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Constitutive Activation of G Protein-Coupled Receptors and Diseases: Insights into Mechanisms of Activation and Therapeutics

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Abstract

The existence of constitutive activity for G protein-coupled receptors (GPCRs) was first described in 1980s. In 1991, the first naturally occurring constitutively active mutations in GPCRs that cause diseases were reported in rhodopsin. Since then, numerous constitutively active mutations that cause human diseases were reported in several additional receptors. More recently, loss of constitutive activity was postulated to also cause diseases. Animal models expressing some of these mutants confirmed the roles of these mutations in the pathogenesis of the diseases. Detailed functional studies of these naturally occurring mutations, combined with homology modeling using rhodopsin crystal structure as the template, lead to important insights into the mechanism of activation in the absence of crystal structure of GPCRs in active state. Search for inverse agonists on these receptors will be critical for correcting the diseases cause by activating mutations in GPCRs. Theoretically, these inverse agonists are better therapeutics than neutral antagonists in treating genetic diseases caused by constitutively activating mutations in GPCRs.

Keywords

G protein-coupled receptor; disease; constitutively active mutation; inverse agonist; mechanism of activation; transgenic model

1. Introduction

G protein-coupled receptors (GPCRs) are versatile signaling molecules at the cell surface. Our sensing of the outside world, including vision, smell, and taste, rely on these receptors. The inter-cellular communications also depend on them (Bockaert & Pin, 1999). They transduce a large variety of extracellular signals, including light, odorants, ions, lipids, catecholamines, neuropeptides and large glycoprotein hormones (Bockaert & Pin, 1999). Analysis of the human genome revealed at least 799 unique GPCRs (Gloriam *et al.*, 2007). One widely adopted scheme classify the GPCRs into five families, with the most important and extensively studied being Family A (Class 1), rhodopsin-like, Family B (Class 2), secretin-like, and Family C (Class 3) metabotropic glutamate receptor-like (Bockaert & Pin, 1999; Kolakowski, 1994).

Another classification scheme, with the acronym GRAFS, divide GPCRs into five families: Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2 and Secretin (Fredriksson *et al.*, 2003).

GPCRs consist of seven transmembrane α-helices (TMs) connected by alternating extracellular and intracellular loops (ELs and ILs), with the N terminus extracellular and the C terminus intracellular. The seven TMs are arranged in a counter-clock fashion as viewed from the extracellular surface. The crystal structures of rhodopsin, opsin, β_1-, and β_2-adrenergic receptor (AR) at high resolution confirmed this topology (Cherezov *et al.*, 2007; Palczewski *et al.*, 2000; Park.*et al*, 2008; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007; Warne *et al.*, 2008).

Since almost all known physiological processes are regulated by GPCRs, it is easy to appreciate that dysfunctions in these signaling pathways will lead to various pathological states. Since the discovery of the first naturally occurring mutation in GPCRs causing human disease (Dryja *et al.*, 1990), the list for diseases caused by GPCR mutations keeps expanding (for an exhaustive list see (Schoneberg *et al.*, 2004)). A number of excellent general reviews on diseased GPCRs have been published (Arvanitakis *et al.*, 1998; Parnot *et al.*, 2002; Rao & Oprian, 1996; Schoneberg *et al.*, 2004; Schoneberg *et al.*, 2002; Shenker, 1995; Spiegel, 1996; Spiegel & Weinstein, 2004; Spiegel *et al.*, 1993; Tao, 2006) together with many reviews on mutations in individual GPCRs.

In addition to the diseases caused by loss-of-function mutations in GPCRs, dysfunction in basal activity can also cause diseases. In this review, I will briefly introduce constitutive activation of GPCRs. I will then summarize several diseased states caused by either constitutive activation or loss of constitutive activity. Selected examples of animal models expressing constitutively active GPCRs will then be reviewed. Insights gained from detailed studies of these naturally occurring mutations in these as well as related GPCRs, by site-directed mutagenesis and homology modeling, are highlighted. Finally, the potential of inverse agonists in treating the diseases caused by activating mutations are reviewed.

2. Constitutive activation of GPCRs

In 1984, Cerione *et al.* first described that β_2-AR was active in the absence of hormone in a reconstituted system consisting of purified receptor and the stimulatory heterotrimeric G protein, Gs (Cerione *et al.*, 1984). Subsequently, Costa and Herz showed that in membranes prepared from NG108-15 cells, a neuroblastoma-glioma cell line expressing high levels of endogenous δ opioid receptor, some competitive antagonists exhibit negative intrinsic activity; they decrease the GTPase activity in the absence of agonist (Costa & Herz, 1989), suggesting the existence of basal activity in the endogenous δ opioid receptor. These negative antagonists are called inverse agonists (for a recent historical review, see (Costa & Cotecchia, 2005)).

With the freshly cloned subtypes of AR cDNAs at hand, Lefkowitz's lab soon showed that mutations in α_{1B}-, α_2-, and β_2-ARs result in dramatic increases in constitutive activity (Cotecchia *et al.*, 1990; Ren *et al.*, 1993; Samama *et al.*, 1993). These studies led them to propose the extended ternary complex model for GPCR activation (Samama et al., 1993). In this model, the wild type (WT) receptor exists in equilibrium between inactive and active conformations. Agonists stabilize the receptor in active conformation, whereas inverse agonists stabilize the receptor in inactive conformation. Neutral antagonists have equal affinities for inactive and active conformations. This model was subsequently modified to more complex models such as cubic ternary complex model (Weiss *et al.*, 1996) to accommodate the existence of multiple active conformations. However, the underlying principals of Lefkowitz's model remain valid.

Not only mutations can cause constitutive activation, many WT receptors also have considerable constitutive activities. In addition to δ opioid receptor described above, other

prominent examples of WT receptors with high constitutive activity include CB1 cannabinoid receptor, growth hormone secretagogue (GHS) receptor (GHSR) (see below), and melancortin-1 receptor (MC1R) (see (Seifert & Wenzel-Seifert, 2002) for an exhaustive list). Members of the same subfamily of related receptors frequently have different constitutive activity. A classical example is that of the dopamine receptors, with the D1B having high constitutive activity (Tiberi & Caron, 1994). Similarly, β_2-AR has significantly higher constitutive activity than β_1-AR (Chakir *et al.*, 2003). The constitutive activity has physiological significance as shown by mutations that selectively eliminates the constitutive activity result in human diseases (see Section 4).

As detailed below, constitutively active mutation in rhodopsin was soon discovered in humans. Cone and colleagues were the first to report a naturally occurring constitutively active mutation in GPCRs that bind diffusible ligands. They showed that the somber allele in mice is due to a constitutively active mutation in the MC1R (Robbins *et al.*, 1993). The mutation, E92K (2.62) (see next paragraph regarding the numbering system), in the extracellular half of TM2, increased the basal signaling to about 60% of the maximal response achieved by the WT receptor. This pioneering report was quickly followed with a series of constitutively active mutations in other GPCRs that cause diseases in humans. This is the focus of this review.

Ballesteros and Weinstein proposed a numbering system that facilitates the comparison of data obtained from different GPCRs (Ballesteros & Weinstein, 1995). In this system, two numbers denote the location of each residue. The first number denotes the helix. The second number denotes the relative position of the residue to the most highly conserved residue in that TM, decreasing towards the N terminus, and increasing towards the C terminus. The most highly conserved residue is given a number of 50. For example, there is a signature motif towards the end of TM3, DRY. Arginine is the most highly conserved residue in TM3, therefore numbered as 3.50. Hence Asp is designated as 3.49, whereas Tyr is designated as 3.51. The mouse MC1R mutation described above, E92K, is designated E92K (2.62). This numbering system is used here to highlight the fact that indeed many mutations affect the same loci, which is not easy to appreciate from the amino acid numbering of the different receptors.

3. Diseases caused by constitutively active mutations in GPCRs

Constitutively active mutations have also been frequently referred to as “gain-of-function mutations”. However, there are in fact three types of gain-of-function mutations (Refetoff *et al.*, 2001). The first one is constitutive activation. The second one is increased sensitivity to the agonist (decreased EC_{50}, concentration of agonist needed to cause 50% maximal response). The third one is lowering of the specificity. Both of the latter types of mutations have been identified. A classical example of the lowering of EC_{50} is the calcium-sensing receptor, where increased sensitivity to the plasma calcium concentration cause autosomal dominant or sporadic hypocalcemia (Baron *et al.*, 1996; Pollak *et al.*, 1994) (recently reviewed in (Hu & Spiegel, 2007)). Only a single mutation has been found to cause constitutive activation in the calcium-sensing receptor (Zhao *et al.*, 1999). Good examples of the lowering of specificity can be found with the glycoprotein hormone receptors. The three receptors, thyroid stimulating hormone (TSH) receptor (TSHR), luteinizing hormone (LH)/chorionic gonadotropin (CG) receptor (LHCGR), and follicle-stimulating hormone (FSH) receptor, usually only recognize their cognate ligands. However, mutations can lower this specificity. A mutation in TSHR was identified that respond to high levels of human CG (hCG) present during early pregnancy, causing familial gestational hyperthyroidism (Rodien *et al.*, 1998). Another example is mutations in FSHR that result in inappropriate response to hCG and in some cases to TSH, causing familial spontaneous ovarian hyperstimulation syndrome (OHSS) and hyperthyroidism during pregnancy (see below).Since the current manuscript is on diseases

associated with the constitutive activation of the GPCRs, the latter two types of mutations will not be discussed in detail herein.

3.1. Rhodopsin mutations and retinitis pigmentosa or congenital stationary night blindness

Rhodopsin, the receptor for vision, is a unique GPCR in that the inverse agonist 11-*cis*-retinal is covalently bound to the protein moiety, opsin, through a protonated Schiff base linkage to the ε-amino group of K296 in TM7 (7.43). The inverse agonist keeps the basal activity of rhodopsin to almost nonexistent. With absorption of a single photon, the inverse agonist 11-*cis*-retinal isomerizes to all-*trans*-retinal, and rhodopsin, after undergoing conformational changes, is converted to metarhodopsin II, the active form of rhodopsin. Consequent activation of transducin, the G protein in rod cells, initiates the photo-transduction cascade.

Keen et al. reported the first constitutively active mutation in rhodopsin, K296E, in autosomal dominant retinitis pigmentosa (ADRP) (Keen *et al.*, 1991). These patients from a British family, compared to the patients caused by loss-of-function mutations in rhodopsin, have a more severe phenotype and earlier onset. They developed cataracts in their thirties and forties. Oprian and colleagues showed that this mutant is constitutively active (Robinson *et al.*, 1992). Because the site of attachment for 11-*cis*-retinal was mutated, the constitutive activity of the mutant is not suppressed by 11-*cis*-retinal, the inverse agonist for rhodopsin, potentially explaining the phenotypes of patients harboring this mutation (Robinson et al., 1992). Constitutive activity interferes with rod sensitivity, which is usually kept quiescent by the covalently bound inverse agonist. This so called “dark-light” (perception of light in darkness) will result in impaired sensing to dim but not bright light (Sieving *et al.*, 1995). Subsequently, another mutation at the same site, K296M, was identified in an American family (Sullivan *et al.*, 1993). This mutation was also shown to cause constitutive activation (Rim & Oprian, 1995).

Constitutive activation of rhodopsin can also cause congenital night blindness (CNB). Dryja and colleagues reported the first rhodopsin mutation in CNB (Dryja *et al.*, 1993). This mutation, A292E (7.39), is located about one helical turn away from K296. The mutant behaves similarly as the WT rhodopsin in the presence of chromophore. However, the mutant apoprotein opsin, in the absence of chromophore, could constitutively activate transducin (Dryja et al., 1993). It was suggested that the newly introduced Glu competes with E113 for ionic interactions with the positively charged nitrogen on K296, therefore break the salt-bridge between K296 and E113 resulting in activation of the protein (Rao & Oprian, 1996; Gross *et al.*, 2003) (Fig. 1)

Another rhodopsin mutation, G90D (2.57), was reported to also cause CNB (Sieving et al., 1995). The patients have extensive night blindness with early-onset. However, unlike patients with retinitis pigmentosa, they do not exhibit any apparent retinal damage until much later in life. Functional studies showed that indeed G90D constitutively activates rhodopsin (Rao *et al.*, 1994). Detailed biochemical and Fourier-transform infrared spectroscopy experiments showed that G90D has important features of metarhodopsin in the dark, such as deprotonation of E113 (Zvyaga *et al.*, 1994). In the crystal structure of rhodopsin, G90 are in close proximity to K296 (Gross et al., 2003) (Fig. 1). The G90D mutation also causes constitutive activation by disrupting the salt bridge between K296 and E113 through competition of the newly introduced Asp with E113 for the positively charged K296 (Rao & Oprian, 1996). Data from the double mutant G90D/E113Q showed that indeed the newly introduce Asp can compensate for the loss of the Schiff base counterion at E113 (Rao et al., 1994).

