# A Serpentine Way to Signaling

## Nobel Prize in Chemistry, 2012

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**The Nobel Prize in Chemistry, 2012 has been awarded to Robert J Lefkowitz and Brian K Kobilka for their studies on G-Protein Coupled Receptors (GPCRs). GPCRs are receptor proteins present on the cell surface, which are involved in sensing molecules outside the cell and initiating a cellular response. They can sense a wide variety of ligands ranging from light to hormones and small peptides, triggering various physiological responses. Today we understand the molecular mechanisms by which these receptors work largely due to the contributions of Lefkowitz and Kobilka. Owing to the role of GPCRs in cell communication and involvement in various disease conditions, they are targets of over 40% of current drugs in the market. These findings have not only revealed how GPCRs work, but have also made a huge impact on the field of GPCR-associated drug discovery.**

### Introduction

The hallmark of life lies in its dynamism; a constant play between the ever-changing landscape of environmental cues and an equally impressive array of adaptive behaviours govern the outcome of survival at all levels, from the unicellular prokaryote to the cells and tissues that make up a multicellular organism. In order to detect environmental conditions, termed 'signal', and produce a 'response', cells use a variety of molecules that are involved in sensing the signal, transmitting it to the interior of the cell and finally bringing about a desired change in its physiology.

Simplistically, a signal transduction cascade involves a *receptor* or 'discriminator', which senses the stimulus, 'transducer' which serves to transmit and amplify the signal generated by the stimulated receptor and 'amplifier', which amplifies the signal,

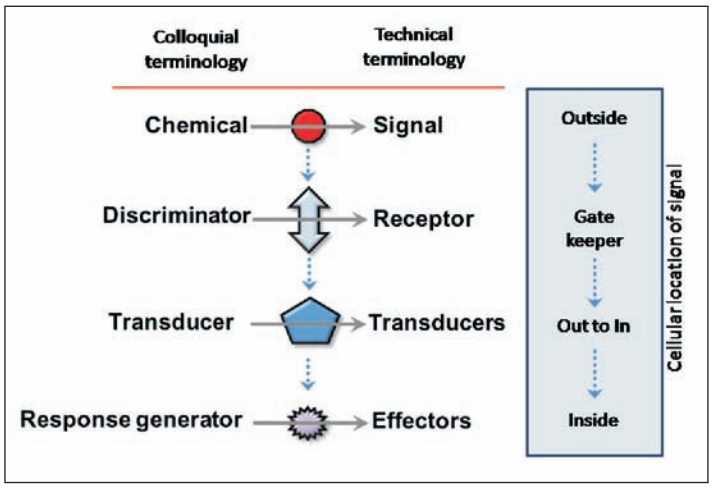


**Figure 1.** Simplified diagram of a signal transduction cascade. A signal transduction cascade represents the route of transfer of information from outside to inside the cell. There are many molecules along this cascade which serve as messengers and amplifiers of the signal. A simplistic representation of a cascade involves a chemical or signal, which activates a discriminator or receptor; the receptor then activates transducers which in turn pass the message onto an amplifier molecule, which passes the signal to the effectors. The effector is the molecule which brings about a cellular response to the signal.

consequently bringing about a change in gene expression or protein behaviour inside the cell (*Figure* 1). Signals are of different types, and may act at short range on cells in the vicinity called paracrine signaling or travel long distances inside the body through the blood, called endocrine signaling. Some signals are self-acting or autocrine where the signaling molecule binds to its receptor on the surface of the secreting cell. Signals can be physical changes such as temperature, pressure, light, or chemicals including gases such as nitric oxide and carbon monoxide, hydrophobic molecules such as steroid hormones or hydrophilic ligands such as growth factors and peptide hormones. In order to sense such a wide variety of signals while maintaining the specificity of the response that is activated, the cell uses a unique receptor for each type of signal. These receptors are localized on the plasma membrane for hydrophilic signals because most signaling molecules cannot cross the hydrophobic plasma membrane barrier to enter the cell. Receptors which recognize hydrophobic or membrane permeable signals are usually present inside the cell. The receptor transfers the signal to intracellular proteins called transducers which convert the stimuli to a chemical form which can be recognised by cellular machinery. Each transducer activates one or more amplifiers, which increase the signal amplitude inside the cells, leading to activation of effectors molecules. Effectors are protein molecules that bind to DNA and cause

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changes in the gene expression of the cell or alter protein activity through protein–protein interactions. Another common function of effectors is to stimulate the movement of ions across the plasma membrane.

