Pharmacogenomic and structural analysis of constitutive G-protein coupled receptor activity

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Abstract

G-protein coupled receptors (GPCRs) respond to a chemically diverse plethora of signal transduction molecules. The notion that GPCRs also signal without an external chemical trigger, i.e. in a constitutive or spontaneous manner, resulted in a paradigm shift in the field of GPCR pharmacology. With the recognition of constitutive GPCR activity and the fact that GPCR binding and signaling can be strongly affected by a single point mutation, GPCR pharmacogenomics obtained a lot of attention. For a variety of GPCRs, point mutations have been convincingly linked to human disease. Mutations within conserved motifs, known to be involved in GPCR activation, might explain the properties of some naturally occurring constitutively active GPCR variants linked to disease. A brief history historical introduction to the present concept of constitutive receptor activity is given and the pharmacogenomic and the structural aspects of constitutive receptor activity are described.

1. Introduction

G-protein coupled receptors (GPCRs) form one of the most versatile families of proteins to respond to the chemically diverse plethora of signal transduction molecules. Hence, for many years this receptor family has been subject of study for human therapeutic benefit. Many top-selling drugs from the past and present target the membrane bound GPCRs and the pipelines of most pharmaceutical industries are filled with GPCR-targeting molecules. With the notion that GPCRs can also signal without an external chemical trigger, i.e. in a constitutive or spontaneous manner, a paradigm shift in the field of GPCR pharmacology was recently initiated. In this overview we aim to give a brief historical introduction to the development of the present concept of constitutive receptor activity, whereafter we will indicate the importance of constitutive GPCR activity in

relation to the present ideas on the structural basis of GPCR (de)activation and to human GPCR pharmacogenomics.

1.1. Early receptor concepts and the molecular basis of drug action.

GPCRs have been subject of study since the early days of pharmacology and many of these investigations have been instrumental to the development of modern concepts of receptor theory. The term *receptors* was initially introduced by Langley (1) and Ehrlich (2) to explain the action of respectively nicotine and toxins. Applying the 'lock – key' model as introduced by Emil Fischer (3), for describing the enzyme-substrate interactions in biochemistry, the founders of early pharmacology suggested 'receptive substances' to exist in order to explain the biological actions of exogenous chemicals on cells. This concept matured with the seminal contribution of Clark, stating that the effect of an agonist is proportional to the number of occupied receptors. His occupancy theory (4, 5) also readily accommodated the difference between an agonist and an antagonist, following the 'lock – key' principle of Fischer.

In the 1960s Ariëns and co-workers published their well-known book "Molecular Pharmacology" (6), in which the work of Clark was extended. Ariëns et al introduced the concept *intrinsic activity* to explain the observation that not every agonist of a given receptor induced the same maximum effect. Compounds reaching the maximum were referred to *full agonist* (intrinsic activity is 1) and other agonists were named *partial agonist*, having an intrinsic activity between 0 and 1. *Competitive antagonists* were supposed to have an affinity for the receptor, but to posses an intrinsic activity of 0. The Clark-Ariëns model was extended first by Stephenson (7) and later Furchgott (8, 9) with the introduction of drug *efficacy* and the system-independent concept of *intrinsic efficacy*.

The developed concepts have had a great impact in the area of pharmacology and drug discovery, especially as the mathematics applied were simple and made it possible to calculate in an easy way the affinity and the activity of agonists as well as the affinity of antagonists. Looking back it is most remarkable that the ideas about receptor activation have been developed during a period of about 75 years, when no real information on the biochemical nature of receptors was available, not to speak about the molecular mechanisms involved in the generation or transfer of a signal. In the Introduction to the book *Molecular Pharmacology* (6) a receptor was compared with a beautiful lady; *you may write a letter to her, sometimes she answers but she never shows up, though some day she may do so*. Moreover, during a conference of the NY Academy of Sciences in 1967 Ariëns admitted in a very clear way: "*when speaking about receptors I am talking about something I do not know anything about*" (10).

It seems that a medicinal chemist (Nauta) and not a pharmacologist, has proposed in 1968 for the first time that a GPCR family member might be a protein adopting a helical conformation, using the receptor for histamine as his model (11) Using

this purely hypothetical model reversible interactions between a ligand and the amino acid side chains of the receptor protein were proposed to be involved in the binding of both agonists and antagonists (Figure 1A).

1.2 From GPCR gene cloning to constitutive, agonist-independent signaling and inverse agonists

With the introduction of the molecular biology in the area of G-protein coupled receptors, it lasted until 1986 before it became clear that the ideas of Nauta were quite close to reality (12). We now have high-resolution X-ray structures of at least one GPCR (figure 1B), rhodopsin {Palczewski, 2000 #4404;Li, 2004 #4403} available and a wealth of information on the structure-function relationships of various GPCRs (13), including drug binding and GPCR activation (see section 2), has been gathered. In addition, genome sequencing projects have permitted to classify the human GPCR sequences into five main families: rhodopsin (Class A or family 1), secretin (Class B or family 2), glutamate (Class C or family 3), adhesion, and frizzled/taste2 (14). The rhodopsin family is the largest and is subdivided in four main groups (α, β, γ, δ) with 13 sub-branches (α: prostaglandin, amine, opsin, melatonin, MECA; β: peptides; γ: SOG, MCH, chemokine; δ: MAS, glycoprotein, purine, olfactory). These groups include orphan GPCRs, receptors for which the ligand and the (patho)physiological function remain unknown. Specialized databases of GPCRs can be found at http://www.gpcr.org/7tm (15) and http://www.iuphar-db.org (16).

Our currents insights in GPCR activation have in the last years strongly relied on the notion that single point mutations could render GPCRs constitutively active, i.e. could signal without the presence of the respective agonist (17-19). At the same time, all these studies have also led to the general concept that constitutive GPCR signaling is an intrinsic property of most (if not all) GPCR family members and that either GPCR ligands or single point mutations can change the equilibrium between inactive and active receptor states (18-20). Looking back, it is interesting to notice that already years before this general acceptance, research with only limited tools had provided convincing experimental evidence for constitutive GPCR signaling. In 1989 Costa and Herz started a shift in the paradigm on drug action with a paper, describing antagonists with negative intrinsic activity at wild type delta opioid receptors, endogenously expressed in NG108-15 neuroblastoma cells (21). In this paper, delta antagonists inhibited the basal GTPase activity with differential negative intrinsic activity and for the first time GPCR pharmacology clearly was faced with intrinsic drug activity going from 1 for agonists to -1 for antagonists with negative efficacy (now also referred to as inverse agonists). Many studies with either wild type or mutant GPCRs have thereafter confirmed the fact that GPCR proteins can signal in an agonist-independent, constitutive way and this has been

extensively reviewed before (19, 22). The notion of constitutive GPCR activity and the bidirectional modulation of GPCR activity by ligands has led to the introduction of a simple two-state model of GPCR action. In this model a GPCR protein can shift spontaneously between an inactive **R** to an active **R*** conformation (23-25). GPCR agonists shift the equilibrium to the active **R*** state, whereas inverse agonists favors the inactive **R** state. The two-state model also explains the observations that some antagonists do not affect constitutive GPCR signaling, since these *neutral antagonists* are considered not to affect the thermodynamic equilibrium between the different protein conformations. The two-state model and the concept of inverse agonism are now generally accepted and have been included in general pharmacology textbooks.

1.3. Constitutive GPCR activity of wild type GPCRs

As discussed above, the concept of constitutive activity was more or less generally accepted following convincing data sets obtained with various constitutively active mutant (CAM) GPCRs, which were generated in the lab. Yet, with the increased efforts in this area it has become clear that many wild type GPCRs also show considerable levels of constitutive activity. This has recently been systematically reviewed by Seifert and Wenzel-Seifert (19) and will therefore only be briefly discussed in this review.

For more than 60 wild type GPCRs from the class A, B and C families constitutive activity has now been documented (19). In particular the GPCRs encoded by herpesviruses exhibit constitutive activity, providing valuable information on this phenomenon that has been linked to the development of disease (see section 3) (26, 27). Especially the availability of recombinant expression systems has been instrumental in this recognition. The extent of constitutive GPCR activity depends on the expression level of the respective receptor and the cellular context (19). Constitutive GPCR activity might e.g. be boosted by increased expression of G proteins or additional downstream effector molecules (19). Nevertheless, for several GPCRs constitutive activity has been observed in native tissue or cells (19). Prominent examples are the histamine H_3 receptor (28, 29) and the melanocortin MCR_1 and MCR_4 receptors, for which endogenous inverse agonists seem to be essential for a proper homeostasis (see also section 3) (30).

