the Rudinger Award from the European Peptide Society (2000). He is a member of the French Academy of Sciences and the European Academy of Sciences, and is Scientific Director of the start-up company Pharmaleads.

one does not expect a robust pharmacological effect and thus one does not pursue the development of a drug. This problem is compounded by the lack of chemical hits in screening for some GPCRs (such as the galanin receptors $GALR_1$ and $GALR_2$, the calcitonin receptor (CALCR) and the FSH receptor (FSHR)), which would be required for the development of selective high-affinity ligands.

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It is very unclear today why all chemical libraries have had hits for the NK_1 receptors, while there have been virtually none for the galanin receptors — all we can surmise is that the libraries in question reflect the chemical history of companies and the design attempts (for new combinatorial libraries) of their chemists. It seems that despite the fact that the peptides have pharmacophores that mainly represent the side chains of amino acids, some receptors (including corticotropin-releasing factor-1 (CRF1) and CRF2 receptors, galanin receptors and some others) may require for recognition of the ligand the formation of specific three-dimensional conformations between these pharmacophores that have not yet been encountered in synthetic chemical libraries.

Roland Seifert. We need to work harder on the human physiology/pathophysiology, get some nice crystal structures and study in detail GPCR polymorphisms, both in humans and in animals.

Susan R. George. I believe that we need a better handle on the physiological roles of the GPCR systems discovered. Only then can rational approaches be developed for targeting the potential pathophysiology.

Akio Inui. Drugs have traditionally been discovered through the screening of numerous chemical structures in a biological system. It seems that the type of receptor screen used to detect biologically active molecules, such as radiolabelled ligands, defines the type of molecule detected. The current emphasis away from radiolabelled-ligand binding and towards high-throughput functional screening is beginning to reveal ligands that can change biological function without exerting apparent effects on radiolabelled-ligand binding[171]. For example, allosteric enhancers potentiate the effects of agonists, either through enhancement of agonist affinity, stabilization of agonist–receptor and receptor–G-protein interaction or other enhancement of efficacy. Similarly, allosteric modulators can block agonist stimulation of the receptor without necessarily interfering with agonist binding to the receptor. Such increased screening capability could cause an increase in the variety of biologically active molecules detected.

Other hurdles include the time-consuming preclinical and clinical evaluations that need to be performed for GPCR-targeted drugs. This could be done more systematically by the cooperation of several pharmaceutical companies and academic groups with shared interests. The information sources that support therapeutic research are quite important; for instance, previous human studies showed some differences in neuroendocrine networking in humans compared with that seen in animals[152,153]. The attempt to include such complexity into drug discovery projects is a crucial initial step.

Olivier Civelli. The large number and diversity of the GPCRs positions them as modulators of reactions that affect higher brain functions, such as memory, feeling, mood, anxiety and so on. Indeed, GPCRs are the primary targets in treating psychiatric disorders, and I

believe that their use in psychiatric therapies will be extended. However, progress is hindered by the fundamental issue of finding animal models that adequately reflect psychiatric disorders. Developing such models is difficult, if not impossible. Genetically engineered strains will undoubtedly be useful, but, ultimately, acute activation and inhibition of the GPCR system will be required if its function is ever to be understood. This is where pharmaceutical research needs to meet academic research. Designing or screening synthetic molecules that can selectively activate or block a GPCR system is needed to find the function of the system and not solely for finding drugs. This is a major, and possibly insurmountable, hurdle for the pharmaceutical industry.

Joël Bockaert. The main hurdle is not to follow the same track as everyone else.

17

What would you describe as the major concerns surrounding the continued development of GPCR-targeted therapies?

Jeffrey L. Benovic. We are now beginning to appreciate the complexity of GPCR biology, which includes the large number of GPCRs, splice variants, polymorphisms, homo- and heterodimerization, multiple interacting partners, differences in localization and trafficking, and mechanisms of regulation. GPCR signalling is incredibly complex, and attempting to integrate this complexity into disease processes and the identification of useful drugs is a major hurdle.