The 2012 Nobel Prize in Chemistry was awarded to Robert J Lefkowitz and Brian K Kobilka for their pioneering work on identification and characterization of the receptor component of the signaling cascades. These receptors are known as G-protein coupled receptors (GPCRs) as they are coupled to GTP-bound transducer molecules called G-proteins. They form a superfamily that is widespread throughout the eukaryotic kingdom, and in humans, approximately 800 different genes have been predicted to encode GPCRs, which is about 3.8% of the entire protein-coding genome. This article will take you through the years of discovery, experimentation and characterization of the structure and molecular mechanisms of GPCR signaling and how this discovery impacts a vast area of research in health and medicine.

**History**: **John Langley, Paul Ehrlich and the birth of the receptor theory**.

In the latter half of the 19th century, ideas of drugs as activators or inhibitors of specific physiological responses emerged. Using animal such as dogs, cats and frogs, Langley studied the effect of a variety of plant-derived alkaloids on heartbeat and salivary secretion. Working at the Cambridge University in England, Langley was the first to discredit the prevailing view that all toxins act on the endings of nerve fibres rather than the nerve cell. Testing preparations of alkaloid poisons on frog hearts, he found that local application of nicotine to nerve cells whose pre-ganglionic fibres were destroyed still produced an effect comparable to that of intact fibres. He observed the same effect using adrenaline and pilocarpine on striated muscles and atropine on gland cells. He deduced that the effects of these toxins were dose-dependent, and that the effect of one compound could be counteracted by addition of a greater amount of another compound having the opposite effect. This antagonistic effect of two poisons was

observed in the case of nicotine and curare in experiments on anesthetized fowl, bearing resemblance to his observation twenty-seven years earlier of the antagonism, between pilocarpine and atropine. These observations led him to propose a theory of a '*receptive substance*' to which nicotine and curare bind competitively to elicit different responses in the cell. He went further to show that different kinds of effects could be elicited by different types of poisons hinting at the presence of different receptive substances for different compounds. He also broadly classified receptive substances as those which stimulate contraction and those which inhibit contraction based on intensive work on nicotine receptors in frog muscles.

At the same time in Germany, an immunologist Paul Ehrlich, was developing his own theory on how immune cells bind to bacterial toxins and release antitoxins or antibodies against them. In order to explain the reactions of antibodies and antigens he developed a 'side-chain theory', wherein certain cells possessed side chains that could specifically bind to certain toxins, thus neutralizing them. Ehrlich later substituted the term 'side chain' with 'receptor' and proposed the existence of many receptors that could bind not only to toxins but also to other molecules inside the system. Despite strong criticism from the scientific community, both Langley and Ehrlich continued to support the receptor theory. These criticisms were finally laid to rest in 1933 with the work of Alfred J Clark, who argued that mathematically, the size of toxin molecules was too small to form a monolayer over the entire cell, and that interactions necessitate a mediator to which these toxins can concentrate their effect. He also showed that the relationship of drug concentration and biological effect followed a hyperbolic curve, indicating a 'one-to-one' interaction between the drug and its receptive substance [1, 2].