For many of the constitutively active GPCRs also inverse agonists have been identified (19). Most of the compounds that were previously characterized as competitive antagonists with intrinsic activities of 0, now turn out to be inverse agonists with negative intrinsic activities between -1 and 0. Common GPCR antagonists and important therapeutic agents, like e.g. adrenergic α_1 and β_1 receptor antagonists (e.g. prazosin and metoprolol) (31), angiotensin AT_1 receptor antagonists (e.g. losartan) (32, 33), dopamine D_2 receptor antagonists (e.g. haloperidol) (34) leukotriene receptor antagonists (e.g. montelukast) (35) and histamine H_1 and H_2 receptor antagonists (e.g.

cetirizine and cimetidine) (36, 37) are now all recognized as inverse agonists at their respective targets. At present, it is however not very clear if the therapeutic success of these molecules is related to their negative intrinsic activity, since neutral antagonists have either not yet been identified or have only been tested in a limited number of studies. At the serotonin $5HT_{2C}$ receptor inverse agonist activity of antagonists did not correlate with their clinical efficacy as antipsychotics (38), but on the other hand the clinical efficacy of serotonin $5HT_{2A}$ receptor ligands was reported to depend on their inverse agonistic activities (39). Similarly, clinical efficacy of the beta blocker metoprolol in heart failure seems to be due to its inverse agonist properties as the neutral antagonists bucindolol is not effective (20, 40). These studies indicate that in certain conditions the therapeutic outcome of inverse agonists and neutral antagonists can indeed be different. In this respect, one also has to consider that long-term exposure to inverse agonists has been found to lead to upregulation of receptors, which might not always be beneficial and a potential reason for e.g. the development of treatment tolerance (37, 41, 42).

2. Structural aspects of (constitutive) GPCR activation.

In contrast to the wealth of available pharmacological data, structural information on GPCRs is still scarce. To date, the only crystal structure available is that of the inactive state of bovine rhodopsin (43, 44). Five structures of rhodopsin are available at the Protein Data Bank, at resolutions of 2.8 Å (PDB identifiers 1F88 and 1HZX), 2.65 Å (1GZM), 2.6 Å (1L9H), and 2.2 Å (1U19). Rhodopsin is formed by an extracellular N–terminus of four β-strands, seven transmembrane helices (TM 1 to TM 7) connected by alternating intracellular (I1 to I3) and extracellular (E1 to E3) hydrophilic loops, a disulfide bridge between E2 and TM3, and a cytoplasmic C–terminus containing an α-helix (HX 8) parallel to the cell membrane. Statistical analysis of the residues forming the TM helices of the rhodopsin family of GPCRs (Class A) shows a large number of conserved sequence patterns (45). This sequence conservation has been used by Ballesteros & Weinstein (46) to define a general numbering scheme consisting of two numbers: the first (1 through 7) corresponds to the helix in which the amino acid of interest is located; the second indicates its position relative to the most conserved residue in the helix, arbitrarily assigned to 50: $N1.50^{55}$ (the superscript represents the residue number of rhodopsin, 100% conserved in the family), $D2.50^{83}$ (94%), $R3.50^{135}$ (96%), $W4.50^{161}$ (96%), $P5.50^{215}$ (77%), $P6.50^{267}$ (100%), and $P7.50^{303}$ (96%). These patterns are easily identifiable on a multiple sequence alignment and allow easy comparison among residues in the 7TM segments of different receptors. This generic numbering scheme of amino acid residues in GPCRs is employed throughout the entire manuscript, when referring to the GPCRs of the class A family.

The molecular actors involved in the mechanisms of GPCR activation are still not fully understood. Farrens et al. have shown that extracellular signals trigger rigid-body motions of several, if not all TMs leading to the active state of the receptor (47). It was, thus, proposed that the inactive conformation of the receptor is maintained through restraining intramolecular interactions impeding these TM motions. Release of these constraints is induced by either agonists or constitutive activity-inducing mutations within the receptor. The discovery of CAM GPCRs, together with homology models constructed from the rhodopsin template has yielded new insights into the mechanism of rhodopsin-like GPCR activation. Importantly, the sequence conservation pattern of GPCRs within this family suggests that this activation mechanism might occur by means of common motifs mainly located at the middle part and cytoplasmic ends of the TM helices (45). This section describes the different motifs that are involved in GPCR activation. This information will be used to explain the properties of naturally occurring GPCR mutants in section 3.

2.1. The ionic lock.

The interaction between R at position 3.50 of the highly conserved (D/E)R(Y/W) motif in TM3 with its adjacent D/E residue at position 3.49 and an additional D/E at position 6.30 near the cytoplasmic end of TM6 (Figure 2c) is known as the ionic lock (48). Charge-neutralizing mutation of D/E6.30 in TM 6 results in increased constitutive activity (48, 49). Removal of the ionic interaction between D/E6.30 and R3.50 in this CAM receptor facilitates the movement of the cytoplasmic end of TM6 away from TM3 by means of the considerable Pro6.50-induced bend angle of TM 6 (44, 48). This type of mutation has been described in patients with spontaneous ovarian hyperstimulation syndrome (see section 3.1.2). Mutation of D/E3.49 in TM3 to either A or N removing the ionic interaction with R3.50 also increases the constitutive activity of rhodopsin (50) and a number of structurally related class A GPCRs (48, 51, 52). Thus, removal of this ionic constrain makes the side chain of R3.50 free to point towards the protein core (the direction of the Cα-Cβ bond).

2.2. The hydrophobic arginine cage.

Ballesteros et al. (53) suggested that this highly conserved R3.50 is also restrained in the inactive conformation by a cage shaped by conserved hydrophobic amino acids at positions 3.46 (L:15%; V:8%; I:58%; M:15%) and 6.37 (L:37%; V:24%; I:20%; M:5%) (Figure 2c). Removal of these interfering bulky constrains by A or G replacement leads to constitutive activity in a number of cases (54-56).

2.3. Intracellular helix 8.

The recent X-ray structure of rhodopsin, revealed the presence of a highly conserved helix 8, suggested to be implicated in G protein coupling (43, 57). Figure 2b shows the interaction of Y7.53 in TM7 with F7.60 in Hx8 and with the side chain and backbone (via water molecule #7) of N2.40 in TM2. Y7.53 and F7.60 are highly conserved in the rhodopsin family of GPCRs (Class A) (92% and 68%, see Figure 2) forming the NPxxYx$_{5,6}$F motif (58). This aromatic-aromatic interaction was proposed to be disrupted during receptor activation, leading to a proper realigning of Hx8 (58, 59). It has also been proposed that the conserved charged (K:17%; R:54%) or polar (Q:11%) side chain at position 7.61 has a specific role in stabilizing the free, helix-ending, carbonyls at positions 7.54 and 7.55 in TM7 through hydrogen bond interactions (Figure 2b). This interaction seems to exert a key role in receptor stabilization, directing in part constitutive receptor activity but also the ligand binding profile of the KSHV-encoded chemokine receptor ORF74 (see section 3) (60).

2.4. The asparagine of the NPxxY motif.

The highly conserved N7.49 of the NPxxY motif in TM 7 acts as an on/off switch by adopting two different conformations in the inactive and active states (61, 62). N7.49 is restrained, in the inactive state, towards TM 6 either via water molecule #9 in rhodopsin (63) and other family A GPCRs (Figure 2d) or through the interaction with the T6.43D6.44 motif in the glycoprotein hormone receptor family (61, 62) (see section 3.1.2). It was proposed that upon receptor activation N7.49 adopts the trans conformation to interact with D2.50 of the (N/S)LxxxD motif in TM2 (62). It was hypothesized that this combination of charged and polar side chains leads to a negative electrostatic landscape, which could force relocation of R3.50 from the ionic lock (62, 64). Any mutation modifying the N7.49 equilibrium, favoring the inactive or active conformation, decreases or increases, respectively, the constitutive activity of the receptor.