Bernard P. Roques. First, GPCRs are easily accessible targets that play a necessary physiological role. Moreover, based on predictions from the human genome, a large number (>200!) of GPCRs remain to be discovered and their functions characterized.

Olivier Civelli. Drug discovery related to GPCRs may, paradoxically, suffer because of its richness. Faced with a plethora of potential therapeutic targets and the inability to decide which ones to target, some will wait until a particular GPCR has been assigned to a particular function before launching a drug discovery programme. This is a self-defeating attitude, as it relies on outside information that everyone can access. However, others may consider starting drug design programmes on a series of new GPCR systems with little information but high hopes. The systems selected should be chosen on the basis of similarities, either in sequence or in function. If the main intention of such a programme is to achieve isolation and *in vivo* analyses of selective, but not necessarily druggable, surrogate ligands, then it could efficiently reach its goal: to define the function of the GPCR system. This would then enable a bona fide drug design endeavour that would have a higher chance of success.

Susan R. George. The approach of looking for a single therapeutic agent that will correct or adequately treat a particular disease condition has to be modified. Most physiological processes are exceedingly complex, and in many situations a multi-pronged approach may have a better likelihood of success. This has been illustrated very nicely in the way treatments of essential hypertension and type II diabetes mellitus have evolved. Optimizing drug treatment for type II diabetes often involves the use of an insulin secretagogue together with a peripheral insulin sensitizer and an inhibitor of hepatic gluconeogenesis. Similarly, in essential hypertension, concurrent therapy may target volume depletion, ACE inhibition, angiotensin-receptor blockade, calcium-channel blockade and β-adrenoceptor blockade, often requiring several drugs for the best control and risk reduction.

Akio Inui. A major concern is receptor desensitization or compensation by other systems, which may decrease the long-term efficacy of the compounds. Almost all anti-obesity agents lead to weight loss for the first four to six months, followed by regain, although weight-loss medications can decrease the rate of regain[172]. A multidrug regimen that targets multiple sites within the body-weight regulatory system might be necessary to achieve and sustain weight loss. Another concern is the multiplicity of GPCR functions in the body that are exerted in different tissues but which can be modified simultaneously by receptor agonists/antagonists. Such compounds might affect, for example, feeding, anxiety and blood-pressure regulation, thereby complicating their use in clinical medicine.

Tamas Bartfai. GPCR agonists and antagonists have been among the safer drugs developed in comparison

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Roland Seifert was a Fellow of the Merit Scholar Foundation of Germany and earned his M.D. degree at the Free University of Berlin, Germany in 1986. He then joined the group of Günter Schultz in the Department of Pharmacology at the Free University of Berlin, completing his ‘Habilitation’ in 1992. His work in Berlin focused on the regulation of nucleotide receptors, histamine H_1 and H_2 receptors, chemoattractant receptors and receptor-independent G-protein activation in human neutrophils and HL-60 leukaemia cells. From 1995 to 1998, he was a research fellow in Brian Kobilka’s laboratory at Stanford University, California, USA. His work at Stanford focused on the molecular analysis of β_2-adrenoceptor/G_s-protein coupling, taking advantage of the GPCR–Gα fusion protein technique. This research provided evidence for the existence of several active GPCR states. In 1998, he became Associate Professor for Pharmacology and Toxicology at The University of Kansas, Lawrence, Kansas. A major focus of his research in Lawrence is the analysis of inter- and intra-species differences in the pharmacological properties of GPCR isoforms, encompassing chemoattractant receptors, histamine receptors and β-adrenoceptors. Another focus of his research is the analysis of differences between the various $G\alpha_s$ isoforms. Recently, the molecular analysis of signal transduction abnormalities in Lesch–Nyhan syndrome (hypoxanthine/guanine phosphoribosyl transferase deficiency), the development of adenylyl-cyclase inhibitors and fluorometric analysis of adenylyl cyclase became part of his research portfolio. Seifert has received research grants from the National Institutes of Health, the Army Research Office and the American Heart Association.