Recently, another mutation, T94I (2.61), was found to cause CNB (al-Jandal *et al.*, 1999). Functional studies showed that T94I rhodopsin could bind 11-*cis*-retinal. The mutant rhodopsin, when bound with 11-*cis*-retinal, is not active in the dark. However, the opsin is constitutively active (Gross et al., 2003; Ramon *et al.*, 2003). Structural analysis showed that

T94 is in close proximity to E113 counterion, therefore involved in maintaining the inactive conformation of the receptor. Fig. 1 showed that indeed all three mutations causing CNB are located near the salt bridge between E113 and K296 (Gross et al., 2003). All five constitutively active mutants disrupt the salt bridge critical for maintaining the ground state of rhodopsin (Kim *et al.*, 2004; Rao & Oprian, 1996).

Why some mutations in rhodopsin cause CNB whereas others cause degenerative ADRP? CNB is a mild disease in which rod cells showed little or no degeneration whereas ADRP is a severe disease that progress from decreased rod cell sensitivity to loss of the photoreceptor cells and total blindness. It was hypothesized that the different phenotypes caused by constitutively active rhodopsin mutations might be explained by their differential abilities to bind 11-*cis*-retinal (Rao et al., 1994). The mutant rhodopsins associated with ADRP (K296E and K296M) cannot bind 11-*cis*-retinal (because K296, the site of chromophore attachment, is mutated), so all the mutant apoprotein are constitutively active, resulting in retinal degeneration. The mutant rhodopsins associated with CNB (G90D, T94I and A292E) can bind to 11-*cis*-retinal; therefore the majority of the mutant rhodopsins are kept inactive by the bound 11-*cis*-retinal. Only a small portion of the mutant proteins is in the active opsin form (not bound to 11-*cis*-retinal). This signaling, while raising the dark-adapted threshold (Sieving et al., 1995), does not result in retinal degeneration (Rao et al., 1994). For K296E and K296M, the constitutive signaling, without intermittent termination by 11-*cis*-retinal binding (as the mutants that cause CNB), ultimately results in apoptosis of the photoreceptor cells. Mutant mice that cannot produce 11-*cis*-retinal (therefore unliganded opsin is constitutively active) also have retinal degeneration (Woodruff *et al.*, 2003). In summary, mutations that cause continuous activation of the photo-transduction pathway will result in ADRP, whereas mutations that can terminate the constitutive signaling led to the mild CNB (Lem & Fain, 2004).

3.2. TSHR mutations and hyperthyroidism

TSH is a large glycoprotein hormone produced by thyrotropes of the anterior pituitary. It is composed of two subunits, an α-subunit identical to that of LH and FSH and a TSH-specific β-subunit. TSH is essential for thyroid hormone synthesis and secretion as well as for cell proliferation and differentiation in the thyroid gland (Rapoport *et al.*, 1998). TSH exerts its effects by interacting with cell surface TSHR. The primary pathway activated by TSHR is G_s and cyclic adenosine monophosphate (cAMP). At high TSH concentrations and high level of TSHR expression, TSHR activation also increases inositol phosphate (IP) and calcium by coupling to $G_{q/11}$ and subsequent activation of phospholipase C (PLC). Indeed, it has been shown that human TSHR can couple to members of all four families of G proteins (Laugwitz *et al.*, 1996). A recent study provided in vivo evidence that the IP/Ca^{2+} pathway is important for hormone synthesis (Grasberger *et al.*, 2007).

Because of the prominent roles of TSH in thyroid gland growth and function, TSHR abnormalities are involved in the pathogenesis of a variety of thyroid diseases. Activating mutations of the TSHR cause toxic adenoma as well as familial and sporadic nonautoimmune hyperthyroidism, whereas inactivating mutations of the TSHR cause hypothyroidism and resistance to TSH (reviewed in (Davies *et al.*, 2005; Kopp, 2001)).

Parma *et al.* were the first to report that constitutively active mutations in the TSHR gene cause functioning thyroid adenoma with hyperthyroidism (Parma *et al.*, 1993). They identified two somatic mutations, D619G (6.30) and A623I (6.34) in TM6 in three out of eleven hyperfunctioning thyroid adenomas. Since this pioneering report, 46 additional constitutively active mutations have been identified in toxic adenoma as well as in familial and sporadic nonautoimmune hyperthyroidism (Table 1). As shown clearly in Table 1, the mutations cluster around IL3 and TM6, consistent with studies in both TSHR and other GPCRs demonstrating the critical importance of this region in maintaining the WT receptor in inactive conformation.

These activating mutations are heterozygous. In the hot nodules, the mutations are somatic. Genomic DNAs isolated from nearby normal tissue or leukocyte revealed WT genotype. The reported frequency of TSHR mutation in hot nodules ranges from 8% in Japanese patients (Takeshita *et al.*, 1995) to 82% in European patients (Parma et al., 1997) (reviewed in (Arturi *et al.*, 2003)). In familial cases, the transmission mode is autosomal dominant. Germline activating TSHR mutations cause hereditary or sporadic (de novo) toxic thyroid hyperplasia in the absence of autoimmunity. Different expressivity was observed, with age of onset of hyperthyroidism ranging from early infancy to adulthood (Van Sande *et al.*, 1995). Some patients with activating TSHR mutations presented with sub-clinical hyperthyroidism. Aggressive treatment such as surgical or radioactive removal of the whole thyroid gland is usually required to prevent relapse (Van Sande et al., 1995). Additional radioiodine therapy is frequently required.

Functional studies showed that all mutants examined constitutively activate the cAMP pathway (see the references cited in Table 1; most of the case reports studied the functional properties of the mutants identified, at least in terms of basal cAMP production). Wild-type TSHR already has a significant level of constitutive activity when expressed heterologously. Expression of these mutant TSHRs further increased basal cAMP levels compared with cells expressing WT TSHR. Most of the mutants do not have increased basal levels of IPs. Only a few mutants, such as I486F, I486M, and I568T (Parma et al., 1995), and Δ613–621 (Wonerow *et al.*, 2000), cause constitutive activation of the PLC-IP pathway, suggesting that activation of cAMP pathway is the cause of these thyroid diseases.

It has been proposed that the TSHR is an oncogene. Several studies investigated the oncogenic potential of the TSHR mutants in vitro. When the constitutively active mutant (CAM) TSHRs were expressed in FRTL-5, a permanent but untransformed rat thyroid cell line, TSH-independent proliferation (Fournes *et al.*, 1998; Ludgate *et al.*, 1999; Porcellini *et al.*, 1997) and neoplastic transformation (anchorage-independence) (Fournes et al., 1998) were observed. (FRTL-5 cells need TSH stimulation to proliferate. In the absence of TSH, these cells become quiescent but viable for several weeks. When these cells are introduced into nude mice, tumorigenesis is observed.) Constitutively active Gsα mutant expressed under the same promoter (bovine thyroglobulin gene promoter), although resulting in TSH-independent cell growth and raised intracellular cAMP level, was not tumorigenic (Fournes et al., 1998). Expression of A263I TSHR by retroviral transduction in human primary thyrocytes also induced formation of colonies, suggesting that signaling of the mutant TSHR is sufficient to initiate thyroid tumorigenesis (Ludgate et al., 1999). Although it is believed that the cAMP-protein kinase A (PKA) signal transduction pathway is the major driver for cell proliferation, other effectors, such as IP, phosphatidyl inositol-3 kinase, Gβγ-mediated signaling, small GTPases (such as Ras, RhoA, and Rap1), and cAMP-dependent but PKA-independent pathway, might also be involved (Fournes et al., 1998; Medina & Santisteban, 2000; Rivas & Santisteban, 2003).

Fuhrer and coworkers compared the constitutive activity measured in COS-7 (monkey embryonic kidney cells as used in most of the previous studies) versus rat FRTL-5 and human thyrocytes (Fuhrer *et al.*, 2003a). They showed that the constitutive activities of the seven TSHR mutants tested in thyroid cells do not correlate with their constitutive activities in COS-7 cells, highlighting the contribution of cellular context to the measured activities. In FRTL-5 cells and human thyrocytes, these mutant TSHRs show a similar order of potency. Furthermore, mutants with the highest constitutive activity (in terms of basal cAMP levels) may fail to induce proliferation, perhaps due to complex counter-regulatory mechanisms at the level of phosphodiesterases and cAMP regulatory element modulator isoforms, as well as receptor desensitization (Fuhrer et al., 2003a; Persani *et al.*, 2000).

Interestingly, activating mutations of TSHR may also be the etiology of feline hyperthyroidism, a common feline endocrinopathy similar to human toxic nodular goiter.Watson et al. screened 134 nodules and found nine somatic mutations (not present in the leukocyte genomic DNA) in the feline TSHR gene (Watson *et al.*, 2005). These mutations include M452T, S504R, V508R, R530Q, V557L, T631A, T631F, D632Y, and D632H. Five of these mutations were identified in human toxic nodular goiter and shown to cause constitutive activation, suggesting that they might be involved in the pathogenesis of feline hyperthyroidism. Functional studies of these mutations in cat TSHR, especially basal cAMP levels, are needed to reach definitive conclusions regarding their roles in the pathogenesis of feline hyperthyroidism.

3.3. LHCGR mutations and male-limited precocious puberty/Leydig cell adenoma

In males, androgen (primarily testosterone) is produced by interstitial Leydig cells in the testis under the stimulation of LH. At puberty, the hypothalamus-pituitary-gonad axis becomes active. Increased pulsatile secretion of gonadotropin-releasing hormone results in increased serum LH level. LH stimulates the testis to produce testosterone, which in turn initiates the development of male secondary sexual characteristics, including growth of testis and penis, growth of pubic hair and beard, and deepening of voice. The receptor mediating LH action, the LHCGR, therefore plays a critical role in pubertal development (Ascoli *et al.*, 2002). Defects in LHCGR signaling cause hypergonadotropic hypogonadism (Latronico & Segaloff, 1999; Themmen & Huhtaniemi, 2000). Because of the critical roles of testosterone in the development of secondary sexual characteristics at puberty and its effect on bone growth, abnormal production of testosterone in pre-pubertal boys will stimulate rapid growth and virilization. However, due to premature epiphyseal closure, the adult heights of these boys are decreased (Shenker, 2002).

Familial male-limited precocious puberty (FMPP) or testotoxicosis is an autosomal-dominant gonadotropin-independent disorder. Robert King Stone described the first case in 1852. He described a boy of four years old with a height of a ten year old and “If the child’s face is concealed, the examiner would declare his figure to be that of miniature man, perfectly developed and at least 21 years of age” (quoted from (Shenker, 1998)). The patient’s father also had precocious puberty: “…I may observe that the father presented extreme precocity, having experienced his first sexual indulgence at the age of 8 years” (also quoted from (Shenker, 1998)). It was more than 140 years later that Shenker and colleagues showed that mutations in the LHCGR gene resulting in constitutive activation could cause premature increase in testosterone production, leading to FMPP (Shenker *et al.*, 1993). The first such mutation identified was D578G (6.44) in TM6 (Shenker et al., 1993). The mutation co-segregates with the disease in multiple families (Kawate *et al.*, 1995; Kremer *et al.*, 1993; Shenker et al., 1993). Functional studies showed that indeed the mutant is constitutively active. When expressed in COS-7 cells, basal cAMP production is dramatically increased (3 – 4.5 fold) with the D578G LHCGR (Shenker et al., 1993; Yano *et al.*, 1994). Since the initial identification of D578G mutation, 14 additional activating mutations have been identified in LHCGR gene causing FMPP (Table 2).