2.5. The hydrophobic asparagine cage.

Alike to the arginine cage, N7.49 is also located in a cage, restraining its conformation towards TM6 in the inactive state, formed by conserved hydrophobic amino acids at positions 2.46 (L:91%) and 6.40 (L:14%; V:42%; I:28%; M:5%) (Figure 2d). Removal (mutation to A or G) of the bulky and β- or γ-branched amino acids at positions 2.46 in rhodopsin (65) and the TSH receptor (62) and 6.40 in rhodopsin (66), serotonin 5HT$_{2A}$R (67), and histamine H_1 receptors (64) induces constitutive activity.

2.6. Extracellular loop 2.

Recently, Klco et al. have shown that the E2 loop, containing a Cys engaged in a disulfide bridge with TM3, acts as a negative regulator of C5a receptor activation (68). Random saturation mutagenesis of the amino acids forming this E2 loop shows in nearly 80% of the functional receptors an increase of constitutive activity. The high variability with respect to length (from 4 to more than 50 residues (45)) and amino acid composition in the different GPCR families suggest a non conserved structure of the E2 loop. As of yet it is not clear by which molecular mechanism the E2 loop stabilizes the inactive conformation of the C5a receptor.

2.7. The W6.48 rotamer toggle switch.

The recent structure of metarhodopsin I, determined by electron crystallography (69), has shown that W6.48 of the CWxP motif in TM 6 undergoes a conformational transition from pointing towards TM7, in the inactive state, to pointing towards TM5, in the active state, as was previously suggested by experimental studies in rhodopsin (70) and computer simulations (71). Rearrangement of W6.48 and the nearby C6.47 decreases the highly conserved Pro6.50-induced bend angle of TM 6 (71), moving the cytoplasmic end of TM 6 away from TM 3 and disrupting the proposed ionic lock between D/E6.30 in TM 6 and R3.50 in TM 3 (48). It has also been suggested that the side chain at position 3.36 acts as a rotamer toggle switch simultaneously with W6.48 (72, 73), modulating the constitutive activity of the receptor.

2.8. A conserved hydrogen bond network linking D2.50 and W6.48.

D2.50 is involved in maintaining W6.48 pointing towards TM7 in the inactive state of the receptor through a conserved hydrogen bond network (44, 69). This conserved network varies among GPCR subfamilies. Rhodopsin forms this network through water molecules #12 and #10 (Figure 2d, top panel) (44). N7.45, present in 67% of the rhodopsin-like sequences but absent in rhodopsin, would be located at the same position as water#10. Thus, N7.45-containing receptors are able to form the D2.50··#12··N7.45··W6.48 network (Figure 2d, middle panel) (72). Similarly, N3.35 (29% of the receptors) would be located at the same position as water molecule#12, thus, N3.35/N7.45-containing receptors would form the D2.50··N3.35··N7.45··W6.48 network of interactions (Figure 2d, bottom panel) (74). Disruption of this network by mutating either N7.45 in the H_1 receptor (72) or N3.35 in the AT1 receptor (75) leads to constitutive activity because it facilitates the reported conformational transition of W6.48 during receptor activation (69).

2.9. Molecular basis of (inverse) agonism.

Many wild-type GPCRs display only moderate constitutive activity under normal conditions and can be significantly activated by addition of agonists. However, GPCRs can in general easily be modified to display enhanced basal activity and often this constitutive activity can be linked to diseases (17, 19) (see section 3). In this respect, inverse agonists are potentially important therapeutics in the treatment of diseases caused by constitutive activity-inducing mutations of the WT receptor.

The motifs described in sections 2.1-2.8 appear crucial determinants for the molecular basis of both agonism as well as inverse agonism. The processes initiated by the recognition of the extracellular ligand by the receptor depends to a large extent on the type of receptor, since they can be activated by a wide range of extracellular ligands, including small neurotransmitters to large hormones. Each subfamily has most likely developed specific structural motifs that allow the receptor to accommodate and respond to its cognate ligand. However, it seems reasonable to propose that in W6.48-containing GPCRs (71% of the rhodopsin-like sequences), ligand binding modifies the conformation of W6.48. Upon activation, either by agonists or constitutive activity inducing mutations, a conformational transition of W6.48 towards TM5 occurs (see section 2.7). Thus, GPCRs possess a small cavity between TMs 5 and 6 to accommodate the side chain of W6.48 in the active conformation. This small cavity is formed by the side chains at positions 3.40 (L:9%; V:25%; I:42%; M:5%), 5.47 (F:70%; Y:11%), and 6.52 (H:29%; F:20%; N:19%). The role of the F/Y5.47 and F6.52 aromatic side chains is to further stabilize the active conformation of W6.48 by aromatic-aromatic interactions in the face-to-edge orientation (Figure 2a, right panel). In addition to the known interaction of aminergic ligands with D3.32 in TM 3 and a series of residues at positions 5.42, 5.43 and 5.46 in TM 5 (76), an interaction with W6.48 is found for agonists in the histamine H_1 receptor (Jongejan, unpublished results) and the 5-HT_{1A}R. We propose that agonists trigger this conformational transition of W6.48 by means of an explicit hydrogen bond or an aromatic-aromatic interaction or both. The right panel of Figure 2a shows a 5-HT_{1A}R agonist in the binding pocket of the receptor (77).

In contrast to the conformational transition triggered by an agonist or a constitutive activity-inducing mutation, an inverse agonist will stabilize or reinforce the constraints that keep the receptor in its inactive state. The left panel of Figure 2a shows the inverse agonist 11-*cis*-retinal located in this cavity between TMs 5 and 6 in rhodopsin. We propose that inverse agonists occupy this small cavity to impede the transition of W6.48 towards TM5, thus, decreasing the constitutive activity of the receptor. The middle panel of Figure 2a shows the inverse agonist ketotifen in the binding pocket of the histamine H_1 receptor. The aromatic phenyl moiety of the ligand favorably interacts with the aromatic F/Y5.47, W6.48, and F6.52 side chains, and is an

important pharmacophoric element of inverse agonists, blocking the conformational transition of W6.48.

3. Pathophysiological consequences of naturally occurring constitutively active GPCR variants.

With the recognition of constitutive GPCR activity and the notion that GPCR binding and signaling can be strongly affected by a single point mutation, GPCR pharmacogenomics recently obtained a lot of attention. For a variety of GPCRs, point mutations have been convincingly linked to human disease. In this section, we will review the present knowledge on naturally occurring mutant GPCRs involved in human disease and linked to constitutive activity. Moreover, we will try to explain the GPCR phenotype in relation to the presented structural motifs that are thought to be involved in GPCR activation.

3.1. Class A GPCRs

3.1.1. Rhodopsin

Vision under dim-light conditions by retinal rod photoreceptor cells is mediated by the visual pigment rhodopsin. Rhodopsin consists of the apoprotein opsin, a class A GPCR, to which an 11-*cis*-retinylidene chromophore is covalently bound through a protonated Schiff-base linkage to the ε-amino group of $K7.43^{296}$ in TM 7 (78), and $E3.28^{113}$ in TM3 acting as a counterion for this linkage (79). Bound 11-*cis*-retinal acts as an inverse agonist by constraining rhodopsin in an inactive conformation in the dark (80). Light absorption photoisomerizes 11-*cis*-retinal into the full agonist all-*trans*-retinal, which initiates consecutive conformational changes in the rhodopsin TM domain (TMD), ultimately leading to G protein transducin coupling and subsequent photoreceptor cell signaling (80). Autocatalyzed hydrolysis of the Schiff-base linkage results in the dissociation of the all-*trans* chromophore, upon which the dark-state (inactive) rhodopsin is regenerated by binding of new 11-*cis*-retinal to the unoccupied binding site.
The apoprotein opsin is constrained in a relatively inactive conformation by a salt bridge interaction between $E3.28^{113}$ and $K7.43^{296}$ (81, 82). Disruption of this salt bridge by mutating either $E3.28^{113}$ or $K7.43^{296}$ results in constitutive activation of opsin (81). A $K7.43^{296}E$ mutation was found in a family with a severe form of autosomal dominant retinitis pigmentosa (ADRP) (Table 1), which manifests itself clinically by night blindness and a progressive loss of vision due to the degeneration of both rod and cone photoreceptor cells (83). This CAM opsin is unable to interact with 11-*cis*-retinal and consequently signals continuously (81). Three other mutations in the opsin gene (*i.e.* $G2.57^{90}D$, $T2.61^{94}I$ and $A7.39^{292}E$) have been identified in families with autosomal-

dominant congenital stationary night blindness (CNSB) (Table 1). Expression of these three opsin mutants in heterologous cells results in constitutively signaling in the absence of a bound chromophore, by interfering with the salt bridge between E3.28^{113} and K7.43^{296} (84). However, all three CAM opsins are inactive in darkness when reconstituted with 11-*cis*-retinal and can be activated upon light absorption (85). Interestingly, the G2.57^{90}D opsin mutant binds 11-*cis*-retinal with an ~80-fold slower rate than wild type opsin and the T2.61^{94}I and A7.39^{292}E mutants (86). CNSB is a less severe retinal disorder in comparison to ADRP, generally resulting in an impaired vision under dim light condition and limited or no degeneration of rods. In contrast, continuous activation of the photosignaling cascade, either caused by an inability to bind 11-*cis*-retinal (*i.e.* CAM opsin K7.43^{296}E) or disrupted biosynthesis of 11-*cis*-retinal (*i.e.* Rpe65 enzyme mutation), is considered to lead to retinal degeneration (84).