with, for example, ion-channel blockers and protein-kinase inhibitors, both on their own and in combination with other drugs. Cross-desensitization of GPCRs in sensory contexts is well known, and the development of cross-tolerance to some GPCR agonists — with or without abuse potential — are among the concerns. However, these mechanism-related toxicities and tolerances do not rank highly compared with the molecule (chemical structure)-related toxicities that some of the new and old GPCR ligands may have.

Roland Seifert. It seems at the moment that if you have no ideas, you just combine your available GPCRs and compound libraries! This is not the way to go. We need to develop specific concepts about the function of GPCRs first.

18

What effect is the deorphanizing of GPCRs likely to have on the field?

Olivier Civelli. The deorphanization of GPCRs started with the application of homology screening approaches to the cloning of receptors. This technique has been with us since 1988, and it is, directly or indirectly, responsible for most pharmacological characterizations of GPCRs. By contrast, the discovery of novel neurotransmitters or neuropeptides that use orphan GPCRs started in 1995 and has led to the discovery of eight novel neuropeptide families (see FIG. 6 (page 621) for an outline of the strategy used to identify novel transmitters for orphan GPCRs). The challenge now is to discover the natural ligands of the last ~100 non-sensory GPCRs that are still orphans. This is still a long task, and one that is increasingly difficult. But any successful deorphanization has, and will continue to have, a great impact on many aspects of biological research, as each success opens a new field for pharmacology, biology, physiology and behavioural studies, and, ultimately, for medicine. This, of course, is of the utmost importance to drug discovery. Indeed, almost all the novel neuropeptide families discovered via orphan GPCRs are, or have been, in drug discovery programmes. One in particular, the orexin/hypocretin family, has the potential to revolutionize narcolepsy therapy and impact related fields.

Joël Bockaert. The deorphanization of GPCRs will be a revolution in terms of obtaining physiological knowledge. The impact on developing therapies should also be great, but will take time and imagination, and involve risk.

Jeffrey L. Benovic. There is little doubt that deorphanizing GPCRs will have a major impact on the field, as it should lead to the identification of novel receptor–ligand combinations and a correlation with important physiological processes. The unknown here is how many of these orphan receptors, if any, will result in the development of blockbuster drugs.

Roland Seifert. We will learn more about the function of these receptors in the long run, leading to new drugs. But we are talking about decades, not years as the industry hopes.

Tamas Bartfai. Deorphanizing of GPCRs will be very rewarding scientifically, as some surprises about the chemical characteristics of the ligands are expected — who knows if there are larger ligands than thrombin, or other lipid metabolites similar to anandamide? Analysing the biosynthesis, metabolism and binding modes of these new ligands will be interesting from a basic science point of view. However, I believe that the effect of deorphanization on the pharma industry will not be terribly large — the relative importance of the pharmacological and physiological effects of a given GPCR can be assessed today without knowing the identity of the endogenous ligand, using techniques such as receptor knockouts, antisense technology and HTS with promiscuous G-protein-containing reporter systems to find exogenous agonists and antagonists.

More importantly, let's not forget the 5,000 years of morphine use in pharmacology, which shows that if a safe and efficacious dose can be established for a compound, people will use it without knowing the identity of one or all of the endogenous ligands! So, the answer to this whole question is best obtained in relation to the question of whether the identification of enkephalins and endorphins enabled large strides forward in the pharma industry. I am talking here about the use of morphine in the management of pain, for which the medical use is proven and the need is large.

Arthur Christopoulos. The most obvious effect of deorphanizing GPCRs will be the potential identification of novel drug targets. However, this also raises questions about the validation and pursuit of the deorphanized receptor as a drug target. For example, what is the normal physiological role of the receptor? What disease state is it involved in? Who owns the intellectual property of the chemical space associated with ligands of the receptor?