**Unravelling GPCR Signaling: One Nobel at a Time**

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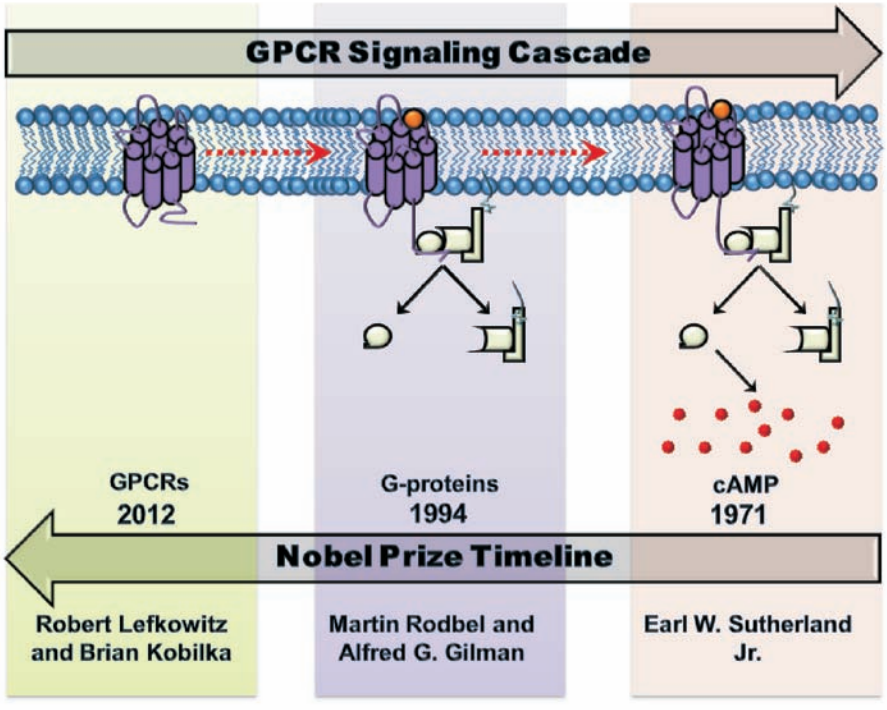
that he was activating a specific 'receptive substance' he had no knowledge of the nature of the receptor, or the mechanism by which receptor activation brought about downstream changes inside the cell. In the case of GPCRs, the pathway from signal to response was characterized in reverse, leaving a trail of Nobel Prizes for each of these discoveries, from 1971 to 2012 (*Box* 1).

In 1958, Earl W Sutherland Jr. discovered the second messenger cyclic adenosine monophosphate (cAMP) which functions as an effector. He was awarded the Nobel Prize in Physiology or Medicine for this work in 1971. Twenty-three years later, the 1994 Nobel Prize in Physiology or Medicine was awarded to Martin Rodbell and Alfred G Gillman for the discovery of heterotrimeric G-proteins in the 1970's. The G-proteins are transducers, acting as the link between the activated receptor and the amplifiers and the effectors. Although by this time the receptor theory was well developed, there was no report of identification

**Box 1. The Timeline of Signaling and the Nobels**



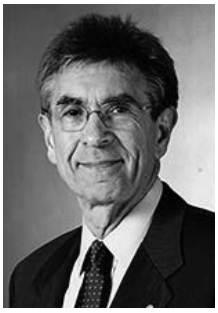
or isolation of receptors. There were even speculations that the receptor and the adenyl cyclase (an amplifier) that had been characterized as part of the pathway were one and the same. The debate on the presence of receptors was settled in1970, when Robert J Lefkowitz used a new technique of ligand radiolabeling to demonstrate the binding of the adreno-corticotropic hormone (ACTH) to its receptors [3] and conclusively proved that GPCRs do exist. The following sections examine the pioneering work of Robert Lefkowitz in detail, and the important contributions of Brian Kobilka, who started as a post-doctoral student in Lefkowitz's laboratory, but went on to carve a niche for himself.

More than 40% of the drugs sold worldwide for various disorders including mental illnesses, heart diseases, allergic responses, gastric disorders are molecules which target GPCRs.

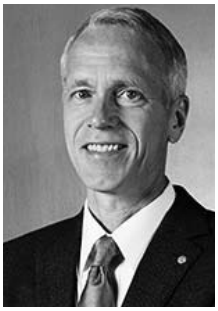
**Challenges of Working with GPCRs**

GPCRs are large membrane-anchored complex proteins that span the membrane seven times. Membrane proteins are generally difficult to study because their surface is relatively hydrophobic as they are integrated in the lipid bilayered cell membrane and need detergent treatment to extract them from membrane preparations. GPCRs are thus difficult to express, solubilise, purify, crystallize and characterize, making working with these receptors an immensely challenging area of research. Lefkowitz and Kobilka's pioneering work on GPCRs has overcome a lot of these challenges and resulted in discoveries which shed light on understanding the molecular mechanisms involved in how a ligand on the outside of the cell can interact with the membrane-spanning GPCR and cause changes that are transmitted to the interior of the cell.