3.1.2. Glycoprotein hormone receptors

The thyroid-stimulating hormone receptor (TSHR), luteinizing hormone/chorionic gonadotropin receptor (LHCGR), and follicle-stimulating hormone receptor (FSHR), form the subfamily of glycoprotein hormone receptors (GpHRs) (87). These receptors distinguish themselves from other class A GPCRs (16) by having a large N-terminal exodomain (NTED), which constitutes the selective hormone binding site (88-91). Disease-causing CAMs have been found for all three members of this subfamily. Interestingly, the TSHR is more susceptible to natural occurring CAMs than the LHCGR and FSHR (92).

Thyroid-stimulating hormone receptor

The thyroid regulates overall body metabolism by secreting thyroid hormones. Pituitary-derived thyroid-stimulating hormone (TSH) controls growth, differentiation and functioning of the thyroid gland by activating the TSHR (93). Autosomal and somatic CAMs in the TSHR gene causes familial nonautoimmune hyperthyroidism and thyroid adenoma, respectively (94). Both pathological conditions are associated with autonomous secretion of thyroid hormones resulting in an acceleration of body metabolism. Hitherto, 38 natural occurring CAMs have been identified in the TMD of the TSHR, with the bottom of TM6 being a hotspot (Figure 3; Table 1) (92). This is rationalized by the fact that the TSHR contains the family-specific T at position 6.43 and D at position 6.44, which are the main partners of N7.49 in the inactive state of the receptor (61, 62) (see section 2.4). Interestingly, also 5 natural occurring CAMs (*e.g.* S^{281} of the conserved TYPSHCCAF motif) have been identified in the so-called hinge-region of the NTED. In fact, designed deletion or mild trypsin digestion of the NTED unmasked the constitutive active character of the TSHR TMD (95, 96). Hence, the NTED acts as a tethered inverse agonist to

constrain the TMD in a relatively inactive conformation, which is released upon TSH binding to the NTED, or can be overruled by point mutations in the hinge region of the NTED (see below).

Luteinizing hormone receptor

The development of the testis and external male genitalia is dependent on testosterone production by Leydig cells (97). During fetal development the proliferation, differentiation and testosterone production of these cells is induced by LHCGR signaling in response to placental-derived chorionic gonadotropin (CG). After birth, the Leydig cells remains largely inactive until the advent of puberty when pituitary-derived luteinizing hormone (LH) stimulates testosterone production by activating the LHCGR (98). At this stage testosterone induces the development of male secondary sex characteristics and contributes together with follicle-stimulating hormone (FSH) to maturation of reproductive organs and the initiation of spermatogenesis. Hitherto, 17 disease-causing CAMs have been identified in the TMD of the LHCGR (Figure 3; Table 1) (92, 99). Most CAMs in the LHCGR affects amino acids in TM6, with *e.g.* D6.30^{564}G releasing the ionic lock (see section 2.1), and T6.43^{577}I and D6.44^{578}E/G/Y affecting the NPxxY motif-mediated receptor activity constraint (see section 2.4). The latter constraint is also perturbed in the LHCGR by L3.43H/K/R substitution, due to the formation of a salt bridge between the positive charge at position 3.43 and D6.44 (100, 101). Notably, while the L3.43H/K mutants are able to respond to the hormone, the L3.43R mutant is unresponsive to further hormonal stimulation (101). Mutational analysis revealed that also the NTED of LHCGR constrains the TMD in an inactive conformation, however, unlike the TSHR no natural CAMs have so far been observed in the LHCGR NTED (102-104). Autosomal CAMs in the LHCGR gene causes familial male-limited precocious puberty (FMPP) or testostoxicosis, whereas somatic CAMs induce sporadic Leydig cell tumors. Male infants inheriting CAMs in the LHCGR gene show accelerated virilization before the age of 4 as a consequence of testosterone hypersecretion by Leydig cells (97). Besides the precocious development of testosterone-driven male secondary sex characteristics, FMPP is associated with an early growth spurt and accelerated bone maturation resulting in a short stature.

Fetal development of female sexual organs, on the other hand, is independent of gonadotropins and sex steroid hormones (97). Pubertal maturation of female reproductive organs and secondary sex characteristics, on the other hand, is driven by estrogens that can only be produced by the ovary upon combined stimulation with FSH and LH (see below) (98). This requirement of both FSHR and LHR activity in regulating ovary function explains the absence of apparent pathophysiological phenotypes in females with a CAM LHCGR (97).

Follicle-stimulating hormone receptor

FSH and testosterone regulate together the secretion of spermatogenesis-supporting paracrine factors by Sertoli cells in the testis (105). A D6.30^{567} to G mutation in TM6 of the FSHR was identified in a hypophysectomized male patient under treatment with testosterone replacement who retained fertility in the absence of FSH and LH (106). This FSHR mutant constitutively elevated cAMP levels in transfected Sertoli cells and mimicked FSH-like activity upon targeted expression on Sertoli cells in transgenic gonadotropin-deficient mice (107-109). The increased constitutive activity caused by this particular mutation is attributed to disruption of the ionic lock (see Section 2.1, Figure 2c).

In females, the cyclic elevation of plasma FSH levels stimulates recruitment and maturation of advanced follicles in the ovary by activating FSHR on the follicle-surrounding granulosa cells (110). FSHR activity upregulates aromatase enzyme expression in these cells allowing the conversion of LH-dependent theca cell-derived androgens into estrogens. Estrogens on their turn stimulate pubertal development in females and play an essential role in the cyclical preparation of the female reproductive tract for conception (98, 110).

Five different CAMs (*i.e.* T3.32^{449}I/A, I5.54^{545}T, and D6.30^{567}G/N) in the FSHR gene were found in the families in which women exhibit familial spontaneous ovarian hyperstimulation syndrome (sOHSS) during pregnancy (49, 111-114). sOHSS is a rare syndrome which may lead to life-threatening complications such as massive ovarian enlargement, multiple ovarian cysts formation, and ascites (115). Mutations at the T3.32 position modify the highly conserved hydrogen bond network linking D2.50 and TM6 (see section 2.8) and the conformational equilibrium of N7.49 (section 2.4), whereas mutations at the D6.30 position modify the ionic lock between TMs 3 and 6 (section 2.1, Figure 2c, Figure 3) (49). Besides being constitutively active, these sOHSS-causing FSHR mutants display increased responsiveness to CG as compared with the wild type FSHR (49, 111-114). This apparent promiscuity is quite surprising given the fact that all CAMs were in the TMD and selectivity of glycoprotein hormone receptors for their cognate hormones is defined by their NTED (89-91). The increased sensitivity to CG explains the clinical manifestation of sOHSS during the first trimester of pregnancy when CG plasma levels are highest. Since the increased responsiveness to CG was not associated with an increase in affinity it was hypothesized that loosening the intramolecular barrier to receptor activation would allow promiscuous receptor activation by low affinity agonists. In fact, mutational analysis revealed a direct relation between the level of constitutive activity and the responsiveness to promiscuous hormones (49).