Susan R. George. Deorphanizing the remaining orphan GPCRs will have an enormous impact on the field of drug discovery. The GPCRs that have recently been identified have each exemplified their importance, in that they have had very unique functions that provide a whole new set of targets for drug discovery. As examples illustrating this, two GPCRs originally cloned by our group and deorphanized recently are the receptors for apelin[173] and for KISS1 (also known as metastin)[174]. The existence of either of these receptor systems was completely unanticipated and unpredicted by physiology or pharmacology, yet each has been shown to have very important and unique functions, and both will prove to be important substrates for drug development. Apelin activation of its receptor has been shown to have hypotensive actions by acting on the vasculature, and marked inotropic action in cardiac contractility. Furthermore, it is DIPSOGENIC, regulates vasopressin secretion and modulates HIV infection of cells.

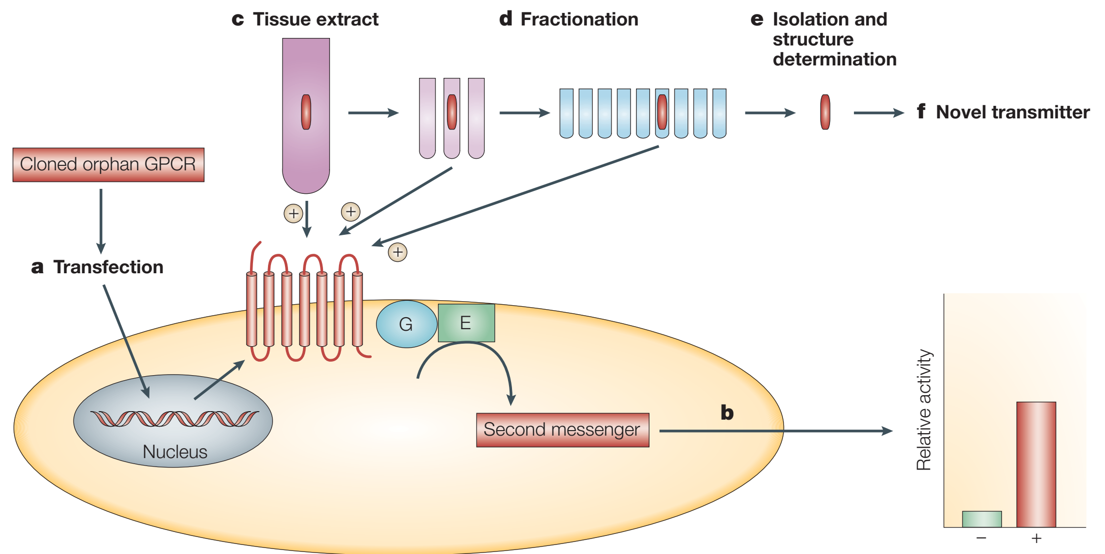

Figure 6 | **Use of orphan receptors to discover novel transmitters.** The search for the endogenous ligands of orphan G-protein-coupled receptors (GPCRs) is based on their expression in heterologous cells and the use of these cells as targets to identify the endogenous ligands. The first orphan GPCRs to be deorphanized were matched to known catecholamines (5-hydroxytryptamine $(5\text{-HT})_{1A}$ and dopamine D_2 receptors). With the application of high-throughput assays, random testing of large compound libraries has led to the matching of numerous GPCRs to known transmitters. However, the disparity between the number of transmitters to be matched and the much larger number of orphan GPCRs led to the hypothesis that many transmitters remain to be discovered. The strategy shown has been developed to identify novel transmitters. **a** | Cells, transfected with an orphan GPCR, will respond to its activation by inducing changes in second messenger levels. **b** | It is the quantitation of these second messenger levels that serves as a monitor for the purification of the transmitter that is activating the orphan GPCR. **c** | Extracts from tissues expected to contain the transmitter are prepared and tested for their abilities to activate the orphan GPCR. **d** | Extracts are fractionated by biochemical approaches, and the activities of the fractions are monitored. **e** | The active fractions are further purified to homogeneity and the structure of the active component is determined through mass spectroscopy. **f** | This isolated compound is a novel transmitter that opens the door to studies on the functional significance of the novel transmitter system in the organism. +, relative activity measured from cells transfected with the orphan GPCR; –, relative activity from control cells (either untransfected or transfected with an unrelated GPCR); E, effector molecule; G, G protein. Figure prepared by Olivier Civelli.