These subjects have normal reproductive function in adulthood so the constitutive activity does not disrupt the normal reproductive axis function. The reason for the lack of abnormality is not well understood. One reason might be that the constitutively active receptor is down regulated due to the inherent instability of constitutively active receptor (Smit *et al.*, 1996). Another reason is that the constitutively active mutant receptor induces phosphodiesterase that might result in decreased signaling in vivo (Shinozaki*et al.*, 2003). There is only one report of an adult patient harboring a LHCGR mutation with testicular seminoma and Leydig cell hyperplasia (Martin *et al.*, 1998). Careful follow-up of the pediatric patients is needed to see whether there are other pathological conditions associated with the constitutively active

mutations. Currently, the main goals for treating these pediatric patients are to delay epiphyseal closure so they can reach their predicted final heights as well as to limit other symptoms associated with high testosterone levels, such as acne, spontaneous erections, psychological and social stress, and aggressive behavior (Jeha *et al.*, 2006). Female carriers of these activating mutations do not have precocious puberty, likely due to the fact that both LH and FSH are needed for ovarian steroidogenesis. For example, the appearance of LHCGR depends on FSH stimulation in ovaries but not in testes. Whether excess androgen production in adult women will cause polycystic ovary syndrome remains to be investigated. So far, the evaluations of the reproductive endocrine status revealed no evidence of sub-clinical abnormalities in older females with constitutively active LHCGR mutations (Latronico *et al.*, 2000a; Rosenthal *et al.*, 1996).

Functional studies of these mutants have shown that they are all constitutively active on the major signaling pathway, G_s. Cells expressing these mutants have increased cAMP production in the absence of agonist. There are some evidences of correlation of the basal activity with age of presentation and severity. D578Y, which has twice the basal activity of D578G, was found in boys with very early-onset and severe testotoxicosis (Kosugi *et al.*, 1996; Laue et al., 1995). Most of the mutants do not cause constitutive activation of the PLC-IP pathway. There are only a few exceptions such as D578Y (Kosugi et al., 1996) and D578H (Liu *et al.*, 1999).

The original mutation, D578G, responds to hCG stimulation with similar EC_{50} and maximal response (Rmax) as the WT LHCGR. Most of the mutants described above also respond to hCG stimulation. The exceptions are L457R, I542L, I575L, and C581R. These four mutants have diminished or absent responses to hCG stimulation, although they bind to hCG normally (Kremer *et al.*, 1999; Latronico et al., 1998; Laue et al., 1995).

Since LH can also stimulate Leydig cell proliferation, it was hypothesized that activating mutations of LHCGR might cause Leydig-cell tumors. Shenker and colleagues first identified D578H mutation from Leydig-cell adenomas in three boys (Liu et al., 1999). This same mutation has since been identified in two other studies from Leydig-cell tumors from three additional boys of different ethnic backgrounds (Canto *et al.*, 2002; Richter-Unruh *et al.*, 2002). In vitro experiments showed that this mutant is extremely active. The basal cAMP level is 28 times that of WT basal cAMP level; that is about 80% of the Rmax in the WT LHCGR. It also increases basal IP production significantly (7-fold, equal to 70% of Rmax) (Liu et al., 1999). It was hypothesized that the dual activation of the cAMP and IP pathways might be a contributor to the neoplastic transformation of Leydig cells by D578H mutation. Ascoli and colleagues performed detailed comparisons of three CAMs: L457R and D578Y (associated with precocious puberty and Leydig cell hyperplasia) and D578H (associated with Leydig cell adenomas) (Hirakawa & Ascoli, 2003; Hirakawa *et al.*, 2002; Min & Ascoli, 2000; Min *et al.*, 2002). These experiments showed that these CAMs constitutively activate the same G proteins (including G_s, $G_{i/o}$, and $G_{q/11}$), resulting in signaling by cAMP, IP and ERK1/2 pathways. They concluded that the signaling pathways activated by these three CAMs are the same, as well as the same as ligand-activated WT receptor (reviewed in (Ascoli, 2007)). Therefore the cause for D578H-induced Leydig-cell adenoma remains to be determined.

After the first laboratory-generated CAMs were reported, it was quickly found that these CAMs are constitutively phosphorylated and desensitized (Pei *et al.*, 1994). What about the naturally occurring mutants? Although there is constitutive desensitization, it is obvious that it cannot compensate the constitutive activation, since the constitutive activity does result in pathological states.

Of the three LHCGR CAMs studied (L457R, D578H, and D578Y), two (L457R and D578H) had increased basal phosphorylation (Min & Ascoli, 2000). After hCG stimulation, they

showed little or no response with increased cAMP production. They also failed to increase phosphorylation after hCG stimulation (Min & Ascoli, 2000). However, all three mutants have increased rates for internalizing hCG which were not decreased by over-expression of arrestin, suggesting that internalization in these mutants bypass many of the steps (such as activation, phosphorylation, and arrestin binding) usually followed by the WT receptor after agonist-binding (Min & Ascoli, 2000). Further studies revealed different postendocytotic fates of receptor-hormone complex between L457R and WT (Galet & Ascoli, 2006). Unlike the WT receptor, most of the L457R is recycled to the cell surface, rather than being routed to the lysosome for degradation, which could contribute to prolonged signaling augmenting the apparent constitutive activity (Galet & Ascoli, 2006).

The LHCGR is known to form dimers or oligomers (Roess *et al.*, 2000; Tao *et al.*, 2004; Urizar *et al.*, 2005) and hCG treatment increases dimer formation (Tao et al., 2004). Using fluorescence resonance energy transfer, it was shown that D578H and D578Y constitutively dimerize. In contrast, in the WT LHCGR, the proportion of the receptor in the dimer form is minimal in the absence of hormone stimulation (Lei *et al.*, 2007). Whether this constitutive dimerization is a common property of other CAMs as well is not known at present.

3.4. FSHR mutations and spontaneous ovarian hyperstimulation syndrome

The FSHR is critically involved in regulating reproductive function (Simoni *et al.*, 1997). In the males, FSH is important for spermatogenesis, whereas in females FSH is absolutely required for follicle growth. Because of the critical importance of FSH in regulating cell proliferation as well as the fact that activating mutations in the TSHR and LHCGR cause thyroid tumors and Leydig cell adenoma (see above), several groups hypothesized that constitutively active mutations in the FSHR might constantly stimulate granulosa cell proliferation, therefore resulting in tumor formation. Unfortunately, no germline or somatic mutations were found in these studies (Fuller *et al.*, 1998; Giacaglia *et al.*, 2000; Latronico & Segaloff, 1999; Ligtenberg *et al.*, 1999). One inactivating (not activating) mutation identified was later found to be due to contamination (Kotlar *et al.*, 1998; Kotlar *et al.*, 1997).

The first constitutively active mutation in FSHR was identified in an unusual case of a hypophysectomized (due to pituitary tumor) but fertile man (Gromoll *et al.*, 1996). This patient, although lacking gonadotropin, had normal testis volume and spermatogenesis with androgen replacement therapy alone. He fathered three children. Since in primates, unlike in rodents, spermatogenesis cannot be achieved with testosterone alone (Schaison *et al.*, 1993), the authors hypothesized that there might be an activating mutation in the FSHR gene in this patient to achieve residual FSH activity. Sequencing showed that indeed this patient harbors a heterozygous D567G (6.30) mutation (corresponding to D564G in LHCGR). Functional studies showed that the mutant receptor resulted in a small but consistent 1.5-fold increase in basal cAMP level compared with WT FSHR (Gromoll et al., 1996). When the mutant was expressed in a mouse Sertoli cell line, a 3-fold increase in basal cAMP level was observed (Simoni et al., 1997). Studies with transgenic mice showed that this small increase does have functional implications in vivo (see below).

Normally, there is strict specificity in the interaction of gonadotropin with their cognate receptors. Therefore, FSHR only interacts with FSH, but not TSH, LH or hCG. However, mutations in the receptors might result in relaxing of this stringency. An earlier example is the TSHR mutation that results in increased response to hCG by the mutant TSHR, resulting in gestational thyrotoxicosis (Rodien et al., 1998) (see above). With the FSHR, recent studies showed that mutations that decrease the specificity of the mutant FSHR were the cause of OHSS (De Leener *et al.*, 2008; De Leener *et al.*, 2006; Montanelli *et al.*, 2004a; Smits *et al.*, 2003; Vasseur *et al.*, 2003). In OHSS, there are two important components: growth of multiple follicles and the luteinization of these follicles (Delbaere *et al.*, 2005). Most cases of OHSS

are iatrogenic due to in vitro fertilization treatments. Only about 5% of the cases are spontaneous.

The mutations recently identified in FSHR gene from OHSS patients include T449A (3.32), T449I, I545T (5.45), D567G (6.30), and D567N. All mutations are heterozygous. The mutant receptors showed a clear dose-dependent response to hCG stimulation. Although the sensitivity is much lower than the cognate LHCGR, the enormous increase in serum hCG during the first trimester of pregnancy can cause significant stimulation of FSHR resulting in over-stimulation of the ovaries. Since the large extracellular domain is responsible for binding to the glycoprotein hormones, these mutations, located in the transmembrane domains, are likely not directly involved in interacting with the ligands. Rather, global conformational changes induced by these mutations might affect the extracellular loops or the amino terminus, resulting in relaxed specificity (Vasseur et al., 2003). In fact, ligand binding studies cannot detect specific binding of the mutant receptors to hCG reflecting the very low affinity for hCG (De Leener et al., 2008; Smits et al., 2003; Vasseur et al., 2003). Some sporadic cases without FSHR mutation might be explained by the promiscuous activation of WT FSHR by the extremely high hCG levels during the first trimester of pregnancy (De Leener et al., 2008).

Interestingly, some of the mutants, including T449A, I545T, D567G, and D567N, are constitutively active. It is likely that constitutive activation is not required for the development of OHSS, since patients with trophoblastic disease (with higher hCG levels than normal pregnancy) also have spontaneous ovarian hyperstimulation (Ludwig *et al.*, 1998; Montanelli et al., 2004a). In addition, T449I and a mutation in the extracellular domain of the FSHR (S128Y) identified in patients with OHSS do not cause constitutive activation (De Leener et al., 2008). Therefore, the loss of specificity is most likely the etiology of spontaneous OHSS in the patients carrying FSHR mutations.

3.5. PTHR1 mutations and Jansen's metaphyseal chondrodysplasia

Parathyroid hormone (PTH)/PTH-related peptide (PTHrP) type 1 receptor (PTHR1) is expressed in bone, kidney, and growth plate chondrocytes, maintaining the circulating concentrations of calcium and phosphorus within narrow limits. Therefore it is a central regulator of both bone development and ion homeostasis. Activating mutations of PTHR1 result in a special bone disorder called Jansen's metaphyseal chondrodysplasia (JMC). This disease is characterized by short limb dwarfism (short stature with extremely short limbs) due to severe abnormalities of growth plate, micrognathia (small lower jaw), hypercalcemia, and hypophosphatemia. The patients have increased bone remodeling. Hence markers for both osteoblastic (such as serum alkaline phosphatase and osteocalcin) and osteoclastic (such as urinary hydroxyproline excretion) activities are increased. These biochemical changes are reminiscent of primary hyperparathyroidism. However, unlike primary hyperparathyroidism, serum PTH and PTHrP levels are normal or undetectable. Urinary excretion of cAMP is increased, suggesting PTH/PTHrP-independent generation of cAMP. The pattern of inheritance is autosomal dominant, although many cases are sporadic (Juppner, 1996).

Since JMC patients have either normal or undetectable serum levels of PTH, yet they have hypercalcemia, constitutively active mutation in PTHR1 was hypothesized to be a potential cause. The first PTHR1 mutation was identified from a JMC patient that changes codon 223 in TM2 from histidine to arginine (Schipani *et al.*, 1995) (PTHR1 is a member of Family B GPCRs. The Ballesteros and Weinstein numbering system are not used for these receptors since they do not have a clear-cut highly conserved residue in each TM as Family A GPCRs). Since that initial report, three additional mutations were identified from JMC patients: T410P (Schipani *et al.*, 1996) and T410R (Bastepe *et al.*, 2004) in TM6, and I458R in TM7 (Schipani *et al.*, 1999).

PTHR1 is primarily coupled to G_s. In vitro expression of these mutants showed that the basal cAMP levels are increased, and these increases are dependent on the amount of plasmids transfected (hence the number of the receptors on the cell surface) (Bastepe et al., 2004; Schipani et al., 1999; Schipani et al., 1996). PTHR1 is also coupled to $G_{q/11}$; therefore receptor activation also increases IP production. However, of the mutants studied (H223R, T410P, and I458R), none causes constitutive activation of this pathway (Bastepe et al., 2004; Schipani et al., 1999; Schipani et al., 1996). H223R also failed to increase IP production after PTH or PTHrP stimulation (Schipani et al., 1996). There seems to have some genotype-phenotype correlation. For example, the constitutive activity of T410R is lower than that of T410P. The phenotype is also less severe in the patients harboring T410R (Bastepe et al., 2004).