Hitherto, patients with nonautoimmune hyperthyroidism receive treatment with antithyroid drugs (*e.g.* carbimazole), that interferes with thyroid hormone synthesis. Although antithyroid drugs are efficient in controlling hyperthyroidism, they do not prevent thyroid enlargement (116). Consequently, antithyroidal drug therapy is usually followed by thyroidectomy. FMPP is currently controlled by either inhibiting adrenal and testicular androgen biosynthesis using the P450 cytochrome inhibitor ketanazole, or combined administration of an androgen receptor antagonist (spironolactone) and aromatase inhibitor (testolactone) (117, 118). Recently, specific non-peptide antagonists and agonists have been identified for the FSHR and LHCGR (119-124), with therapeutic potential for anticonception and assisted-reproduction, respectively. Hence, identification of non-peptide inverse agonists specifically inhibiting constitutive signaling of LHCGR, FSHR, or TSHR may therefore be a matter of time.

3.1.3. Growth hormone secretagogue receptor (GHSR) type 1a

The GHSR-1a receptor modulates growth hormone release upon activation by the peptide ghrelin, a potent hunger signal that stimulates appetite (125). The GHSR-1a has attracted considerable interest in recent years for its role in satiety. Modulators of GHSR-1a activity or GHSR-1a expression (126) are consequently investigated as potential therapies for the treatment of obesity (127).

The GHSR-1a has been shown to possess a high level of constitutive activity in vitro (128). The recent discovery of a naturally occurring mutant GHSR-1a receptor ($A^{204}E$) that lacks constitutive activity, but not its capacity to mediate agonist induced signaling, support a physiological role for the constitutive GHSR-1a activity. The GHSR-1a $A^{204}E$ mutation results in a functional receptor that lacks constitutive activity and leads to a syndrome characterized by a short stature and might be related to obesity that develops during puberty (129). The presence of this mutation in the GHSR-1a receptor, as well as a GHSR-1a $F6.51^{279}L$ mutation were described previously to occur in obese individuals (130). Both the $A^{204}E$ and $F6.51^{279}L$ mutant GHSR-1a receptors exhibit loss of constitutive receptor activity, while maintaining the capacity to mediate ghrelin-induced signaling events (128, 129). Whereas the $A^{204}E$ mutation occurs in the E2 loop (see section 2.6), the $F6.51^{279}L$ mutation modifies the conserved aromatic cluster CWxPFF motif in TM 6 (section 2.7).

The loss of constitutive activity results in the expected phenotype with respect to growth, while this is not the case with respect to appetite and energy expenditure (131). These findings indicate the existence of complex interactions and potential compensatory pathways to compensate for this loss of function in the GHSR-1a receptor. Possibly, activation of GPR39, recently deorphanized as the receptor for the peptide hormone obestatin, derived from the same gene as ghrelin, might account for this compensatory

pathway. Activation of GPR39 has opposite effects on food intake and weight gain compared to stimulation of the GHSR-1a receptor (132).

3.1.4. Melanocortin receptors

The family of melanocortin receptors and the existence of endogenous inverse agonists acting at these receptors have greatly strengthened the concept of constitutive receptor activity and have further supported its physiological relevance. The melanocortin system, which controls pigmentation and body weight, encompasses a family of five receptors. Both the melanocortin-1 receptor (MC1R) and 4 (MC4R) illustrate the occurrence and relevance of constitutive receptor activity in vivo. MC1R was originally called melanocyte stimulating hormone (MSH) receptor and is expressed in cutaneous and hair follicle melanocytes. Stimulation of MC1R by MSH as well as by adrenocorticotrophin (ACTH) leads to a stimulation of melanogenesis, through an increased transcription of genes involved in the production of eumelanic dark pigments. In contrast, modulation of the MC1R by the naturally occurring inverse MC1R agonist agouti, a paracrine factor expressed in the skin, induces the production of the yellow pigment pheomelanin (133). The MC1R exhibits a high level of constitutive activity accounting for the phenomenon of inverse agonism. Over 60 naturally occurring MC1R variants, located throughout the receptor, have been described (reviewed in (134, 135)). These mutant receptors are often associated with clearly visible phenotypes, showing abarrent cell surface expression, decrease or further increase in constitutive activity. The latter unmasks inverse agonistic properties of endogenous peptides (reviewed in (134, 135)).
The MC4R controls body weight and in the brain constitutive MC4R activity is inhibited by agouti-related protein (AgRP) (136). MC4R knock-out, the presence of inactivating mutations in the NTED ($R^{7}H$, $T^{11}A$, $T^{11}S$, $R^{18}C$, $R^{18}H$, $R^{18}L$), in the I2 loop ($A^{154}D$, $Q^{156}P$) or pharmacological inhibition of the MC4R results in obesity (137, 138). A cluster of naturally occurring mutations in the NTED of the MC4R in obese patients have been identified. The resultant mutant MC4R receptors exhibit a reduced constitutive activity, which led to the suggestion that the NTED in the receptor functions as an intrinsic partial agonist that contributes to the level of constitutive MC4R activity (30, 139). The activity of the MC4R is modulated through the opposing effects of the anorexigenic agonist α-MSH and the orexigenic inverse agonist AgRP (139, 140). Whereas MC4R agonists are of interest for the potential treatment of obesity, inverse MC4R agonists might exhibit favorable characteristics for the treatment of *e.g.* cancer-associated cachexia (141).
While no constitutively activating MC4R mutations have been reported to date from anorexia nervosa patients (142), one AgRP gene polymorphism has been associated with anorexia nervosa (143, 144). In fact mutations in MC4R are thought to be the most common genetic cause of obesity. Besides the inactivating mutations within MCR4, there

are also mutant MC4Rs found to be retained in the cytoplasm and poorly expressed (145), or that respond poorly to MSH (146). Yet, the overall influence of mutations in the MC4R on obesity is not clear, a variety of MC4R mutations found in non-obese individuals also exhibit loss-of-function characteristics, while impairment of cell surface expression for some mutant MC4R linked to the occurrence of obesity was not confirmed (142).

3.1.5. Virus-encoded GPCRs

Besides the naturally occurring GPCR variants described in previous sections 3.1.1-3.1.4 a relatively novel and intriguing class of GPCRs, encoded by the herpesviruses, exhibit marked constitutive activity. Altogether, the herpes- and poxviruses encode over 40 GPCRs, most of them displaying homology to chemokine receptors (147), known to be implicated in the regulation of the immune response (148). Although the roles of these viral-encoded receptors have not been revealed yet, they are believed not only to subvert the immune system but also to contribute to virus-induced pathogenesis. A large number of these viral GPCRs have acquired additional properties compared to their cellular counterparts, including the ability to bind a broad spectrum of chemokines, couple to a variety of G proteins and display high constitutive activity (26, 27, 149). In particular, the GPCRs encoded by the Kaposi's sarcoma associated herpes virus (KSHV), Epstein-Barr virus (EBV) and human cytomegalovirus (HCMV) illustrate well the (patho)physiological importance of constitutive receptor activity.

KSHV, implicated in the development of Kaposi Sarcoma (KS), encodes the GPCR ORF74 (150). ORF74 shows highest homology to the human chemokine receptor CXCR2. This viral GPCR binds a broad spectrum of chemokines and is unlike CXCR2 able to constitutively activate a variety of signal transduction cascades linked to proliferation (see (151) for references). Expression of ORF74 in vivo within haemopoetic or endothelial cells leads to the development of angioproliferative lesions that morphologically resemble KS lesions (152). Not only modulation of ORF74 activity by endogenous chemokines, but also constitutive activity of ORF74 appears to play an important role in the progression of Kaposi's Sarcoma-like lesions in ORF74 transgenic mice. The constitutive activity of ORF74 has been attributed to the absence of residues which normally are thought to be involved in GPCR activation (see section 2) (153). Re-introduction of these motifs did however not result in significant changes in basal activity of the receptor. Mutation of N2.50^{92} to the corresponding D (see section 2.8) did not lead inactivation of the ORF74 receptor. Interestingly, substitution of the neighboring L2.49^{91} with an Asp did result in loss of constitutive activity (153). Expression of this constitutively inactive mutant in vivo (L2.49^{91}D) completely prevented development of a KS-like disease in transgenic mice (154), emphasizing the relevance of constitutive receptor activity. Of particular interest are the mutations within helix 8 that besides influencing constitutive activity also have a

remarkable effect on the ligand binding properties of KSHV-ORF74 (section 2.3, Figure 2b) (60). Constitutive activation of Akt by ORF74 e.g. plays a crucial role in ORF74 mediated sarcomagenesis (155, 156). Moreover, ORF74-induced upregulation and release of pro-angiogenic factors, including proinflammatory cytokines and chemokines appear sufficient to drive angioproliferative tumor formation by autocrine or paracrine stimulation (156-159). Hence, this constitutively active viral chemokine receptor ORF74 seems to be implicated in the pathology of KS.