Metastin and its receptor have been shown to be involved in the suppression of cancer metastasis with an important tumour-suppressor role, and have also been shown to have crucial roles in placental implantation and in the initiation of puberty in both sexes. This is a small illustration of the huge potential for drug discovery afforded by the newly deorphanized GPCRs, providing drug targets in clinical areas where no treatments are available at present, and novel targets for complex disorders to augment currently available therapeutics.

Rémi Quirion. Endogenous ligands for various GPCRs have been identified over the past few years, and more are still to come. I believe that this is of the utmost importance, as it will give us a more complete picture of the possible role of GPCRs; for example, in anxious behaviour or obesity. Until now, we have attempted to 'fit' a hypothesis to our preferred GPCR or transmitter system. However, in the near future, with the identification of ligands for most orphan GPCRs, we should be able to generate better hypotheses for the role of a given peptide and its receptor in disorders such as anxiety, obesity and so on, which take into account the more global picture. The identification of the endogenous ligands allows for pharmacological studies aimed at establishing the biological effects of the ligand and its receptors, and so on. Moreover, recent data have shown that some of these orphan GPCRs are uniquely distributed in the CNS and the peripheral nervous system (for example, only in the dorsal root ganglion, implying a possible role in pain, such as the receptor for bovine adrenal medulla (BAM) peptides[2,175]). Identification of their ligands will clearly open up therapeutic avenues.

Jean-Philippe Pin. The first effect of deorphanization is obviously the discovery of new important physiological players, and consequently new possible targets and new physiological regulation processes. In terms of drug development, one important issue is the determination of the specific cellular and subcellular location of these receptors, as well as their expression under different pathological conditions. One issue I would like to discuss here is whether all orphan receptors have a natural ligand. For example, the $GABA_{B2}$ subunit of the $GABA_B$ receptor is not only insensitive to $GABA_B$ agonists (they all bind to the $GABA_{B1}$ subunit), but, according to evolutionary trace analysis, is unlikely to bind any natural ligand[176]. As such, the $GABA_{B2}$ subunit can be considered as an orphan receptor that cannot be deorphanized. Similarly, other receptors may be difficult to deorphanize using a functional assay, as they may require an associated subunit to function, as exemplified by the RAMPS, the $GABA_B$ receptor or the SMOOTHENED RECEPTORS.

Akio Inui. Recent analysis of the human genome sequence predicts the presence of 720 GPCRs, half of which are thought to be sensory receptors[113,177–179]. Of the remaining 360 receptors, the natural ligand has been identified for approximately 210 receptors, leaving around 150 orphan receptors[179]. Approximately 40% of orphan GPCRs have been paired with peptides, and if this proportion continues to be maintained, around 60 further peptides might be paired with those orphan GPCRs. Deorphanizing of GPCRs will therefore have a major impact on the field. Ghrelin is an example of a ligand that was recently discovered in the stomach as an endogenous ligand for the growth hormone secretagogue receptor[180]. Ghrelin is a novel hunger signal from the stomach to the brain with antagonistic, yet complementary actions to leptin, and has an integrative role for the regulation of energy balance and growth[181]. Recently identified ligands for orphan GPCRs are in fact as diverse as peptides, growth factors and bile acids. Elucidation of the ligands and their physiological effects will not only deepen our understanding of physiology and diseases but also lead to innovative new drugs.