Expression of H223R or T410P PTHR1 with arrestin3-green fluorescent protein in HEK-293T cells resulted in agonist-independent recruitment of arrestin3 to the cell surface, whereas arrestin3 was distributed uniformly in cytoplasm in cells expressing WT PTHR1 (Ferrari & Bisello, 2001). In the absence of ligand, H223R and WT were predominantly localized on the cell surface, whereas T410P was partly localized intracellularly, indicating constitutive intracellular trafficking. T410P PTHR1 also internalizes antagonists, whereas WT or H223R do not. Therefore, cellular localization and internalization characteristics are different between the two naturally occurring CAMs, H223R and T410P (Ferrari & Bisello, 2001).

In summary, constitutively active mutations in GPCRs were clearly shown to cause several diseases. Most of the diseases are associated with the endocrine system with the exception of rhodopsin. It should be noted that several receptors were hypothesized to be potential causes of some diseases if constitutively activated, according to their physiological roles. However, extensive search for these constitutively active mutations have failed. The case for FSHR was mentioned before. There are a few other examples. No activating mutations were found in angiotensin II AT_{1A} receptor in hyperfunctioning adrenal Conn adenoma (Davies *et al.*, 1997), despite the critical importance of angiotensin II in regulating aldosterone secretion in adrenal gland. Similarly, in the melanocortin-2 receptor, no constitutively active mutations were identified in a variety of adrenal tumors (Latronico *et al.*, 1995b; Light *et al.*, 1995), although melanocortin-2 receptor mediates adrenocorticotropin's function in stimulating cell proliferation. In the gonadotropin-releasing hormone receptor, no activating mutations were identified from pituitary gonadotroph adenomas (Kaye *et al.*, 1997).

4. Diseases caused by loss of constitutive activity in GPCRs

4.1. MC4R mutations and obesity

Mutations in the melanocrtin-4 receptor (MC4R) gene are the most common monogenic form of obesity. Amazingly, in a small protein with 332 amino acids, more than 130 mutations, mostly missense mutations, have been identified from various patient populations (see (Tao, 2005, 2006) for earlier reviews). Roughly one third of the codons were found to be mutated. The MC4R, coupled to G_s, has some constitutive activity. In most of the earlier studies, the basal cAMP levels of the mutants were not studied (Donohoue *et al.*, 2003; Gu *et al.*, 1999; G. Ho & MacKenzie, 1999; Lubrano-Berthelier *et al.*, 2003; Nijenhuis *et al.*, 2003; Tao & Segaloff, 2003; Vaisse *et al.*, 2000; VanLeeuwen *et al.*, 2003; Yeo *et al.*, 2003).

In 2004, Vaisse and coworkers suggested that loss of constitutive activity in the mutant MC4Rs is one cause of obesity (Srinivasan *et al.*, 2004). They showed that several mutations in the extracellular N terminus resulted in no changes in other functions except for the loss of basal activity when compared with the WT MC4R. They suggested that the constitutive activity of the MC4R transmits a tonic satiety signal. Defects in this tonic signaling could cause obesity (Srinivasan et al., 2004).

Subsequently, we and others studied the basal activities in other MC4R mutants. In our studies of ten MC4R mutants identified from obese patients with binge eating disorder as well as other obese or non-obese subjects, we showed that five mutants, I102S (2.62) and I102T in TM2, A154D in IL2, F202L (5.48) in TM5, and N240S (6.30) in TM6 have decreased basal activities, whereas the other four mutants, including T11A in the extracellular domain, F51L (1.39) in TM1, T112M in EL1, and S295P (7.46) in TM7, have normal basal activities (Tao & Segaloff, 2005). Of the three mutants identified from obese Chinese subjects, including Y35C and C40R in the extracellular domain and M218T in IL3, all have normal basal activities (Rong *et al.*, 2006).

Another evidence supporting the potential importance of the constitutive activity of the MC4R in regulating energy balance is the unique existence of endogenous inverse agonists in the melanocortin system. For the MC4R, Agouti-related protein (AgRP) is an inverse agonist (Haskell-Luevano & Monck, 2001; Nijenhuis *et al*., 2001) (reviewed in (Adan, 2006; Adan & Kas, 2003)). The balance between the actions of the agonists and the inverse agonist determines the activity of the MC4R. For a mutant receptor, even if the agonist action were not altered, increased affinity for AgRP would be predicted to result in defective receptor activity. Therefore several studies have also investigated the affinity of the mutant receptors for AgRP. O'Rahilly and colleagues first showed that a mutant in the carboxyl terminus (I316S) had decreased affinity for the agonist but unchanged affinity for the antagonist, therefore with an altered balance for them (Yeo et al., 2003). Of the three MC4R variants identified in Chinese subjects, all had normal binding affinities towards AgRP (Rong et al., 2006). Haskell-Luevano and colleagues systematically studied the binding of 40 naturally occurring MC4R mutants towards multiple agonists and the antagonist AgRP. They identified mutants with both increased and decreased AgRP potency as measured by AgRP ability to antagonizing agonist signaling (Xiang *et al.*, 2006).

Paradoxically, some of the mutant MC4Rs identified from obese patients have increased constitutive activity compared with WT MC4R. The first CAM MC4R identified, L250Q (6.40) in TM6, has very high constitutive activity (Vaisse et al., 2000). Even when the expression of the mutant receptor is only half of the WT receptor, it still exerts high constitutive activity (Xiang et al., 2006). Subsequently, other mutations with high constitutive activity were identified from either obese or overweight subjects (Hinney *et al.*, 2006; Hinney *et al.*, 2003; Valli-Jaakola *et al.*, 2004). Theses mutants, including S127L (3.30) in TM3, H158R in IL2, and P230L in IL3, all significantly increased basal cAMP levels compared with WT MC4R. As explained above, loss of constitutive activity is consistent with obesity pathogenesis. Increased constitutive activity is expected to result in lean phenotype or perhaps even associated with anorexia nervosa. Indeed, studies have shown that some variants such as I251L and V103I are associated with protection from obesity (Stutzmann *et al.*, 2007; Young *et al.*, 2007). Therefore whether these constitutively active mutations are indeed the cause of obesity remains unknown. For L250Q, slightly decreased expression of the mutant receptor was reported (Vaisse et al., 2000). For S127L, impaired signaling to the agonist was reported (Valli-Jaakola et al., 2004). These defects might balance the constitutive activity in vivo. One caveat is that the functional studies were performed in heterologous cells and may not faithfully mimic the response in neuronal cells. Furthermore, constitutive desensitization or down-regulation might decrease the constitutive signaling in vivo. Another reason might be the induction of phosphodiesterases in vivo that degrades the cAMP produced therefore attenuates the signal generated by these neurons (Persani et al., 2000; Shinozaki et al., 2003).

Interestingly, two naturally occurring variants were also identified from different strains of pigs. We showed that one mutation, D298N (7.49), which mutates a highly conserved Asp in TM7 (of the N/DPXXY motif), cause a small decrease in basal activity, although it does not change the affinity for AgRP (Fan *et al.*, 2008). The other mutant, R236H in IL3, has the same

basal activity and binding affinity for AgRP as WT porcine MC4R (Fan et al., 2008). The relevance of these two variants in modulating pig growth traits is doubtful, consistent with the genetic studies (reviewed in (Fan et al., 2008)).

It should be pointed out that for the related melanocortin-3 receptor (MC3R), the WT receptor has no constitutive activity (Tao, 2007). Of the mutant MC3Rs identified so far, none changed the basal activity (Tao, 2007; Tao & Segaloff, 2004).

4.2. GHSR mutations and familial short stature/obesity

The field of GHS and ghrelin is a fascinating story. In the 1970s, Bowers and colleagues developed a series of small synthetic peptides that stimulate growth hormone (GH) secretion (Bowers, 1998). Subsequently, Merck developed peptidomimetics such as MK-0677 that could be administered orally (Patchett *et al.*, 1995). This group then cloned the GHSR from pituitary and hypothalamus in 1996 as an orphan receptor with the endogenous ligand unknown (Howard *et al.*, 1996). Subsequently, ghrelin was shown to be the endogenous cognate ligand for GHSR (Kojima *et al.*, 1999). Ghrelin is unique in having a special post-translational modification at the third residue, Ser^3, octanoylation, that is essential for binding to the receptor and its biological activities.

Extensive studies showed that ghrelin/GHSR system is important in regulating both energy homeostasis and growth hormone secretion (reviewed in (Davenport *et al.*, 2005)). As one way of brain-gut communication, ghrelin regulates both short-term energy balance, through its stimulatory effect on food intake and inhibitory effect on fatty acid oxidation, as well as long-term energy homeostasis. In the arcuate nucleus, ghrelin activates the neuropeptide Y (NPY)/AgRP neuron (Nakazato *et al.*, 2001). Ghrelin is the first circulating hormone that can stimulate food intake with peripheral administration. The other orexigenic hormones, such as NPY, AgRP, orexins, and melanin-concentrating hormone, all failed to stimulate food intake following systemic administration (Hosoda *et al.*, 2002). In addition, ghrelin has many other functions, such as stimulation of gastric acid secretion and motility, anti-proliferative actions on cancer cells, and vasodilatory effect likely by acting on the smooth muscle (Davenport et al., 2005).

Because of the critical importance of ghrelin on regulating GH secretion and energy homeostasis, dysfunctional GHSR was expected to cause short stature due to deficient GH secretion and/or obesity. The first screening effort for mutations in GHSR identified two missense variants, A204E in EL2 from an obese patient and F279L (6.51) in TM6 from a child with short stature (Wang *et al.*, 2004). Subsequently, A204E mutation was identified in two unrelated families with familial short stature (Pantel *et al.*, 2006). The mode of inheritance is dominant. Heterozygous individuals already exhibit the phenotype. However, there is incomplete penetrance: not all individuals harboring the heterozygous mutation have short stature (Pantel et al., 2006). The causative role of GHSR in obesity is less conclusive. Although there is genetic linkage and association of GHSR with obesity in humans (Baessler *et al.*, 2005), the first screening effort obtained inconclusive evidence regarding GHSR mutation and obesity (Wang et al., 2004). In the patients described in the Pantel et al. study, some with A204E mutations were not obese (Pantel et al., 2006). The mechanism of any loss-of-function mutations that are associated with obesity is not clear. Ghrelin is a potent hunger signal postulated to be a meal initiation factor (Cummings *et al.*, 2001). Loss-of-function would be predicted to protect from obesity leading to a lean phenotype, such as in ghrelin and/or GHSR knockout mice (Pfluger *et al.*, 2008; Wortley *et al.*, 2005; Zigman *et al.*, 2005).

GHSR is coupled to $G_{q/11}$, therefore receptor activation leads to increased levels of IP and Ca^{++}. Schwartz and colleagues showed that human GHSR has very high constitutive activity, at about 50% of ghrelin-stimulated signaling (Holst *et al.*, 2003). This constitutive activity is

likely to be relevant in vivo. For example, GHSR knockout leads to more severe defect in energy balance than ghrelin knockout (Pfluger et al., 2008). Functional studies showed that A204E GHSR can signal normally to GHSR but have diminished constitutive activity (Pantel et al., 2006). Although binding capacity was decreased due to low cell surface expression, the binding affinity for ghrelin was not affected. A previous study showed that F279L had decreased binding for MK-0677 (Feighner *et al*., 1998). Schwartz and colleagues showed that F279 is part of a cluster of aromatic residues located at the cytoplasmic ends of TM6 and TM7 that is important for maintaining the active conformation of the WT receptor, mutations of these residues result in loss of the constitutive activity (Holst *et al.*, 2004). Kopin and colleagues recently performed extensive functional characterization of the two mutants and two variants reported in the single nucleotide polymorphism database, I134T (3.43) in TM3 and V160M (4.42) in TM4 (G. Liu *et al*., 2007). They showed that V160M and F279L have significantly reduced constitutive activity whereas A204E is devoid of constitutive activity (basal signaling is similar to mock transfected cells) (Liu et al., 2007).

5. Lessons learned from animal models expressing constitutively active GPCRs

Expression of the mutant GPCRs in mice not only confirms the involvement of the mutations in the pathogenesis of the diseases, but also it can reveal novel physiological and pathological roles of the receptors.