At a time when even the existence of a separate GPCR was doubted, Lefkowitz's research work proved GPCR activity and his group also found the gene which actually coded for a GPCR, the β-adrenergic receptor (receptor for epinephrine). The next challenge was to understand the structure and molecular details of this receptor; this was the major contribution of Kobilka. GPCRs are naturally mobile and transmit signals inside the cell by moving and undergoing conformational changes. It was therefore considered nearly impossible to crystallize them and identify



Robert J Lefkowitz



Brian K Kobilka

their structure in active and inactive forms. Different GPCRs associate with different ligands and different G-protein transducers; hence GPCRs have floppy regions in the ligand-binding site and G-protein interacting residues which make it even more complicated to study the structure of active GPCRs in cells. Most of the crystal structures elucidated during that time were of water soluble proteins, for which obtaining diffracting crystals was much simpler. Kobilka's team was able to design strategies to overcome all these challenges, a difficult task that took them almost two decades.

## The Work of Lefkowitz and Kobilka

The approach, methodology and experiments of Lefkowitz and Kobilka ranged from cloning and purification to modification and crystallization of these receptors, revealing the complex molecular properties of GPCRs. Lefkowitz used chemically synthesized, biologically active adrenocorticotropic hormone (ACTH) and observed binding of this ligand to adrenal membrane preparations. He then shifted focus to understanding the receptors for epinephrine (adrenaline), which is an important hormone and neurotransmitter involved in the regulation of various physiological processes. Lefkowitz used the β-adrenergic receptor as a model system for studying the diverse family of GPCRs, which eventually fetched him and Kobilka the Nobel Prize.

The Nobel Prize winning contribution of Lefkowitz began with the tedious process of isolating, purifying and sequencing these receptors. Lefkowtiz's group solubilised membrane proteins using digitonin and developed ion exchange and affinity chromatography to isolate β-adrenergic receptors in the purest way possible. Phenomenal amount of effort was required to identify suitable ligands to be used in their affinity chromatography columns so that they could isolate these GPCRs [4]. They were successful in purifying huge amounts of β-adrenergic receptor protein to carry out amino acid sequencing of discrete peptides. Sequences of these peptides helped them construct degenerate oligonucleotides using which Kobilka, who joined Lefkowtiz's

group as a post-doctoral fellow, generated a genomic library to screen and clone the elusive β-adrenergic receptor gene [5].

Along the same time, the visual pigment rhodopsin had been isolated and purified in a functional form by other groups and the sequence of the gene encoding bovine rhodopsin was known. The cloning of β-adrenergic receptor gene by Kobilka led to many phenomenal conclusions that led to classifying GPCR into the seven transmembrane receptor family, which gives it the title 'serpentine receptor'. Lefkowitz's group was able to deduce the whole amino acid sequence of the gene from a single exon[1] since they figured out the receptor gene was intronless. Furthermore they went ahead to show that the β-adrenergic receptor shared sequence homology with rhodopsin. Based on this information, they predicted a 7-transmembrane (7-TM) architecture for the β-adrenergic receptor. Lefkowitz and co-workers subsequently cloned a series of related adrenergic receptor and various other groups cloned GPCRs like acetylcholine receptors, which led to the inference that all GPCRs shared the same 7-TM structural arrangement (*Figure* 2) [6].