In addition, EBV, known to be associated with many lymphoproliferative diseases such as infectious mononucleosis, Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC), encodes a GPCR referred to as BILF1 (160, 161). Like ORF74, BILF1 constitutively activates signaling to NFκB and CRE, both implicated in proliferative signaling (161). The increased activation of signaling pathways was also apparent in EBV-positive lymphoblastoid B cell lines (161), suggesting a role for BILF1 in EBV-related proliferative diseases.

The human cytomegalovirus (HCMV) encodes even four GPCRs (US27, US28, UL33 and UL78), which also show highest homology to chemokine receptors. Both US28 and UL33 alter cellular signaling in a constitutively active manner, when ectopically expressed and more importantly after HCMV infection, as shown using HCMV US28 and UL33 deletion strains (162-165). Through promiscuous G protein coupling US28 and UL33 activate multiple signaling pathways, including effectors and transcription factors within infected cells. In contrast to other GPCRs, US28 does not possess 'the ionic lock', offering a possible explanation for the observed constitutive activity. Substitution of R3.50[129] of the conserved DRY-motif with an Ala, removes an important determinant for G protein coupling, resulting in the loss of constitutive activation of G protein-mediated signaling pathways (Waldhoer 2003 3879). Moreover, nonpeptidergic ligands have been identified that acts as inverse agonist (*e.g.* VUF2274), inhibiting basal US28 signaling in heterologous systems but also infected cells. In addition, VUF2274 was shown to partially inhibit HIV-1 entry into US28-expressing cells (166).

HCMV has been associated with chronic diseases including e.g. vascular diseases (167) and malignancies (168, 169). Since the CMV-encoded receptors US28 and UL33 constitutively activate transcription factors, implicated in inflammatory events associated with e.g. atherosclerosis and tumorigenesis (170), these receptors are believed to play a role in onset or progression of these HCMV-related pathologies. Recently, we have observed that expression of US28 induces transformation and tumorigenesis in vivo, suggesting that US28 might act in a concerted manner with other oncogenic HCMV-encoded proteins (171) to enhance tumorigenesis (172). The use of constitutively inactive mutants, the development of adequate disease model systems and use of recently identified inverse agonists targeting the HCMV-encoded receptors (173) will

serve as important tools to determine the (patho)physiological relevance of constitutive receptor activity of these receptors in vivo model systems.

3.2. Class B/C and Frizzled family GPCRs

In contrast to GPCRs belonging to the rhodopsin family of GPCRs (class A), little information is available on structural determinants involved in GPCR activation and inverse agonism of the class B/C and Frizzled GPCR families. Nonetheless, examples of natural occurring CAMs in these receptor families will be discussed below.

3.2.1 Parathyroid hormone (PTH)-related peptide (PTHrP) type 1 receptor (PTHR1)

The PTHR1 for PTH and PTHrP belongs to the class B GPCRs. This receptor is highly expressed in bone and kidney and mediates the PTH-dependent regulation of mineral ion homeostasis, including the circulating concentrations of calcium and phosphorous (174). Mutation of a histidine at the bottom of TM2 ($H^{223}R$), of a threonine in TM6 ($T^{410}R$ and $T^{410}P$) and of an isoleucine in TM7 ($I^{458}R$) of the human PTH-receptor have been reported to be associated with constitutive PTH receptor activation in Jansen-type metaphyseal chondrodysplasia, a rare disorder that is typically characterized by severe growth plate abnormalities that lead to short-limbed dwarfism (175). The high level of constitutive activity of the mutant receptor is thought to result in hypercalcemia and hypophosphatemia, and most likely the abnormal formation of endochondral bone (175).

3.2.2 Ca^{2+}-sensing receptor (CaSR)

Extracellular Ca^{2+} (Ca^{2+}_o) homeostasis is regulated by the parathyroid hormone (PTH), which is produced by parathyroid glands. PTH stimulates the (re-)absorption of Ca^{2+} by the kidney and intestine, and Ca^{2+} mobilization from bone. Fluctuation in Ca^{2+}_o levels is detected by the Ca^{2+}-sensing receptor (CaSR), which is abundantly expressed on PTH-producing chief cells in the parathyroid gland and tubular cells in the kidney (176). Like most other members of the class C GPCR family (16), the CaSR contains a Venus-flytrap-like ligand-binding domain within its large NTED (177, 178). Activation of the CaSR by elevated Ca^{2+}_o levels inhibits PTH secretion by the parathyroid chief cells and stimulates the urinary Ca^{2+} excretion, as such restoring the homeostatic Ca^{2+}_o concentration (176). Hence, the CaSR is crucial for the negative feedback regulation of the Ca^{2+}_o homeostatis. Activational mutations in the CaSR results in hypoparathyroidism, which is clinically manifested as autosomal dominant hypocalcemia (ADH). To date, 35 sporadic or familial ADH-causing mutations have been identified in the CaSR (179). Only one of these 35 activating mutations (*i.e.* $A^{843}E$ in TM 7) appeared to be a true CAM by inducing ligand-independent basal signaling (180, 181). The other activating mutations involve increased

receptor sensitivity to Ca^{2+}_o, without affecting basal signaling, showing receptor activation at inappropriately low Ca^{2+}_o concentrations (178).

3.2.3. Smoothened

Smoothened is a GPCR that is thought to signal via families of heterotrimeric G proteins and possibly via non-G protein signaling pathways (182) and forms a distinct group within the family of GPCRs together with the frizzled receptors. This receptor is kept in an inactive state through its interaction with the transmembrane protein Patched (Ptc), the receptor for lipid-modified secreted glycoproteins of the Hedgehog (Hh) family. Binding of Hh to Ptc activates Ptc to release the catalytic repression of Ptc on smoothened activity to unleash the constitutive activity of smoothened (183). Whereas the Hh pathway plays fundamental roles during pattern formation in animal development, the dysfunction of Hh-pathway components are frequently associated with congenital disorders and cancer (184-187). Cyclopamide, a teratogen found in the *Veratrum californicum* plant that blocks cholesterol synthesis, is an Hh/smoothened inhibitor, and induces regression of skin tumors (188). The effects of oncogenic mutations in TM7 ($S^{537}N$ and $W^{539}L$) and constitutively activating mutations in TMs 6 ($G^{460}Y$) and 7 ($S^{537}T$, $G^{533}L$, $G^{533}H$, $G^{533}T$, $G^{533}Y$, $G^{533}S$, and $G^{533}A$) in smoothened and Ptc can be reversed by cyclopamide treatment (189). Hence, modulators of smoothened activity appear promising for the treatment of a variety of cancers as well as psoriasis (188-191). Consequently, several smoothened modulators have recently been described (192-194).

4. Concluding remarks

Not only mutational analysis of wild type GPCRs, but also those natural occurring CAMs that are associated with disease, have contributed to our knowledge on constitutive receptor activity. Specific conserved domains within the receptor appear essential in the regulation of (spontaneous) signaling. In particular, mutational changes near the interface at the bottom of TM3 or top of TM6 often result in increases of constitutive activity (19). Analogous to these observations, an agonist is believed to relieve the receptor from these intrinsic constraints, inducing a movement of the bottom of TM6 (47, 195). Mutations in these regions are found in a number of GPCRs, e.g. of the glycoprotein family, that are associated with human diseases. Disruption of the constraint within those receptors, leads to constitutive receptor activity and consequently to the development of disease. In particular, in these cases the use of inverse agonists is in particular definitely the choice of treatment.

For some GPCRs, autoantibodies have been identified that recognize epitopes on the second, most variable, extracellular loop, causing pathologies (see for refs (20, 22). The fact that the second extracellular loop seems to be in part implicated in constitutive

activity of some receptors (68), might explain the observed effects of autoantibodies. These antibodies appear indeed to influence receptor activity and some even display agonsitic activity (196) that can be counteracted by inverse agonists.