The mutation associated with ADRP, K296E, was shown to be constitutively active in vitro (see above). However, the mutant receptor is not constitutively active in transgenic animals (Li *et al.*, 1995). It was shown that the mutant rhodopsin is phosphorylated (likely by rhodopsin kinase) and bound with arrestin forming inactive complexes (Li et al., 1995). In vitro studies produced conflicting results. One study suggested that constitutively active rhodopsins are not constitutively phosphorylated (Robinson *et al.*, 1994) whereas another study showed that these mutants are constitutively phosphorylated by rhodopsin kinase (Rim & Oprian, 1995).

Haywood et al. devised a clever strategy to investigate the functional relevance of the first naturally occurring constitutively active mutation in FSHR, D567G (Haywood *et al.*, 2002). Transgenic mice expressing D567G-FSHR were produced in gonadotropin-deficient background, the *hpg* mice. These mice cannot produce gonadotropin-releasing hormone; therefore there is no LH or FSH production. Transgenic *hpg* mice expressing D567G-FSHR had increased testis weight, containing mature Sertoli cells and postmeiotic germ cells absent in *hpg* controls. Sertoli cells isolated from transgenic mice had increased (2-fold) basal cAMP level demonstrating that indeed the mutant FSHR is constitutively active. Comparison of transgenic mice expressing FSH or D567G-FSHR with LHCGR knockout mice showed that in mice, full Sertoli cell proliferation could be accomplished by FSH activity alone (Allan *et al.*, 2004). To investigate its action in maintaining rather than initiating spermatogenesis, D567G-FSHR was expressed in normal mice. Analysis of the testes from these mice showed that total Sertoli and spermatogonia cell numbers were increased, indicating constitutive FSH-like activity (Allan *et al.*, 2006).

A transgenic mouse line in which expression of a constitutively active PTHR1, H223R, targeted to the growth plate by the rat α1 (II) collagen promoter, was established (Schipani *et al.*, 1997b). These mice recapitulate the phenotypes of JMC patients with short and deformed limbs. They showed delayed mineralization, decelerated chondrocyte maturation in skeletal segments that are formed by the endochondral process, and prolonged presence of hypertrophic chondrocytes with delay of vascular invasion (Schipani et al., 1997b). The transgene also corrects most of the bone abnormalities in PTHR1 knockout mice, although the PTHR1 null mice with the transgene still die perinatally (Soegiarto *et al.*, 2001). Expression of the same

CAM in osteoblasts by placing its expression under the control of the type I collagen promoter showed that both the osteoblast and osteoclast numbers were increased, suggesting that PTH activation of PTHR1 in osteoblasts increase both bone-forming and bone-resorbing activities (Calvi *et al.*, 2001). In another transgenic mouse line with the CAM expression targeted to the osteoblasts under the control of Col1a1 promoter, mutant mice have decreased disuse-induced bone loss due to decreased osteoclastic activity (Ono *et al.*, 2007). Analysis of the bone marrow system of these mice revealed that PTHR1 regulates the establishment of the hematopoietic stroma compartment and of the skeletal stem cells (Kuznetsov *et al.* 2004).

6. Insights into mechanism of activation of GPCRs

Detailed characterizations of the naturally occurring constitutively active mutations of GPCRs that cause diseases described above not only provide novel insights into the physiological and pathophysiological roles of the underlying system, but also shed lights on the structure-function relationships of the GPCRs, especially the mechanism of activation. In addition, using the clues provided by the experiments of nature, scientists have generated multiple mutations at the diseased loci to examine which amino acids can cause constitutive activation. In addition, homologous mutations in other related GPCRs or in the same GPCRs from other species were also made to gain a better understanding of the mechanism of activation of related receptors. In many of these studies, experimental data were combined with homology modeling using the crystal structure of inactive rhodopsin as the template. With the recent reports of the β_1- and β_2-AR crystal structures, some of the future studies will likely also use these structures as templates. As mentioned earlier, rhodopsin is a unique receptor with the ligand covalently bound to the opsin moiety. All other receptors, including β_2-AR, bind diffusible ligands. Although the crystal structures of rhodopsin and β_2-AR are very similar, with the root mean squared deviation for the alpha carbon backbone of the TMs at 1.56 Å(Rasmussen et al., 2007), there are some significant differences, such as a more open conformation for the β_2-AR, weaker interactions between the cytoplasmic ends of TM3 and TM6, the lack of “ionic lock” between D/E (3.49) and R (3.50) as well as E (6.30). Modeling based on information from multiple structures might provide a better representation of other GPCRs that still wait for direct structural studies.

One question that remains to be addressed unequivocally is how accurate a representation the constitutively active mutation is of the ligand-induced active conformation. The consensus is that CAM is an intermediate conformation (R’) between the inactive (R) and fully activated (R*) conformations. In fact, it is widely accepted that there is an ensemble of active conformations and different agonists may induce and/or stabilize different active conformations (Maudsley *et al.*, 2005; Whistler *et al.*, 2002). The conformational changes associated with ligand binding involve both disruption of interactions that constrain the WT receptor in inactive state and the establishment of new interactions maintaining the receptor in active conformation. Some CAMs mimic the breakage of interactions that constrain the WT receptor (for example, mutations of A293 in IL3 of the α_{1B}-AR into any other residue will cause constitutive activation (Kjelsberg *et al.*, 1992)), whereas some mutants mimic the establishment of new interactions (for example, only the introduction of Arg in L460 in human FSHR cause constitutive activation, other mutations at this locus do not cause constitutive activation (Tao $_{\text{et al}}$., 2000). In the related LHCGR, mutations that induce a positive charge (Lys, Arg and His) cause constitutive activation (Shinozaki *et al.*, 2001).

With these caveats in mind, I summarize below some of the studies that exploited the naturally occurring mutations in more detail and lessons learned.

6.1. Multiple mutations of the diseased locus

To gain a better understanding of the mechanism of activation in the naturally occurring mutants, investigators have generated multiple mutations at the diseased loci to see which amino acids are tolerated and which ones are not. From these results, the potential interactions involved could be predicted.

Since LHCGR activating mutations cluster around the IL3 and TM6 (Table 2), several investigators have studied in detail the importane of this region in LHCGR activation. Kosugi et al. mutated D578 to multiple residues (Kosugi et al., 1996). They showed that replacement of D578 with Gly, Ser, Leu, Tyr, or Phe cause constitutive activation, whereas replacement to Asn do not cause constitutive activation, suggesting that the ability of D578 acting as a hydrogen bond acceptor, rather than its negative charge, is critical for constraining the receptor in inactive conformation (Kosugi et al., 1996). Computer modeling and further mutagenesis experiments suggested that D578 forms hydrogen bonds with both N615 (7.45) and N619 (7.49) (Angelova *et al.*, 2002;Lin *et al.*, 1997). Mutations that disrupt these hydrogen bonds cause constitutive activation (Angelova *et al.*, 2000).

Fanelli performed extensive molecular dynamic simulations of the WT and CAMs of the LHCGR (Angelova et al., 2002; Fanelli, 2000; Fanelli *et al.*, 2004; Zhang *et al.*, 2005; Zhang *et al.*, 2007). These computational experiments suggested that the activation of the LHCGR is accompanied by the breakage of a salt bridge between the cytoplasmic ends of TM3 and TM6 (R3.50-D6.30). The result is that several residues at the cytoplasmic ends of TM3 and TM6 are more exposed, leading to increased solvent accessible surface area (SAS value). The residues used for calculating SAS values were revised several times. The most recently used residues include R464 (3.50), T467 (3.53), I468 (3.54), and K563 (6.29). For example, for the L457R, molecular dynamic simulations suggested that the introduced Arg forms a salt bridge with D578 (6.44), the locus of the first constitutively active mutation reported. Segaloff and colleagues generated disruptive and reciprocal mutants to test this hypothesis. The results of these experiments showed that a mutant designed to disrupt this salt bridge (L457R/D578N) resulted in dramatic decrease in constitutive activity, whereas a reciprocal mutant L457D/D578R (the salt bridge can still form in this mutant except that the positions of the two residues were switched) has similar high constitutive activity as L457R, suggesting that indeed there might be a salt bridge in L457R mutant that contributes to the constitutive activation of the mutant receptor (Zhang et al., 2005) (reviewed in (Latronico & Segaloff, 2007)).

Multiple mutations of some of the FSHR loci identified that result in constitutive activation have been performed. Combination of multiple mutagenesis and molecular modeling suggested that weakening of interhelical locks between TM6 and TM3 or TM6 and TM7 was responsible for the constitutive activity of D567 (6.30) and T449 (3.32) mutants (Montanelli *et al.*, 2004b). A common feature of several members of Family A GPCRs is an ionic lock between D (6.30) and R (3.50) (Angelova et al., 2002; Ballesteros *et al.*, 2001; Gether, 2000; Greasley *et al.*, 2002). Any mutation that weakens or disrupts this interaction results in constitutive activation. The recently reported opsin structure showed that indeed this ionic lock is broken in opsin (Park et al., 2008). For FSHR, a negatively charged residue is needed at D (6.30) to maintain the receptor in inactive conformation. Especially, mutations introducing a basic residue such as Arg or Lys cause in repulsion of the newly introduced residue and R (3.50), resulting in high constitutive activity (Montanelli et al., 2004b). Similarly, T449 (3.32), together with S (3.36), forms hydrogen bonds with H (7.42). Mutations that disrupt these interactions result in constitutive activation (Montanelli et al., 2004b). I545 (5.54) was mutated to Ala, Phe, Leu, Asn, and Val in addition to the naturally occurring mutation Thr (De Leener et al., 2008). After correcting for differences in cell surface expression, it was shown that some mutants such as I545L, I545N, I545F, and I545T cause constitutive activation, whereas others such as I545V and I545A do not cause constitutive activation. Molecular modeling suggested

that I545, at the intracellular end of TM3, is part of a tight packing involving residues from TM3 (L460 and T464) and TM6 (I579). Mutations that disrupt the packing either by establishing new interactions (I545T or I545N) or by disrupting the local structure (I545F and I545L) cause constitutive activation, where those that do not are not constitutively active (De Leener et al., 2008). Our previous data showing that L460R mutation can cause constitutive activation is consistent with this hypothesis (Tao et al., 2000).

Saturation mutagenesis (mutation of a residue to all other 19 natural amino acids) was also performed at the H223 and T410 loci in PTHR1 (Schipani *et al*., 1997a). Mutation of H223 to a basic residue (Arg or Lys) causes constitutive activation, whereas all the other mutants were not constitutively active. Substitution of T410 with any other residue causes a certain degree of constitutive activation, suggesting that mutation of T410 results in disruption of an interaction that constrains the WT receptor in inactive conformation (Schipani et al., 1997a).

6.2. Homologous mutations in other GPCRs

Members of the superfamily of GPCRs are divided into families and subfamilies. Members of subfamilies usually share high homology. For example, of the five melanocortin receptors, all can bind adrenocorticotropin and are about 50% homologous. However, they do exhibit important differences in signaling. For example, the WT MC4R has some constitutive activity, whereas the WT MC3R has no basal activity. Closely related receptors also have different susceptibilities to mutation-induced constitutive activation, as shown by the examples described here.

Another fruitful approach employed by numerous investigators is to generate homologous mutations in related receptors corresponding to the naturally occurring mutations that we know cause constitutive activation. Indeed, naturally occurring activating mutations have been found in the same locations in related receptors. For example, L3.43 were mutated to Arg in both LHCGR (Latronico et al., 1998) and TSHR (Kosugi et al., 2000; Trulzsch et al., 2001). D6.30 were mutated in LHCGR, FSHR, and TSHR (see above). Similarly, I/V5.54 was mutated in LHCGR (to Leu) (Laue et al., 1995) or TSHR (to Phe or Leu) (Esapa et al., 1999). Some of the positions mutated in LHCGR were also sites for constitutively active mutations in other Family A GPCRs (reviewed in (Shenker, 2002)).

Inspection of the naturally occurring activating mutations in the glycoprotein hormone receptors revealed that the TSHR has the most mutations, followed by the LHCGR, and finally the FSHR (see above). This is the same rank order of the basal activity of the WT glycoprotein hormone receptors, with the TSHR the noisiest and the FSHR the most quiescent (Feng *et al.*, 2008; Van Sande et al., 1995). This may not be a coincidence. WT TSHR is the least constrained, therefore a minor disturbance in the interactions constraining the WT receptor can further destabilize the receptor, resulting in constitutive activation, even against the noisy WT state (Van Sande et al., 1995). For the FSHR, the WT receptor is highly constrained. Small disturbance in the interactions constraining the WT receptor may not be able to destabilize the receptor enough to generate measurable constitutive activity. Laboratory-designed mutants are consistent with this suggestion.

