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Inactivating mutations of G protein-coupled receptors and diseases: Structure-function insights and therapeutic implications

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Abstract

Since the discovery of the first rhodopsin mutation that causes retinitis pigmentosa in 1990, significant progresses have been made in elucidating the pathophysiology of diseases caused by inactivating mutations of G protein-coupled receptors (GPCRs). This review aims to compile the compelling evidence accumulated during the past 15 years demonstrating the etiologies of more than a dozen diseases caused by inactivating GPCR mutations. A generalized classification scheme, based on the life cycle of GPCRs, is proposed. Insights gained through detailed studies of these naturally occurring mutations into the structure-function relationship of these receptors are reviewed. Therapeutic approaches directed against the different classes of mutants are being developed. Since intracellular retention emerges as the most common defect, recent progresses aimed at correcting this defect through membrane permeable pharmacological chaperones are highlighted.

Keywords: G protein-coupled receptor; Disease; Loss-of-function mutation; Trafficking; Pharmacological chaperone; Classification

Abbreviations: ACTH, adrenocorticotropin; ADRP, autosomal dominant retinitis pigmentosa; cAMP, cyclic adenosine monophosphate; CaR, calcium-sensing receptor; CFTR, cystic fibrosis transmembrane conductance regulator; EC_{50}, the concentration that results in 50% maximal response; ECD, extracellular domain; EL, extracellular loop; ET_BR, endothelin-B receptor; FGD, familial glucocorticoid deficiency; FHH, familial hypocalciuric hypercalcemia; fs, frameshift; FSH, follicle stimulating hormone; FSHR, FSH receptor; GFP, green fluorescent protein; GH, growth hormone; GHRH, growth-hormone releasing hormone; GHRHR, GHRH receptor; GnRH, gonadotropin-releasing hormone; GnRHR, GnRH receptor; GPCR, G protein-coupled receptor; hCG, human chorionic gonadotropin; HIV, human immunodeficiency virus; IL, intracellular loop; LH, luteinizing hormone; LHR, LH receptor; MCR, melanocortin receptor; MC1R, melanocortin-1 receptor; MC2R, melanocortin-2 receptor; MC3R, melanocortin-3 receptor; MC4R, melanocortin-4 receptor; MIP, macrophage inhibitory protein; NSHPT, neonatal severe hyperparathyroidism; POMC, pro-opiomelanocortin; PTH, parathyroid hormone; PTHR1, PTH/PTH-related peptide receptor type 1; RHC, red hair color; TM, transmembrane α-helix; TSH, thyroid stimulating hormone; TSHR, TSH receptor; V2R, V2 vasopressin receptor; wt, wild-type.

Contents

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1. Introduction

G protein-coupled receptors (GPCRs) comprise the largest family of cell surface proteins, with more than 1000 members in humans. They transduce a large variety of extracellular signals, including light, smell, ions, catecholamines, neuropeptides, and large glycoprotein hormones (Bockaert & Pin, 1999). 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 crystal structure of rhodopsin at high resolution confirmed this topology (Palczewski et al., 2000). Almost all known physiological processes are regulated by GPCRs. Therefore it is easy to understand that defects in these signaling pathways will lead to various dysfunctions and diseases. 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 reviews on diseased GPCRs have been published (Spiegel et al., 1993; Shenker, 1995; Spiegel, 1996; Schoneberg et al., 2002; ; Spiegel & Weinstein, 2004).

The purpose of this review is to summarize the studies in 15 GPCRs where multiple pathogenic loss-of-function mutations have been reported. Only overt mutations are covered. Polymorphic variants are not discussed herein. Interested readers are referred to recent reviews (Rana et al., 2001; Sadee et al., 2001). Based on the life cycle of the GPCRs, a general classification scheme is suggested for categorizing the ever-increasing mutations in these GPCRs. Insights that can be learned from the functional studies of the naturally occurring mutations in these GPCRs as well as the therapeutic implications are also highlighted.

2. Diseases caused by inactivating GPCR mutations

2.1. Rhodopsin mutations and retinitis pigmentosa

Rhodopsin, the dim-light receptor, is one of the best-studied model systems in GPCRs. After absorption of light (photons), rhodopsin undergoes conformational changes and is converted to metarhodopsin II, the active form of rhodopsin. Metarhodopsin II activates the heterotrimeric G protein in rod cells, transducin, initiating the photo-transduction cascade.