Numerous of the clinically used drugs acting on GPCRs, in particular those of the bioaminergic families, are in fact inverse agonists. Their inverse agonistic properties have in particular been shown in recombinant systems, but also in native systems (21, 29) Inverse agonists inhibit constitutive signaling initially, yet chronic use might lead to receptor upregulation and sensitization. Taken together, the importance of inverse agonism for the clinical efficacy of drugs targeting receptors with low or high constitutive activity is just beginning to emerge and requires further attention.

Figure Legends

Figure 1. A) Early model of histamine receptor and the binding of 4-methyldiphenhydramine to a phenylalanine residue through a π - π interaction mechanism by Nauta (197). B) Crystal structure of bovine rhodopsin (PDB code 1GZM) (44). Retinal shown using CPK representation.

Figure 2. Crystal structure of bovine rhodopsin (PDB code 1GZM) (44). The color code of the α-carbon ribbons is: TMs 1 (crimson), 2 (goldenrod), 3 (dark red), 4 (gray), 5 (red), 6 (orange), and 7 (blue), and Hx8 (blue). The positions of the amino acids involved in receptor activation, together with their conservation pattern in the rhodopsin family of GPCRs (45) are shown. The standardized nomenclature of Ballesteros and Weinstein is employed (46). (a) Molecular basis of agonism and inverse agonism. Detailed view of the inverse agonists 11-*cis*-retinal (left panel) and ketotifen (middle panel) in a cavity between TMs 5 and 6 as observed in the crystal structure of rhodopsin (44) and a computational model of the histamine H_1 receptor, respectively. The 5-HT_{1A}R agonist (the naphtyl ring of the ligand is not shown for clarity) triggers the conformational transition of W6.48 towards TM5 by an explicit hydrogen bond (right panel) (77). Ligands are shown in dark green. (b) Network of interactions involving highly conserved amino acids within TM2, TM7 and Hx8 in rhodopsin (59, 60). (c) The ionic lock (48) and the hydrophobic arginine cage (53) between TMs 3 and 6 in rhodopsin. (d) Proposed hydrogen-bond network linking D2.50 and W6.48 in the inactive conformation of rhodopsin (top panel) (44), the histamine H_1 receptor (middle panel) (72), and δ opioid receptor (bottom panel) (74). N7.49 of the NPxxY motif is restrained towards TM6 via water molecule #9 in rhodopsin (63) and hydrophobic amino acids at positions 2.46 and 6.40 forming the asparagine cage (62, 64).
These figures were created using MolScript v2.1.1 (198) and Raster3D v2.5 (199).

Figure 3. Snake plot of a consensus GpHR showing the NTED to which glycoprotein hormones bind and the TMD. Amino acid residues that are conserved in FSHR, LHCGR and TSHR are indicated. Conserved cysteine residues in the TMD, the N-terminal Cys domain and C-terminal Cys domain of the NTED that are involved in disulfide bridges, are indicated by circles with a black background. The hormone binding domain in the NTED is boxed and β-strands of the leucine-rich repeats and N-terminal Cysteine rich domain that form the binding surface are represented by arrows (89). The hormone-binding domain is connected to the TMD by the so-called hinge region, which is of variable length between the 2nd and 4th conserved C-terminal Cys residues in the TSHR, LHCGR, and FSHR. The TM helix boundaries correspond to the bovine rhodopsin crystal structure (PDB code 1GZM) (44). The conserved amino acid in each TM helix of class A GPCRs is indicated

according to the Ballesteros & Weinstein numbering scheme (See section 2) (46). Locations of natural occurring GpHR CAMs (see Table 1) are indicated: yellow – FSHR; orange – LHCGR; pink – TSHR; blue – LHCGR/TSHR; green – LHCGR/TSHR/FSHR.

Table 1. Constitutive active GPCR mutants associated with pathophysiological conditions

Family	GPCR	OMIM[1]	inheritance	phenotype[2]	CAM[2,3]
A	rhodopsin	180380	autosomal dominant	congenital stationary night blindness	G2.57D, T2.61I, A7.39E
			autosomal dominant	retinitis pigmentosa	K7.43E
A	TSHR	603372	autosomal dominant	nonautoimmune hyperthyroidism	G1.49S, M2.53V, S3.36R, V3.40A, A6.34V, L6.40F, P6.50S, N650Y [EL3], N7.45Y, C7.47Y
			de novo (germline)	nonautoimmune hyperthyroidism	S281N [NTED], M2.43T, S3.36N, V5.54L/F, F6.42L
			somatic	hyperfunctioning thyroid adenoma	S281T/N/I [NTED], M2.43T, I486M [EL1] L3.43R, I568T [EL2], Y5.58N, D6.30G, A6.34V/I/S L6.40F, F6.42L/C/I, T6.43A/P/I, D6.44A/E/H/Y P6.50S, V656F [EL3]
			somatic	hyperfunctioning thyroid carcinoma	M2.43T, I486F [EL1], A6.34V, T6.43A/I, D6.44H, L7.52V
			autosomal dominant	euthyroid hyperthyrotropinemia	R310C [NTED], C390W [NTED]
A	LHCGR	152790	autosomal dominant	familial male-limited precocious puberty	L1.41P, A1.46V, M2.43T, L3.43R, I5.54L, D6.30G, A6.34V M6.37I, A6.38V, I6.41L, T6.43I, D6.44E/G/Y, C6.47R, M6.48G
			somatic	Leydig cell adenoma	D6.44H
A	FSHR	136435	autosomal dominant	spontaneous ovarian hyperstimulation syndrome	T3.32A/I, I.5.54T, D6.30N
				FSH-independent spermatogenesis	D6.30G
B	PTHR1	168468	autosomal dominant	Jansen's metaphyseal chonfrodysplasia	H223R [TM2], T410R/P [TM6], I458R [TM7]
C	CaSR	601199	autosomal dominant	autosomal dominant hypocalcemia	A843E [TM7]

[1]OMIM: Online Mendelian Inheritance in Man, OMIM (TM). McKusick-Nathans Institute for Genetic Medicine, Johns Hopkins University (Baltimore, MD) and National Center for Biotechnology Information, National Library of Medicine (Bethesda, MD), World Wide Web URL: http://www.ncbi.nlm.nih.gov/omim/

[2]Pathophysiological conditions associated with constitutively active mutant (CAM) GPCRs were collected from the OMIM, Glycoprotein-hormone Receptor Information System (GRIS) (92), and Human Gene Mutation Database (HGMD) (200) databases, see references herein for more specific details.

[3]Amino acid mutations are indicated using the Ballesteros & Weinstein numbering if situated in the TM helices of class A GPCRs (see section 2 for details on this numbering scheme). Amino acid mutations that are situated elsewhere in class A GPCRs, or mutations in class B or C GPCRs are represented by residue number and the location is indicated between brackets.

TSHR = thyroid-stimulating hormone receptor; LHCGR = luteinizing hormone/chorionic gonadotropin receptor; FSHR = follicle-stimulating hormone receptor; PTHR1 = parathyroid hormone -related peptide type 1 receptor; CaSR = Ca^{2+}-sensing receptor; NTED = N-terminal exodomain; EL1, -2, -3 = extracellular loop 1, 2, and 3, respectively; TM1-7 = transmembrane helices 1 to 7.