As described earlier, several activating mutations were identified in LHCGR that cause male-imited precocious puberty. Hsueh's group generated four mutants in the FSHR corresponding to those activating mutations in LHCGR. Surprisingly, they showed that all four mutations do not cause constitutive activation in FSHR (Kudo *et al.*, 1996). After the identification of the LHCGR L457R (3.43) activating mutation (Latronico et al., 1998), we generated the corresponding mutation in the FSHR (Tao et al., 2000). We showed that the corresponding mutation L460R causes a strong constitutive activation, increasing basal cAMP level five-fold (Fig. 2). When L460 was mutated to Lys, Ala, or Asp, none of the mutants are constitutively

active. L (3.43) is highly conserved in Family A GPCRs, present in 74% family members (Mirzadegan *et al.*, 2003). Therefore we generated additional mutants in the β_2-AR. We showed that mutations of the homologous residue (L124) to Arg, Lys, or Ala all resulted in constitutive activation (Tao et al., 2000). These results suggest that L3.43 is important in stabilizing the inactive conformation of GPCRs. This conclusion is further supported by the following studies. In the TSHR, the corresponding mutation (L512R) has been identified from patients with hyperthyroidism (Kosugi et al., 2000; Trulzsch et al., 2001). L512R is constitutively active. In addition, alanine-scanning mutagenesis from Hulme and colleagues also showed that Leu3.43 is important for maintaining the inactive conformation in the rat M1 muscarinic receptor (Lu & Hulme, 1999). Mutation in C5a receptor at this locus also causes constitutive activation (Baranski *et al.*, 1999). Recent crystal structure of β_2-AR showed that this Leu, as well as L272 (6.34), are linked through packing interactions with those residues that when mutated cause defective agonist-induced activation (indicating these residues are important for forming intramolecular interactions that stabilize active conformation) (Fig. 2). It was suggested that signaling generated by ligand binding might be propagated through this packing interaction, mediated by rotameric toggle switch at W286 (6.48), to the cytoplasmic half of the receptor, resulting in G protein coupling/activation. However, as always, there are exceptions. I generated the corresponding mutation in MC4R. The mutant receptor (L140R) was not constitutively active. In fact, the mutant receptor has lower basal activity compared with WT MC4R (Tao YX, unpublished observations) (Fig. 2). This result suggests that there are important differences in the role of L3.43 in maintaining the inactive conformation in the MC4R versus the other GPCRs cited above.

The roles of L3.43 in mediating agonist-induced activation of the receptors seem to be different among the different receptors. For example, in both LHCGR and FSHR, L(3.43)R mutation leads to severely impaired hormone-induced signaling (Latronico et al., 1998; Tao et al., 2000; Shinozaki *et al.*, 2001). However, in β_2-AR, the mutation does not affect agonist-induced signaling (Tao et al., 2000).

For the TSHR, it was hypothesized that the large extracellular domain acts as an inverse agonist constraining the unliganded receptor in inactive conformation. Extensive studies have shown that a serine in the extracellular domain (S281 in the TSHR or S277 in the LHCGR) is critical for constraining the WT receptors in inactive conformation (Ho *et al.*, 2001; Nakabayashi *et al.*, 2000; Parma et al., 1997; Vlaeminck-Guillem *et al.*, 2002). It was shown that in the LHCGR, constitutive activation induced by S277N mutation requires all the leucine rich repeats of the ectodomain (Sangkuhl *et al.*, 2002). A corresponding mutation (S273I) in the FSHR has been shown to also cause constitutive activation (Montanelli et al., 2004b).

It was assumed that the human FSHR (hFSHR) is more resistant to mutation-induced constitutive activation than the TSHR or LHCGR. To study this further, we recently performed a detailed comparison of the susceptibility to mutation-induced constitutive activation in the LHCGR versus the FSHR (Zhang et al., 2007). Based on data from LHCGR, nine homologous mutations were made in FSHR. These experiments showed that only three FSHR mutants have significant constitutive activity. The levels of constitutive activities in the FSHR CAMs are also lower than those in the LHCGR. However, modeling suggested that the mechanisms through which homologous mutations cause constitutive activation are similar between the FSHR and LHCGR (Zhang et al., 2007).

Although we confirmed Hsueh's data on the hFSHR D581G (6.44) (not constitutively active), we found unexpectedly the corresponding mutation in rat FSHR (rFSHR) (D580G) caused very robust constitutive activation (Tao *et al.*, 2002). The basal cAMP of D580G rFSHR is increased ~10-fold compared with the WT rFSHR. Since the rat and human FSHRs are highly homologous, at 89% over the full-length receptors, and 95% in the TMs, we sought to identify

the molecular determinants of this dramatic difference in the two receptors in susceptibility to D(6.44)G mutation-induced constitutive activation. Using chimera, we showed that switching the extracellular domains do not affect the mutation-induced constitutive activation. Hence, the chimera with the extracellular domain of hFSHR and TMs of rFSHR were constitutively active when the D(6.44)G was introduced. The chimera with the extracellular domain of rFSHR and the TMs of hFSHR was not constitutively active when the D(6.44)G mutation was introduced. These results suggest that the molecular determinants dictating the two receptors to D(6.44)G-induced constitutive activation lie in the serpentine region of the receptors. We therefore used reciprocal site-directed mutagenesis to identify these molecular determinants. We showed that two residues, one each in TM6 and TM7, could account for this dramatic difference. The double mutant M576T/H615Y hFSHR, although not constitutively active, becomes constitutively active when the D(6.44)G mutation was introduced. Similarly, when Y614 in rFSHR was mutated to the corresponding residue in hFSHR, His, the D(6.44)G mutant receptor is not constitutively active any more (Fig. 3). Homology modeling suggested that differences in hydrophobic interactions between TM6 and TM7 of the two FSHRs might account for the different susceptibility to D(6.44)G mutation-induced constitutive activation (Tao et al., 2002) (Fig. 3). In hFSHR, hydrophobic interaction between I588 in TM6 and V612 in TM7 is prevented in the rFSHR by the steric and polar interference from H615. Stronger hydrophobic interactions in the cytoplasmic ends of TM6 and TM7 are necessary for D(6.44) G mutation-induced constitutive activation. In rFSHR, four residues, including T575 and L576 in TM6 and L624 and F628 in TM7, have hydrophobic interactions. However, in hFSHR, the side chain of M576 is predicted to project to the lipid bilayer therefore cannot participate in the hydrophobic interaction. Hence in hFSHR, only three residues form hydrophobic interactions (Tao et al., 2002). Hsueh's group showed that different interactions between TM5 and TM6 are responsible for the different susceptibility to D(6.30)G mutation-induced constitutive activation in hFSHR (where the mutant is not constitutively active) and LHCGR (where the mutant is constitutively active) (Kudo et al., 1996).

Homologous mutations of H223R and T410P mutations were also made in other PTHR1 as well as related Family B GPCRs (Schipani et al., 1997a). Interestingly, these mutations result in constitutive activation in the opossum receptor but not in rat receptor. The molecular determinants of this difference in susceptibility to mutation-induced activation have not been elucidated. When these mutations were introduced into PTHR2, calcitonin receptor, secretin receptor, growth hormone releasing hormone receptor, glucagon-like peptide 1 receptor, and corticotropin-releasing hormone receptor, no constitutive activation was observed (Schipani et al., 1997a). However, when the homologous mutations were introduced into rat glucagon receptor, H178R (corresponding to H223R in PTHR1) was constitutively active, whereas T352P (corresponding to T410P in PTHR1) was not (T352A is constitutively active) (Hjorth *et al.*, 1998).

7. Therapeutic implications for inverse agonists

When the inverse agonism was first described, there was reservation on whether it was of therapeutic relevance or just an interesting laboratory observation (Milligan *et al.*, 1995). Studies performed during the past decade or so have provided unequivocal evidences on the physiological relevance of constitutive activity, suggesting the potential therapeutic values of modulating the constitutive activity. Kenakin reviewed the activity of 380 antagonists for 73 receptors that show constitutive activity and found 85% of these antagonists were inverse agonists (Kenakin, 2004). Some of the most highly prescribed drugs are inverse agonists. Prominent examples include the beta-blockers propranolol, alprenolol, pindolol, and timolol used for treating hypertension, angina pectoris, and arrhythmia that act on the β_2-AR, metoprolol and bisoprolol used for treating hypertension, coronary heart disease, and arrhythmias by acting on β_1-AR, cetirizine and loratadine for treating allergies and hay fever

by acting on histamine H_1 receptor, cimetidine and ranitidine acting on the histamine H_2 receptor for treating heartburn and peptic ulcers, prazosin and phentolamine for treating hypertension by acting on α_1-adrenergic receptors, atropine used as premedication for anesthesia, and pirenzepine used for treatment of peptic ulcer, respectively, that act on the muscarinic receptors, and naloxone used for treating heroin overdose by acting on μ-opioid receptor (Kenakin, 2004). As recently suggested by Kenakin, translational research of inverse agonism will show whether new therapeutic options will emerge (Kenakin, 2006), especially for silencing the constitutively active mutant receptors that cause the diseases described herein.

Govardhan and Oprian (Govardhan & Oprian, 1994) did the first proof-of-principal experiment on the therapeutic potential of inverse agonist in treating diseases due to constitutively active mutations in GPCRs. In that study, they set out to find the inhibitor that can fit three criteria: it should inactivate the mutant rhodopsins in the dark and in light, it should not affect the WT rhodopsin, and it should be able to cross the blood-retina barrier. They showed that amine derivatives of 11-*cis*-retinal could accomplish this on K296G, a laboratory-generated CAM. However, they are not active on the naturally occurring mutants K296E and K296M, likely due to the greater steric bulk of Glu and Met side chains in comparison to Gly (Govardhan & Oprian, 1994). Subsequently, they synthesized a retinylamine analog that is one carbon shorter than the parent 11-*cis*-retinal and showed that this compound is indeed an effective inhibitor of both the K296E and K296M mutants. The analog inhibited constitutive activation of transducin as well as their constitutive phosphorylation by rhodopsin kinase (Yang *et al.*, 1997).

Currently, the treatments for diseases caused by constitutively active mutations in the TSHR gene include removal of the thyroid gland with additional radiotherapy. For diseases caused by activating LHCGR gene mutations, current treatments include antiandrogens such as cyproterone acetate and bicalutamide, aromatase inhibitors such as testolactone and anastrozole, and cytochrome P450 inhibitor such as ketoconazole (Almeida *et al.*, 2008). Again, the major goal is to decrease the skeleton maturation to increase the adult height of the affected boys. Variable success was observed. For OHSS patients with constitutively active FSHR gene mutations, the treatment was careful observation or termination of pregnancy to remove the source of hCG. No inverse agonists have been tested in these systems. Recently, small molecule agonists and antagonists have been identified for LHCGR and FSHR (reviewed in (Blakeney *et al.*, 2007; Heitman & IJzerman, 2008)) although it is not known whether any of the antagonists are inverse agonists and their potential utility in treating these genetic diseases.

In the MC4R, activation of the receptor results in decreased food intake and increased energy expenditure and inhibition of the receptor results in increased food intake and decreased energy expenditure. Several groups have tried to use MC4R antagonists for treating cancer cachexia. Marks and coworkers showed that intracerebroventricular injection of the natural inverse agonist AgRP prevented anorexia and loss of lean mass in mice bearing a syngenic sarcoma or caused by uremia (Cheung *et al.*, 2005; Marks *et al.*, 2001). Wisse and coworkers showed that intracerebroventricular injection of the MC4R antagonist SHU9119 (a peptide analogue of α-MSH) completely reversed cancer anorexia in mice with prostate cancer (Wisse *et al.*, 2001). Both AgRP and SHU9119 are non-specific antagonists for the neural MCRs, blocking the action of both the MC3R and MC4R. Further studies showed that the effects of these antagonists are mediated by the MC4R not the MC3R (Marks *et al.*, 2003). These studies established the principle that MC4R antagonism results in increased food intake and decreased energy expenditure, resulting in less lean mass loss in chronic disease state. However, peptides are not practical therapeutic approach. Subsequently, a small molecule antagonist acting as inverse agonist for the MC4R developed by Vos and colleagues, ML00253764, was also shown to prevent tumor-induced anorexia (Nicholson *et al.*, 2006; Vos *et al.*, 2004). Another small

molecule compound developed by Neurocrine Biosciences, NBI-12i, was also shown to ameliorate tumor-induced anorexia and loss of lean mass (Markison *et al.*, 2005). However, this compound was shown to be a competitive MC4R antagonist. Recently, it was shown that oral administration of a novel MC4R antagonist 1-[2-[(1S)-(3-dimethylaminopropionyl) amino-2-methylpropyl]-6-methylphenyl]-4-[(2R)-methyl-3-(4-chlorophenyl)propionyl] piperazine increased food intake in tumor-bearing mice (Chen *et al.*, 2007). Several excellent reviews were published recently summarizing the studies on MC4R antagonism as potential therapeutic approach for cachexia, acute inflammation, and renal failure (Deboer & Marks, 2006; DeBoer & Marks, 2006; Foster & Chen, 2007). Further studies are needed to see whether there is any advantage of using inverse agonist versus neutral competitive antagonist in treating cachexia caused by cancer or other chronic diseases. It would also be of great interest to see whether small molecule mimetics of AgRP can be used for enhancing appetite in diseased states.