The first mutation in GPCRs that cause human diseases was found in rhodopisn, P23H, causing autosomal dominant retinitis pigmentosa (ADRP) (Dryja et al., 1990). Since then, up to 150 distinct mutations, most of them missense mutations and several small in-frame deletions, have been reported (reviewed in (Farrar et al., 2002; Stojanovic & Hwa, 2002; Mendes et al., 2005)). Stojanovic and Hwa provided an exhaustive table summarizing the rhodopsin mutations and the references known in 2002 (Stojanovic & Hwa, 2002). Novel mutations continue to be reported. For example, three novel mutations, S176F, R314fs16, and V20G, were recently reported from ADRP patients in Germany (Schuster et al., 2005). Mutations in rhodopsin represent the most frequent single cause of ADRP, contributing to about 25% of cases in Caucasian population (Dryja et al., 2000).

Although these mutations are scattered throughout the receptor, careful examination revealed that they cluster in three domains, namely, those that surround the conserved disulfide bond linking EL1 and EL2, those that surround the chromophore binding pocket, and those that reside in the cytoplasmic tail (Stojanovic & Hwa, 2002). Extensive biochemical and biophysical studies have been done to identify the molecular defects of the rhodopsin mutations associated with

ADRP (see (Sung et al., 1991, 1993; Kaushal & Khorana, 1994) for earlier references). The first rhodopsin mutation reported and the most common one in North America, P23H, was retained in the ER due to misfolding. It is prone to aggregation and accumulated in aggresomes and destined for degradation by the ubiquitin-proteasome pathway (Illing et al., 2002; Saliba et al., 2002). Indeed, mutations that result in intracellular retention of the mutant rhodopsins are the most common defect. One important mechanism is the disruption of the highly conserved disulfide bond linking EL1 and EL2 or the formation of alternate disulfide bond C185-C187 (reviewed in (Stojanovic & Hwa, 2002)). It is interesting to note that some mutant rhodopsins have no abnormal biochemical or cellular defect (Mendes et al., 2005) resembling the Class V mutants in GPCRs that bind diffusible ligands (see below).

2.2. GnRHR mutations and hypogonadotropic hypogonadism

Goandotropin-releasing hormone (GnRH) is a decapeptide hormone secreted by the hypothalamus. GnRH is essential for the neuroendocrine control of reproduction by acting on the gonadotrophs in the pituitary gland to stimulate the secretion of gonadotropins. Defect in GnRH production or action results in hypogonadotropic hypogonadism. Male patients have small testes. Female patients present with primary amenorrhea, with the absence of breast development frequently observed. So far 19 mutations in GnRHR gene have been reported from patients with idiopathic hypogonadotropic hypogonadism, including M1T (Wolczynski et al., 2003), N10K (Costa et al., 2001), Q11K (Meysing et al., 2004), and T32I (Beranova et al., 2001) (all in the extracellular domain (ECD)), E90K (Soderlund et al., 2001) in TM2, Q106R (de Roux et al., 1997) in EL1, A129D (Caron et al., 1999) and R139H (Costa et al., 2001) in TM3, S168R (Pralong et al., 1999) and A171T (Karges et al., 2003) in TM4, C200Y (Beranova et al., 2001) in EL2, S217R (de Roux et al., 1999) in TM5, R262Q (de Roux et al., 1997) and L266R (Beranova et al., 2001) in IL3, C279Y (Beranova et al., 2001) and Y284C (Layman et al., 1998b) in TM6, L314X (Kottler et al., 2000) and P320L (Meysing et al., 2004) in TM7, and a splice acceptor site mutation resulting in truncation of the receptor (Silveira et al., 2002). The mode of inheritance is autosomal recessive. Most of the patients are compound heterozygotes, whereas only a few patients are homozygotes (reviewed in (Bhagavath et al., 2005)). Several studies investigated the prevalence of GnRHR gene mutations in normosmic idiopathic hypogonadotropic hypogonadism patients, ranging from 2.2% (Layman et al., 1998b) to 10.4% (Beranova et al., 2001).