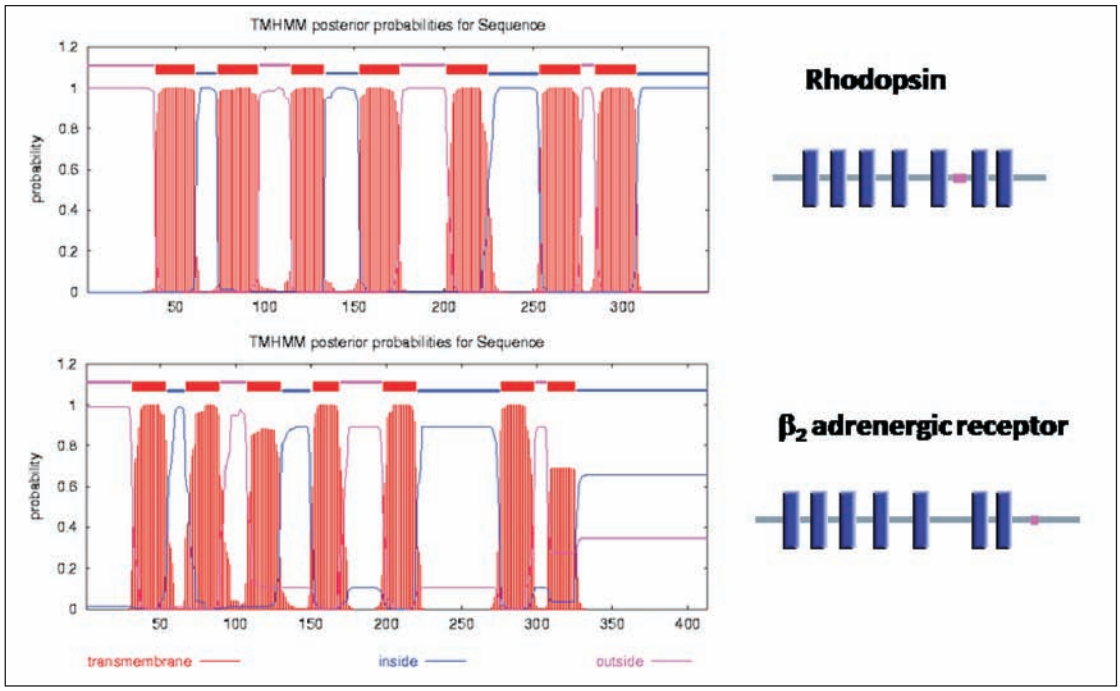



**Figure 2.** Identifying GPCRs using homology to rhodopsin. The amino acid sequences of rhodopsin and β2-adrenergic receptor obtained from NCBI were analysed on the TMHMM server (Transmembrane prediction using Hidden Markov Model; http://www.cbs.dtu.dk/services/TMHMM/ for TM prediction) and the SMART server (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/) for structural similarity. A similar approach was used by Lefkowitz's group to predict structural homology of β-adrenergic receptor with rhodopsin.

[1] Exons are gene regions that code for protein. In eukaryotes, most gene exons are interrupted by intervening non-coding sequences called introns, that are removed prior to translation by a process called gene splicing. Prokaryotic genes lack introns and are translated directly from their mRNA sequence.

[2] An agonist is a molecule that can bind to and activate a receptor.

Lefkowitz and his co-workers had also proposed the ternary complex model for receptor activation, which involves the binding of an 'agonist[2]' to the extracellular part of the 'receptor' and the binding of 'G-proteins' to its intracellular region [7]. The signaling mechanism associated with GPCRs involves 'ligand-induced conformational changes in the receptor' transferring the signal to the cellular interior. Therefore it was important to identify the structural details of inactive and active receptors in order to understand the molecular details of GPCR signaling. During the 1990s, the projection structure of rhodopsin and the presence of seven transmembrane helices were revealed by Gebhard Schertler and Richard Henderson [8]. In 2000, Palczewski and co-workers published the first X-ray crystal structure of a GPCR, that of non-activated rhodopsin [9]. After the discovery by Lefkowitz's group that GPCRs constitute a vast family of receptors sharing the 7-TM architecture (using sequence analyses), it took almost twenty years to crystallize and reveal the structural details of this receptor, a task which was carried out by Kobilka and co-workers at Stanford University.

The crystallization of active ligand-bound GPCRs was extremely challenging because of the dynamic nature of these 7-TM receptors and also because of their size and complexity. Overcoming these challenges, Kobilka designed various ingenious strategies to crystallize and identify the structural activity of these GPCRs. Kobilka first crystallized the β-adrenergic receptor in a complex with a partial inverse agonist and an antibody against the third intracellular loop of the receptor [10]. He then generated a new structure using a receptor fusion protein, replacing most of the third intracellular loop with T4 lysozyme, which is an easily crystallisable protein, to stabilize the floppy regions of the receptor [11]. More recently, they used a nanobody-assisted crystallographic approach for stabilizing GPCR structures in various conformations. Nanobodies are recombinant minimal-sized intact antigen-binding domains of a heavy chain antibody, raised against a purified antigen. In this case, they raised nanobodies against the agonist-bound β-adrenergic receptor. They identified