Because of the critical importance of ghrelin/GHSR in regulating GH secretion and energy balance, low molecular weight agonists for GHSR could be used to treat GH deficiency and cachexia due to chronic diseases. It was proposed that these agonists might be used to restore a youthful GH profile in senior citizens. GHSR antagonists could be used for treating obesity. Holst and Schwartz hypothesized that GHSR antagonists could be used before meals to antagonize the action of high levels of endogenous ghrelin and inverse agonists could be used to decrease the constitutive activity of GHSR between meals so there would be no craving for snack (Holst & Schwartz, 2004). Both approaches reduced hunger signal from GHSR (Holst & Schwartz, 2004). They first reported that [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P is a full inverse agonist decreasing the basal activity of the WT GHSR to the level in untransfected cells (Holst et al., 2003). Another peptide, D-Lys^{3}-GHRP-6, was shown to be an inverse agonist in seabream GHSR (Chan *et al.*, 2004). Several recent studies reported a number of small molecule GHSR antagonists (Demange *et al.*, 2007; Esler *et al.*, 2007; Liu *et al.*, 2004; Liu *et al.*, 2006; Moulin *et al.*, 2007; Moulin *et al.*, 2008a; Moulin *et al.*, 2008b; Rudolph *et al.*, 2007; Serby *et al.*, 2006; Xin *et al.*, 2006; Xin *et al.*, 2005; Zhao *et al.*, 2004; Zhao *et al.*, 2005). Some of these compounds were found to decrease food intake and body weight. In addition, since ghrelin were shown to also play a role in controlling glucose-induced insulin secretion (Dezaki *et al.*, 2008), some of these compounds were reported to have glucose-lowering effects mediated by enhanced glucose-dependent insulin secretion (Esler et al., 2007; Rudolph et al., 2007). However, none of them were reported to be inverse agonists. Many of these studies did not investigate whether the compounds reported are inverse agonists or not. Functional studies measured intracellular calcium level rather than IP production that fail to detect the constitutive activity of GHSR (Holst et al., 2003) therefore masking any potential inverse agonistic activity. Inverse agonism can only be measured in a system with constitutive activity. Clinical trial to test Holst and Schwartz's hypothesis of combining neutral antagonists and inverse agonists to combat obesity has to wait for the development of safe inverse agonists.

One concern for using inverse agonists as therapeutics is the up-regulation of receptor numbers resulting in tolerance (Milligan & Bond, 1997). This was first observed with the H_2 histamine receptor (Smit et al., 1996). The inverse agonists cimetidine and ranitidine but not the neutral antagonist burimamide induced up-regulation of the exogenously expressed H_2 receptor, providing a plausible explanation for the tolerance to the inverse agonists after prolonged clinical use (Smit et al., 1996). Milligan and colleagues showed that inverse agonists also up-regulate β_2-and α_{1B}-ARs (Lee *et al.*, 1997; MacEwan & Milligan, 1996). Therefore achieving a high degree of suppressing the constitutive activity without the undesired effect of up-regulating receptor expression (with its corresponding increase in basal signaling) is the challenge for any drug discovery efforts trying to exploit inverse agonists as treatment modalities for the diseases caused by constitutively active mutations in GPCRs.

8. Conclusions

Since the pioneering reports of constitutively active mutations associated with either special phenotype (dominant black color in mice with MC1R mutations) or diseases (retinitis pigmentosa, hyperthyroidism or male-limited precocious puberty) in early 1990s, tremendous progresses have been made during the past 15 years or so. Constitutively active mutations in several additional receptors were found to cause other diseases. In addition, the loss of constitutive activity in the mutant receptors can also cause disease, suggesting the critical importance of the basal activity in the WT receptor in normal physiology. The detailed functional studies of these naturally occurring mutations, in combination with homology modeling using rhodopsin as the template, provided novel insights into the mechanism of activation of these GPCRs. Transgenic animals expressing some of these mutants were generated. The studies of these humanized animal models yielded novel insights into the physiological functions of these receptors. Clinical studies, combined with in vitro functional expression studies, as well as studies with genetically modified animals, significantly advanced our understanding of these genetic diseases and provided models for identifying new therapeutic options. For the diseases caused by constitutively active mutations, inverse agonists are expected to have better therapeutic values compared with neutral antagonists. Further medicinal chemistry studies are expected to yield better drugs for treating these genetic diseases.

Abbreviations

ADRP, autosomal dominant retinitis pigmentosa
AgRP, Agouti-related protein
AR, adrenergic receptor
CAM, constitutively active mutant
CNB, congenital night blindness
EC_{50}, the concentration that results in 50% maximal response
EL, extracellular loop
FMPP, familial male-limited precocious puberty
FSH, follicle stimulating hormone
FSHR, FSH receptor
GH, growth hormone
GHS, growth hormone secretagogue
GHSR, growth hormone secretagogue receptor
GPCR, G protein-coupled receptor
hCG, human chorionic gonadotropin
hFSHR, human FSHR
IL, intracellular loop
IP, inositol phosphate
JMC, Jansen's metaphyseal chondrodysplasia
LH, luteinizing hormone
LHCGR, LH/CG receptor
MCR, melanocortin receptor
MC1R, melanocortin-1 receptor
MC3R, melanocortin-3 receptor
MC4R, melanocortin-4 receptor
OHSS, ovarian hyperstimulation syndrome
PLC, phospholipase C
POMC, pro-opiomelanocortin
PTH, parathyroid hormone
PTHrP, PTH related peptide

PTHR1, PTH/PTHrP receptor type 1
rFSHR, rat FSHR
SAS, solvent accessible surface
TM, transmembrane α-helix
TSH, thyroid stimulating hormone
TSHR, TSH receptor
WT, wild type

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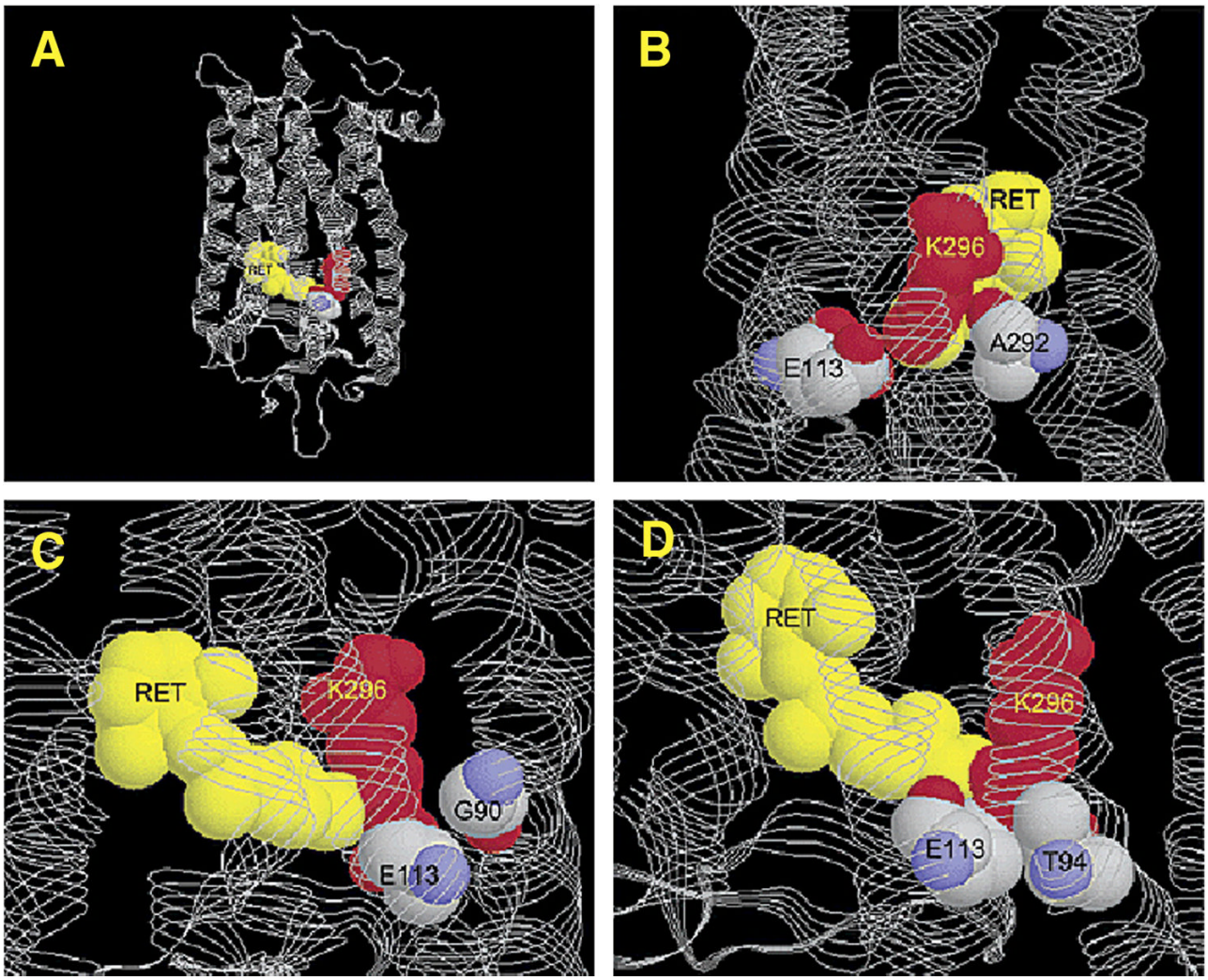

Fig. 1.

Structure of bovine rhodopsin, with coordinates from PDB entry 1L9H. Shown in A is the overall structure of rhodopsin. Shown in B–D are close-up views of the active site highlighting the locations of the three night blindness mutations: (B) A292, (C) G90, and (D) T94. Reprinted with permission from A. K. Gross et al. 2003. Biochemistry 42:2009–2015. Copyright (2003). American Chemical Society.