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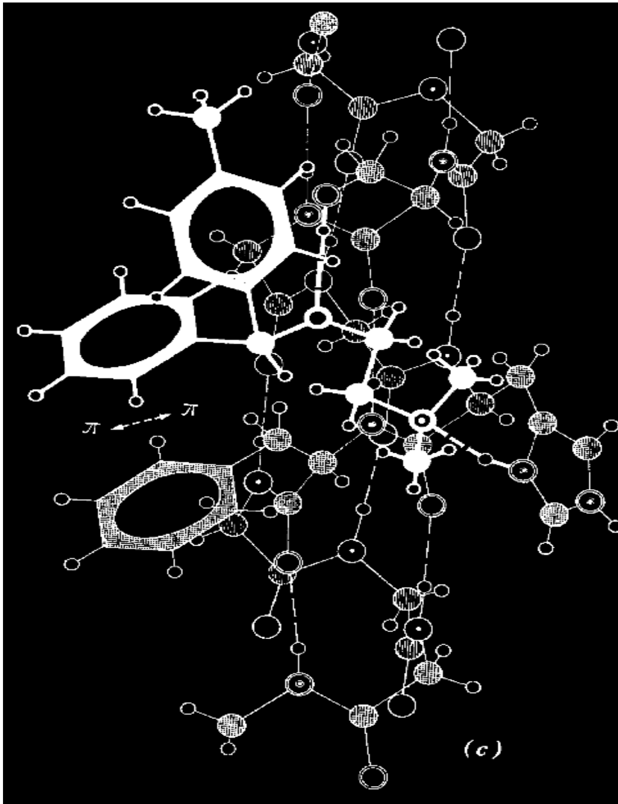

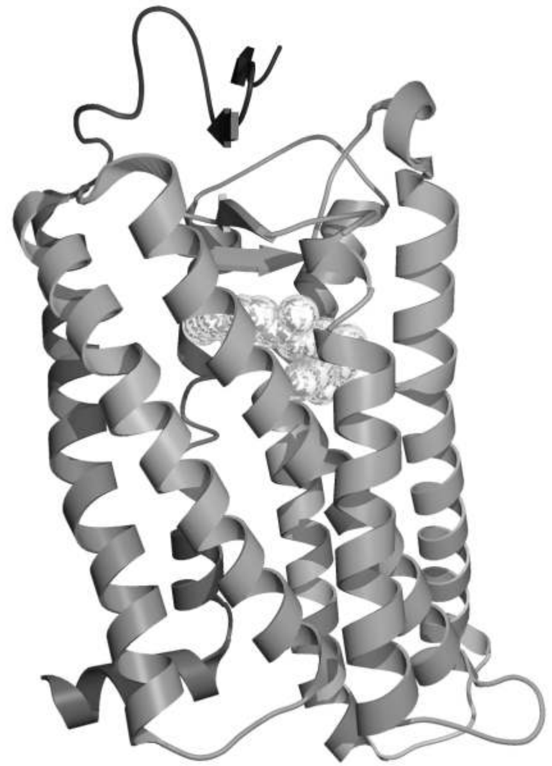

Figure 1

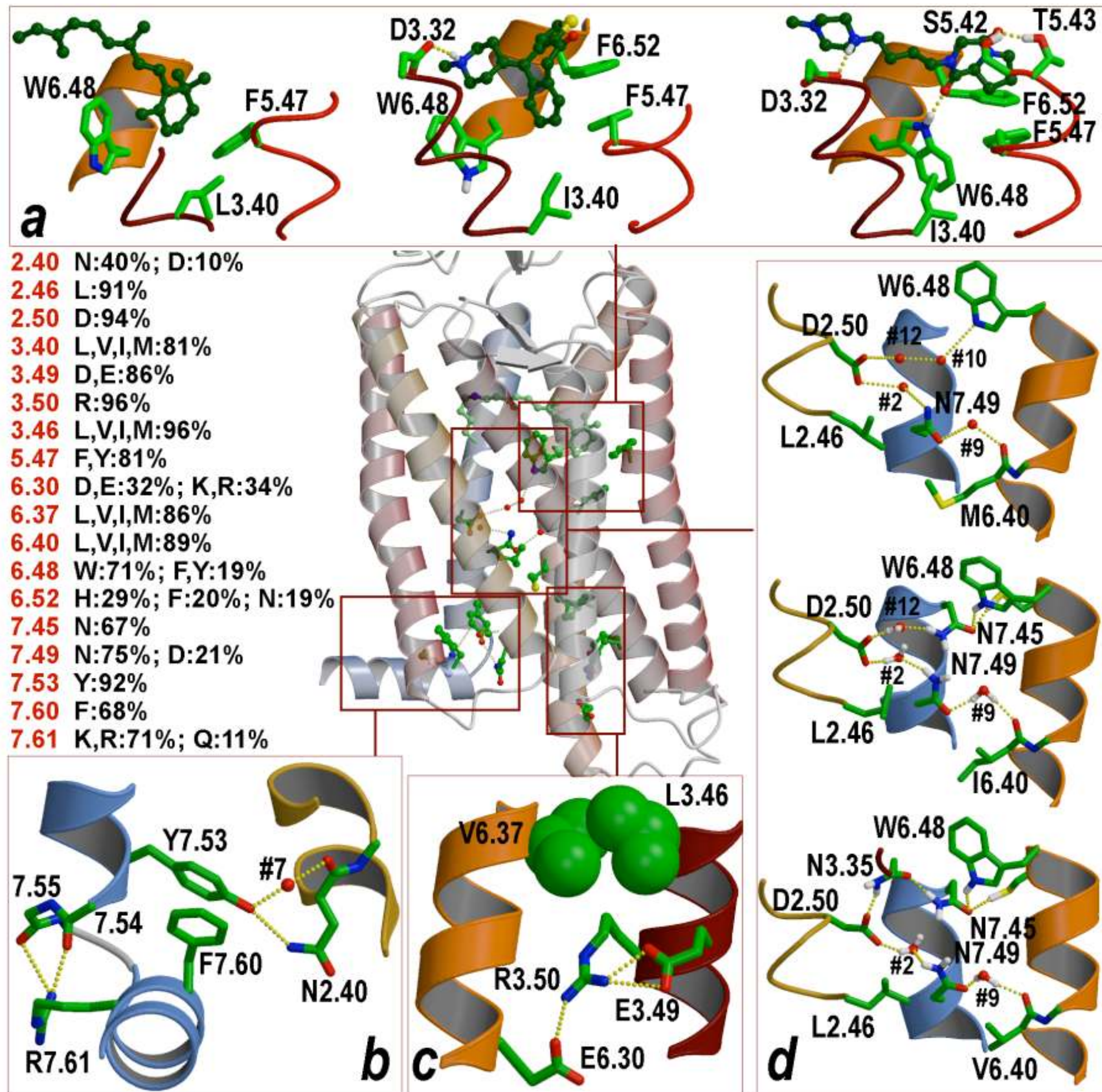

Figure 2

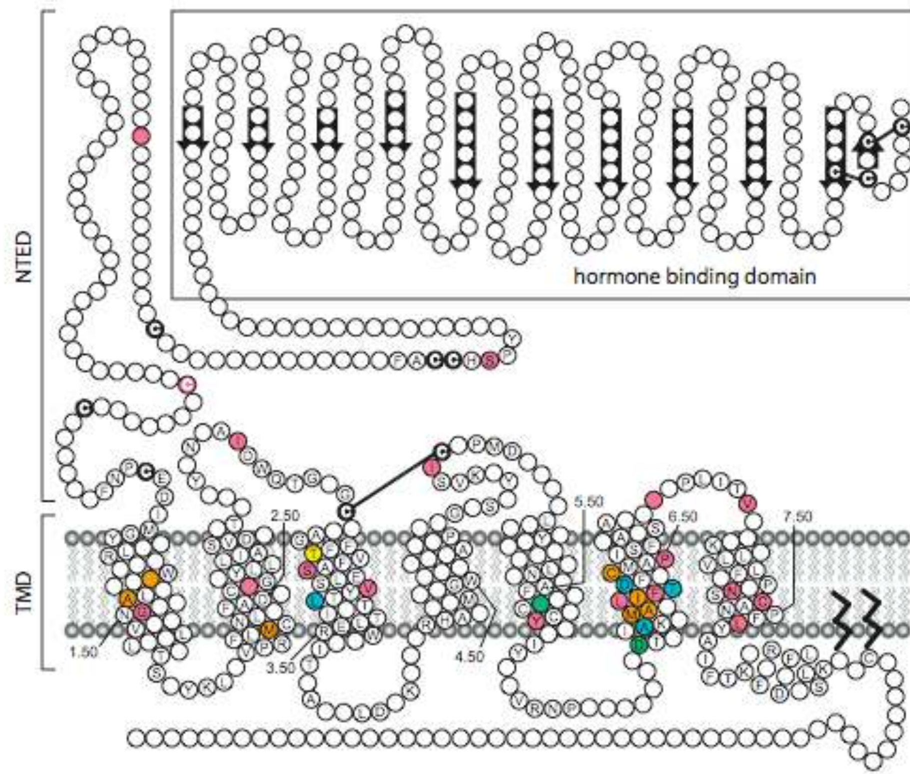

Figure 3