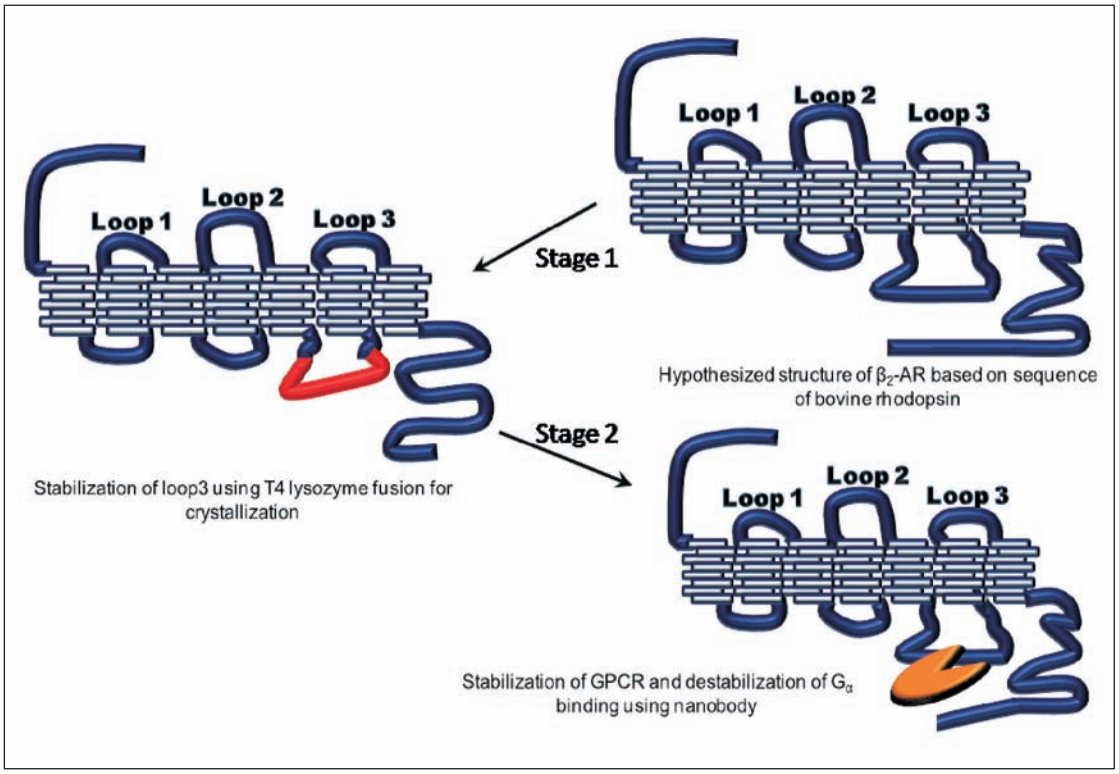



**Figure 3.** GPCR structure elucidation stages. Kobilka and co-workers used two main approaches to stabilize the GPCR structure: they replaced a large portion of the intracellular loop 3 with a fragment from T4 lysozyme (shown in red) to aid crystallization. They also generated nanobodies against ligand-bound receptor (shown in orange) to reduce the movement of the activated GPCR.

an antibody fragment that exhibits G-protein-like behaviour towards the β-adrenergic receptor and were thus able to crystallize the agonist-bound active β-adrenergic receptor along with the nanobody complex for the first time. Kobilka's group went ahead to compare the subtle changes in the GPCR's complex structure during its inactive and active states (*Figure* 3) [12]. Subsequently, they again used nanobodies as molecular 'pliers' to stabilize GPCR–G-protein interaction and resolve dynamics of their interaction in both resting and activated states. In a landmark paper published in 2011 [13], they reported the changes in the GPCR and G-proteins structures during their activation which consequently allowed them to initiate effector activation (*Figure* 4). These discoveries have provided new insights into the area of GPCR–G-protein biology wherein critical domains and regions as well as molecular mechanisms responsible for GPCR signaling have been identified. It is interesting to note here that at the same time another group led by Raymond Stevens was also