Bernard P. Roques. Deorphanizing a GPCR is very important, because it opens the way to other possible pharmacological (clinical) approaches, such as changing the effector level of a peptide by inhibiting its metabolizing pathway. Moreover, the physiological role of the GPCR is indirectly investigated by knocking out the gene that generates the endogenous effector[182]. The methods that are being used at present to find the endogenous effector need to be optimized. Thus, we could imagine using the metabolizing system instead of the orphan receptor to 'fish' for an endogenous peptide effector. This strategy is now under investigation in the biotechnology start-up company PharmaLeads.

Rob Leurs. As can already been noticed in the field, the deorphanization of GPCRs results in increased interest in these new targets, both in the area of receptor pharmacology/physiology and in drug discovery. It is of huge interest to learn which (new) chemical mediators act through GPCRs and to investigate their effect on physiological processes. It is a big opportunity!

19

What outstanding questions in GPCR research are likely to be clarified in the next few years?

Thomas P. Sakmar. With the sequence of the human genome in hand, it might be expected that the complete repertoire of human GPCRs and their ligands will be known in the near future. A longer-term hope would be to know the expression pattern for each GPCR in each different tissue type of the body, depending on the functional state of the tissue. The question of post-translational receptor modification is also finally starting to be addressed.

Jeffrey L. Benovic. Over the next several years, one of the major areas that should be better clarified involves the correlation of receptor structure and function. Current efforts should lead to the crystallization of additional GPCRs and provide insight into conformational differences that occur during GPCR activation, as well as surfaces between GPCRs and interacting partners. Such studies have the potential for shaping the future of drug design.

Roland Seifert. I am concerned that we will not come by GPCR crystals easily. I am not sure whether this is an outstanding question, but if we could figure out the functional differences between intra- and interspecies GPCR polymorphisms, that would be an important link between genomics and molecular pharmacology. To achieve this goal, we will need to conduct an extensive and systematic analysis of all available natural and synthetic ligands for a given receptor. The target receptor will have to be analysed in all of its isoforms. Without detailed knowledge about the three-dimensional structure of a target GPCR, it may be difficult to predict the effects of a given ligand on a given GPCR isoform. Thus, systematic analysis cannot be avoided in this field. However, once we have identified ligands that exhibit differential effects on various GPCR isoforms, we can then use this information for the rational design of more selective drugs and establish, step-by-step, a detailed understanding of ligand–receptor interactions. Such an approach has already been successfully applied to various GPCR families; for example, the histamine receptors[25,58,183].

Another avenue of research will be to identify associations of specific GPCR isoforms with defined disease states or, more probably, subtypes of disease states. Again, we have to be systematic because we may have looked at the wrong receptors in disease states in which we are interested. Related to this, we will have to deal (or, more correctly, struggle) with the functional analysis of GPCR polymorphisms. This is not a trivial task, as the properties of a given GPCR polymorphism may vary in different laboratories[184,185]. Ultimately, however, this approach, hopefully bolstered by good pathophysiological concepts and perhaps facilitated by

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Philip G. Strange earned his Bachelor of Natural Sciences degree at the University of Cambridge, Cambridge, UK, and did his Ph.D. in Organic Chemistry in the lab of Sir Alan Battersby, also in Cambridge. He did his postdoctoral training in the lab of Daniel E. Koshland Jr in Berkeley, California, USA, where he was a Fulbright Fellow and Fellow of the Salters' Institute of Industrial Chemistry. After a further postdoctoral year in the lab of Sir Arnold Burgen in London, he held positions at the Universities of Nottingham, Kent and Reading, where he is now Professor of Neuroscience. He has worked on the structure and function of GPCRs, particularly for neurotransmitters such as dopamine. Highlights of his research include purification of the dopamine D_2 receptor from brain in 1988, showing that antipsychotic drugs are inverse agonists at the D_2 receptor in 1997, and showing that the D_2 receptor is a functional dimer in 2001. In 1992, he published the book *Brain Biochemistry and Brain Disorders*.

serendipity, could result in the development of GPCR therapies targeted to the genotype of an individual. In the area of the drug-metabolizing cytochrome P450 isoenzymes, this individualized approach is already clinically relevant[186]. The methodologies and strategies for accomplishing the above-discussed research aims are already in place. Therefore, I am confident that we will see considerable progress towards these aims in the next few years.