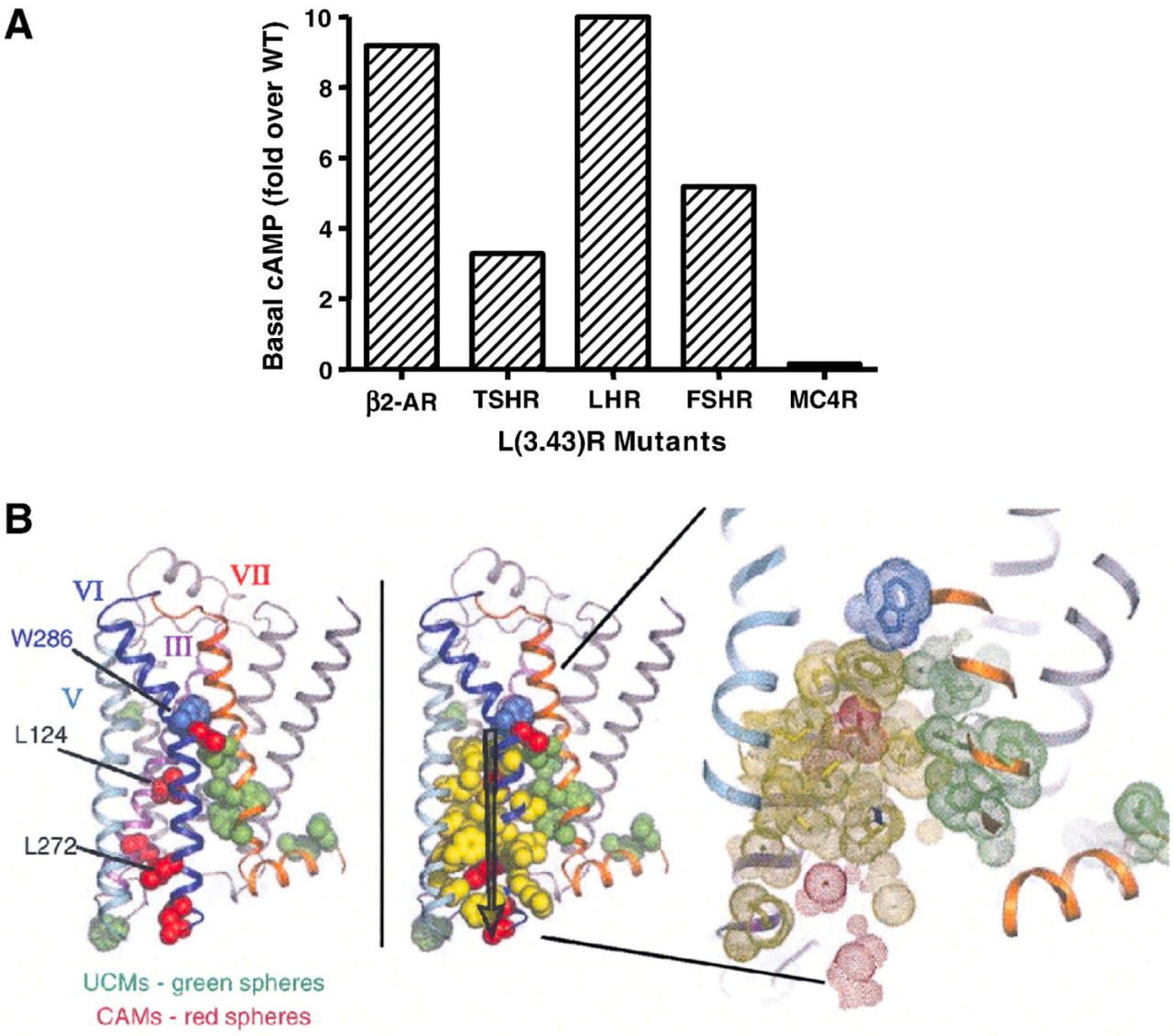

Fig. 2.

A. Basal activities of L(3.43)R mutants in several GPCRs. Data were obtained from references listed in the article with the exception of MC4R that is my own unpublished data. B. Packing interactions of CAMs, including L124 (3.43), with uncoupling mutations in the β_2-AR. Panel B is reprinted with permission from D. M. Rosenbaum et al. 2007. Science 318:1266–1273. Copyright (2007). AAAS.

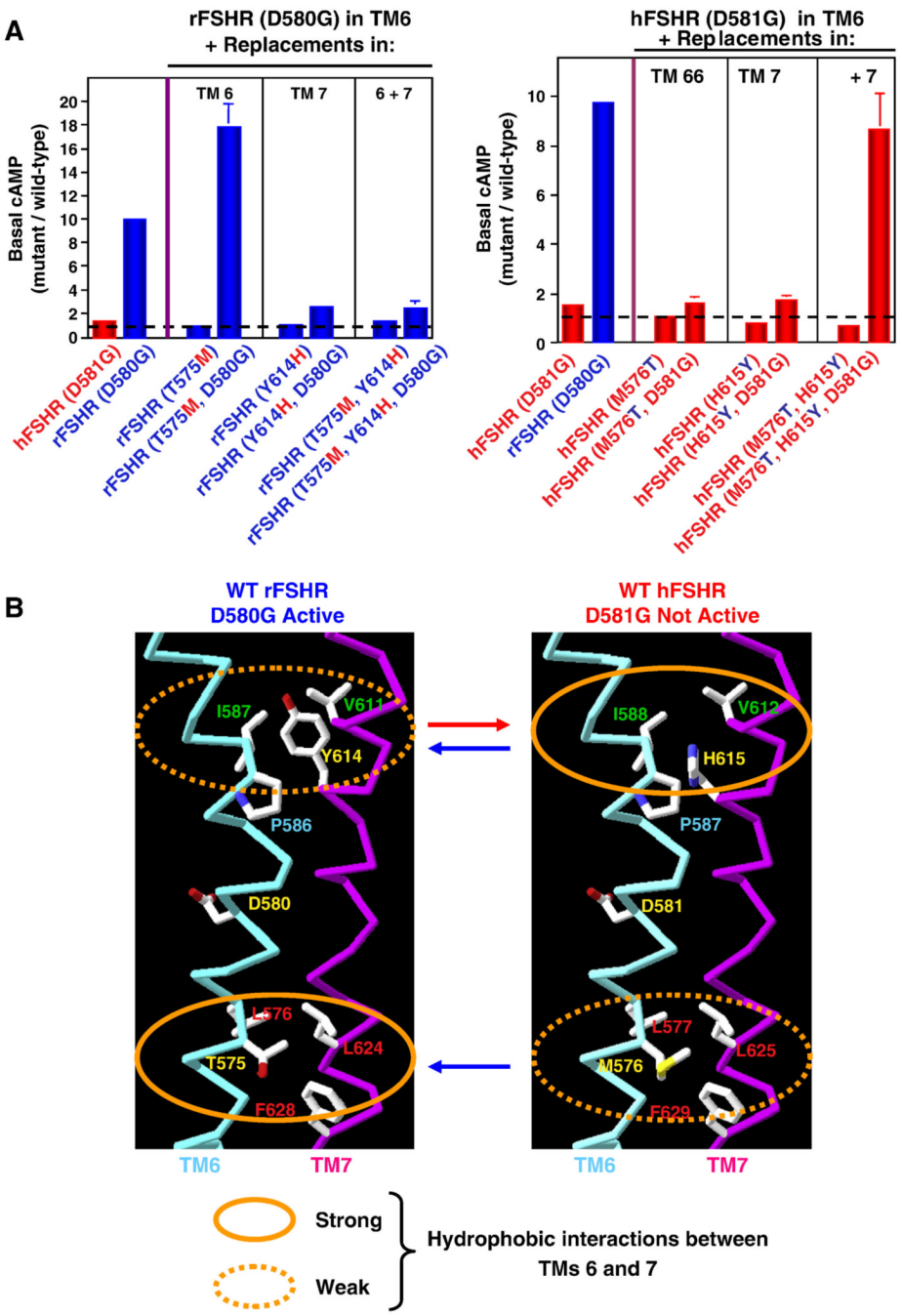

Fig. 3.

Different susceptibility in rFSHR and hFSHR to D(6.44)G-mutation induced constitutive activation. Panel A showed that two mutations, one in TM6 and one in TM7, can reverse the susceptibility to D(6.44)G-mutation induced constitutive activation. Panel B is a model of the two receptors suggesting that different hydrophobic interactions in TM6 and TM7 might be responsible for the different susceptibility to D(6.44)G-mutation induced constitutive activation. These data and models were modified and reprinted with permission from Tao et. al. 2002. Mol. Endocrinol. 16:1881–1892. Copyright (2002). The Endocrine Society.

Table 1

Naturally occurring constitutively active mutations in TSHR

Mutation	Location (Ballesteros numbering when applicable)	References
S281I	Extracellular domain	(Kopp *et al.*, 1997b)
S281N	Extracellular domain	(Biebermann *et al.*, 2000; Duprez *et al.*, 1997b)
S281T	Extracellular domain	(Duprez et al., 1997b)
S425I	TM1 (1.43)	(Trulzsch *et al.*, 2001)
G431S	TM1 (1.49)	(Biebermann *et al.*, 2001)
M453T	TM2 (2.43)	(de Roux *et al.*, 1996; Duprez *et al.*, 1997a)
M463V	TM2 (2.53)	(Fuhrer *et al.*, 2000)
A485V	IL1	(Akcurin *et al.*, 2008)
I486F	IL1	(Camacho *et al.*, 2000; Parma *et al.*, 1995)
I486M	IL1	(Parma et al., 1995)
S505N	TM3 (3.36)	(Holzapfel *et al.*, 1997)
S505R	TM3 (3.36)	(Tonacchera *et al.*, 1996),
V509A	TM3 (3.40)	(Duprez *et al.*, 1994)
L512E	TM3 (3.43)	(Trulzsch et al., 2001)
L512Q	TM3 (3.43)	(Nishihara *et al.*, 2006)
L512R	TM3 (3.43)	(Kosugi *et al.*, 2000; Trulzsch et al., 2001)
I568T	EL2	(Parma et al., 1995, 1997; Tonacchera *et al.*, 2000)
V597F	TM5 (5.54)	(Alberti *et al.*, 2001)
V597L	TM5 (5.54)	(Esapa *et al.*, 1999)
Y601N	TM5 (5.58)	(Arseven *et al.*, 2000)
Δ613–621	IL3	(Wonerow *et al.*, 1998)
D617Y	IL3	(Nishihara *et al.*, 2007)
D619G	TM6 (6.30)	(Nogueira *et al.*, 1999; Parma et al., 1993, 1997; Russo*et al.*, 1996)
A623I	TM6 (6.34)	(Parma *et al.*, 1993)
A623S	TM6 (6.34)	(Russo *et al.*, 1995)
A623V	TM6 (6.34)	(Nogueira et al., 1999; Paschke *et al.*, 1994; Schwab *etal.*, 1997)
M626I	TM6 (6.37)	(Ringkananont *et al.*, 2006)
L629F	TM6 (6.40)	(Parma et al., 1997)
I630L	TM6 (6.41)	(Parma et al., 1997)
F631C	TM6 (6.42)	(Porcellini *et al.*, 1994)
F631I	TM6 (6.42)	(Fuhrer *et al.*, 2003b)
F631L	TM6 (6.42)	(Kopp *et al.*, 1995; Parma et al., 1997)
T632A	TM6 (6.43)	(Spambalg *et al.*, 1996)
T632I	TM6 (6.42)	(Kopp *et al.*, 1997a; Parma et al., 1997; Paschke et al., 1994; Porcellini et al., 1994)
D633A	TM6 (6.44)	(Parma et al., 1997)
D633E	TM6 (6.44)	(Parma et al., 1997; Porcellini et al., 1994)
D633H	TM6 (6.44)	(Parma et al., 1997; Russo et al., 1996, 1997)
D633Y	TM6 (6.44)	(Fuhrer et al., 2003b; Nogueira et al., 1999; Parma et al.,1997; Porcellini et al., 1994)

Mutation	Location (Ballesteros numbering when applicable)	References
P639S	TM6 (6.50)	(Khoo et al., 1999; Tonacchera et al., 1998)
N650Y	EL3	(Tonacchera et al., 1996)
V656F	EL3	(Fuhrer *et al.*, 1997)
Δ658–661	TM7	(Parma et al., 1997)
F666L	TM7 (7.41)	(Tassi *et al.*, 1999)
N670S	TM7 (7.45)	(Tonacchera et al., 1996)
C672Y	TM7 (7.47)	(Duprez et al., 1994)
L677V	TM7 (7.52)	(Russo *et al.*, 1999)
I691F	C terminal tail	(Liu *et al.*, 2008)

Table 2

Naturally occurring constitutively active mutations in LHCGR

Mutation	Location (Ballesteros numbering when applicable)	References
L368P	TM1 (1.41)	(Latronico *et al.*, 2000b)
A373V	TM1 (1.46)	(Gromoll *et al.*, 1998)
M398T	TM2 (2.42)	(Evans *et al.*, 1996; Kraaij *et al.*, 1995; Yano *et al.*, 1996)
L457R	TM3 (3.43)	(Latronico *et al.*, 1998)
I542L	TM5 (5.54)	(Laue *et al.*, 1995)
D564G	TM6 (6.30)	(Laue et al., 1995)
A568V	TM6 (6.34)	(Latronico *et al.*, 1995a)
M571I	TM6 (6.37)	(Kosugi *et al.*, 1995 Kremer et al., 1993)
A572V	TM6 (6.38)	(Yano *et al.*, 1995)
I575L	TM6 (6.41)	(Laue *et al.*, 1996)
T577I	TM6 (6.43)	(Kosugi et al., 1995)
D578E	TM6 (6.44)	(Wu *et al.*, 1999)
D578G	TM6 (6.44)	(Shenker et al., 1993)
D578Y	TM6 (6.44)	(Laue et al., 1995)
C581R	TM6 (6.45)	(Laue et al., 1995)