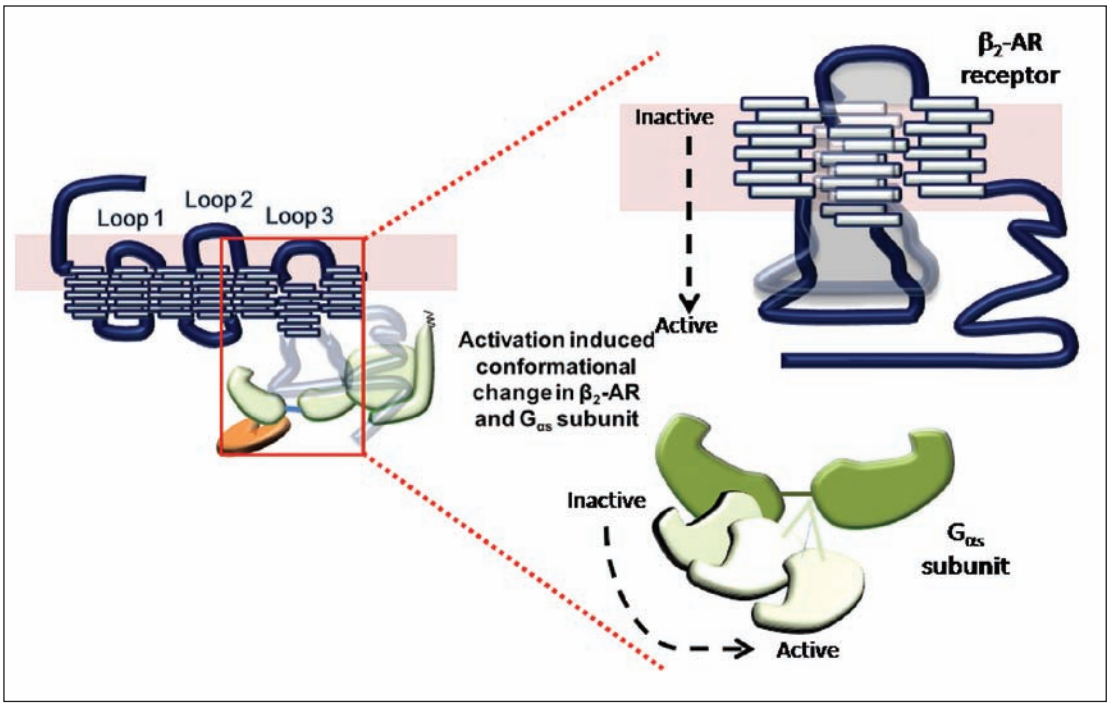



**Figure 4.** Identification of GPCR–G-protein interaction induced changes. The central diagram shows how the stabilization of the GPCR–G-protein interaction complex using nanobodies revealed conformational changes in the loop 3 of the receptor (upper panel) and the movement of the G-protein as subunit (shown in green, lower panel) upon activation.

using similar techniques to elucidate the structure of another GPCR, CXCR4 [14], but it was Kobilka's team which solved the GPCR–G-protein interaction mystery.

## The Future of GPCRs

GPCRs are mediators of most of the physiological responses in our body and their dysfunction is responsible for a large number of disorders, making them attractive targets for drug discovery. Kobilka's study which led to identification of the structure and function of GPCRs at the molecular level has made structure-based drug screening for GPCRs even more attractive. It is a well- known fact that more than 40% of the drugs sold worldwide for various disorders including mental illnesses, heart diseases, allergic responses and gastric disorders are molecules that target GPCRs (*Box* 2). In 1988, Sir James W Black shared the Nobel Prize in Physiology or Medicine for his discovery of the GPCR inhibitor propranolol (used in treatment for heart diseases) and cimetidine (used to treat gastric ulcers).

**Box 2. GPCRs as Drug Targets: The Top Sellers**

| GPCR | Disorder | Drugs in clinical use |
|---|---|---|
| β2 adrenergic receptor | Asthma | Salmeterol |
| | Heart disease | Alprenolol |
| Angiotensin receptor | Hypertension | Losartan |
| Opioid receptor | Pain management | Oxycontin |
| Histamine receptor | Allergies | Fexofenadine |
| | Acidity, Ulcers | Ranitidine |
| Dopamine receptor | Schizophrenia, | Olanzapine, |
| | Bipolar disorder | Clozapine |
| | Parkinson's | |
| Serotonin (5HT) receptor | Schizophrenia, Autism | Risperidone, |
| | Depression, Migraine | Rizatriptan |
| Adenosine (P2Y) receptor | Coronary artery disease | Clopidogrel |
| | Cerebro vascular disease | |

Crystal structures of more GPCRs are expected to widen the understanding of these receptors and their unique signaling mechanisms. But GPCRs being inherently flexible proteins, exhibit conformational dynamism depending on interactions with ligands, transducers or other cellular proteins, making it important to stabilize receptor conformations and understand the rate of conversion between various conformational states of these receptors. Apart from crystal structures, such information can be gleaned from solution-based or membrane-compatible biophysical tools which can actually track molecular motions of these receptors, providing more insight into GPCR dynamics. It is hence anticipated that though all the components of the GPCR signaling cascade have been 'nobely' recognised, it is not the end but beginning of a new inning of GPCR biology.

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