Tamas Bartfai. More GPCR–ligand X-ray structures will be solved, which should enable a better molecular understanding of the definitions of partial agonists, inverse agonists and antagonists. The relevance of oligomerization for GPCR function and regulation of desensitization is also likely to be clarified.

Joël Bockaert. The fine-tuning of GPCR signalling, the deorphanization of GPCRs and the determination of GPCR structures are likely to be clarified in the next few years.

Ronald E. Stenkamp. We will be fortunate if we can obtain answers in the next few years to the structure/function questions I listed for question 7 (page 599). The next set of questions will then be concerned with the connections between GPCR functions and the biology of cells and tissues. Creating effective overviews of all the interconnected signalling pathways and their outputs to provide a useful model of a living system looks very challenging at this point, but is a very important task.

Olivier Civelli. Over the next few years, we will gain significant understanding of the diversity and roles of the sensory GPCRs. We will discover how some of the sensory GPCRs impact brain responses that are now viewed as unrelated to sensory function. We will also enrich our repertoire of novel transmitters and peptides that act on orphan GPCRs. We are going to find, with the help of genomic analyses, an increasing number of links between particular GPCRs and human disorders. But many of these successes will rely on serendipity.

Bernard P. Roques. The fate of GPCRs under various conditions (for example, normal, disease, drug treatment and so on) may be solved. This could result in an improvement in disease treatment (in terms of better efficacy, reduction of tolerance and resistance). In addition, if successful in the clinic, NK_1 receptor antagonists could revolutionize the development of drugs directed towards other neuropeptide GPCRs for analgesia, depression, memory and/or vigilance improvement[187], reduction of food intake, cardiovascular diseases and so on.

Jean-Philippe Pin. In light of the possible formation of dimers, receptor–G-protein stoichiometry and structure are likely to be illuminated in the next few years.

Rick Neubig. The question of whether dimerization is of key importance to the mechanism of G-protein activation, or in determining function or specificity of drugs, will need to be clarified.

20 What areas are unlikely to be clarified by research over the next few years?

Rick Neubig. I think that gaining an understanding of the full contribution of GPCR-interacting proteins will probably require more than a decade. Similarly, the *in vivo* differences between inverse agonists and pure antagonists will also require substantial work, as I predict that this will not be a universal truth, but will be highly system-specific.

Thomas P. Sakmar. On the molecular level, we should achieve a better understanding of receptor activation and receptor–G-protein coupling, but I would not expect a complete clarification anytime soon. With the possible exception of rhodopsin, for which we are beginning to understand how CHROMOPHORE photochemistry is coupled to protein movements, we are still far from understanding the structural basis of GPCR function. As there are no true prokaryotic homologues of GPCRs, the rapid progress seen in the ion channel and transporter fields is not likely to happen with GPCRs. On the physiological side, our knowledge of signalling networks and regulation of G-protein function is becoming more complex every day, and new experimental and theoretical approaches will be required to integrate the vast amount of new information.

Roland Seifert. As I said in question 19 (page 622), the crystals will not show up soon. I also think that the gene-knockout approach in mice will turn out to be less useful for defining receptor functions than we had hoped (see my response to question 13 (page 612)).

Joël Bockaert. The integration of the role of GPCRs in physiology and pathology will probably remain unclarified.

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Competing interests statement

Some of the authors declare competing financial interests: see Web version for details.

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