Functional studies showed that all missense GnRHR mutations tested have absent or decreased signaling. E90K does not bind to GnRH or generate second messenger in response to GnRH stimulation, likely due to defect in cell surface expression (Maya-Nunez et al., 2002). Q106R, A129D, and S217R are defective in ligand binding and signaling (de Roux et al., 1997; Caron et al., 1999; de Roux et al., 1999) although it is not known whether the mutant receptors are expressed on the cell surface. On the other hand, it was shown that S168R and A171T in TM4, expressed on the cell surface, are defective in ligand binding per se (Pralong et al., 1999; Karges et al., 2003). It is interesting that R139H is expressed on the cell surface, but cannot bind to GnRH therefore there is no signaling (Costa et al., 2001). R139 belongs to the highly conserved DRY motif (in GnRHR, the motif becomes DRS) in TM3. The most conserved Arg residue in this triplet is believed to be important for signaling. The molecular basis for its effect on ligand binding remains to be investigated. Similarly, P320L, where the highly conserved Pro in N/DPxxY motif in TM7 was mutated, is also expressed on the cell surface but lacks ligand binding and signaling (Meysing et al., 2004). R262Q in IL3 and Y284C in TM7 exhibited normal binding affinity but reduced signaling (increased EC_{50} and decreased maximal response) (EC_{50} is the concentration of the ligand that causes 50% maximal stimulation), suggesting that they are likely involved in G-protein coupling/activation or confer conformational change that are important for signaling (de Roux et al., 1997; Layman et al., 1998b). Analysis of four mutants, including T32I, C200Y, L266R, and C279Y, showed that they are all expressed on the cell surface but have no ligand binding and little (T32I) or no (C200Y, L266R, and C279Y) signaling (Beranova et al., 2001; Bedecarrats et al., 2003).

Recently, Conn and colleagues suggested that many mutant GnRHRs are misrouted as evidenced by their abilities to be rescued by pharmacological chaperones (see below) (Janovick et al., 2002; Leanos-Miranda et al., 2002). They suggested that studies using HA-tagged and green fluorescent protein (GFP)-tagged GnRHR might have reached wrong conclusions regarding the trafficking of the mutants, since these epitope tags can affect the expression of the receptor (Brothers et al., 2003). This controversy remains to be further investigated.

2.3. GHRHR mutations and dwarfism

Growth hormone releasing hormone (GHRH) is a 44-amino acid peptide hormone synthesized and secreted by the arcuate nucleus of the hypothalamus. GHRH stimulates the synthesis and secretion of growth hormone (GH) from the pituitary somatotrophs. GHRH also regulates the proliferation or differentiation of the somatotrophs. Defective production or action of GHRH leads to somatotroph hypoplasia, decreased GH secretion, and deficient growth. The receptor for GHRH (GHRHR) is coupled to the stimulatory heterotrimeric G protein Gs. Hence activation of GHRHR results in increased production of cyclic adenosine monophosphate (cAMP).

The first mutation in GHRHR was reported from a mutant mouse strain called *little* mouse. The *little* mouse, first identified in 1976, has pituitary hypoplasia and decreased GH level. In vitro studies showed that somatotrophs from *little* mice do not release GH after GHRH stimulation, but cAMP or other agents that increase intracellular cAMP levels can stimulate GH release (Jansson et al., 1986), suggesting that the defect in *little* mouse somatotrophs lies not in GH secretion, but in responding to GHRH, with GHRHR a potential target. Following the cloning of the GHRHR, two independent groups identified the mutation in *little* mouse as D60G (Godfrey et al., 1993; Lin et al., 1993).

These studies suggested that patients with isolated GH deficiency might also harbor GHRHR mutations. Wajnrajch and colleagues were the first to identify a mutation in GHRHR from two children with profound GH deficiency (Wajnrajch et al., 1996). These patients are homozygous for a nonsense mutation, E72X (changing Glu72 to stop codon), predicting a mutant protein lacking all seven TMs. Subsequent studies identified 8 additional mutations, including missense mutations such as H137L in TM1 (Salvatori et al., 2001b), L144H (Salvatori et al., 2001a), A176V (Carakushansky et al., 2003), A222E (Salvatori et al., 2001a) in TM3, F242C in TM4 (Salvatori et al., 2001a), K329E (Alba & Salvatori, 2005), and two deletions in TM7 resulting in premature stop codons (Salvatori et al., 2001b). The mode of inheritance is autosomal recessive. Patients are either homozygous or compound heterozygous.

Functional studies of the mutant receptors were also performed. Extensive studies on the murine *little* mutant (D60G) showed that the mutant is defective in ligand binding (Gaylinn et al., 1999). Hence the mutant receptor is expressed on the cell surface with normal glycosylation, but lacks ligand binding and signaling. Of the mutations observed in humans, all the mutant receptors were found to be defective in signaling, although the mechanism of the dysfunction is not clear in most cases. One reason is technical difficulties. Most of the studies did not perform ligand binding analysis. Studies using receptors tagged at the C terminus with epitope tag cannot differentiate cell surface vs intracellular expression, since permeabilization was required to detect the epitope. One study showed that A176V is expressed on the cell surface but with no signaling in response to GHRH stimulation (Carakushansky et al., 2003) suggesting that the mutant is defective in either ligand binding or signaling.

2.4. LHR mutations and hypergonadotropic hypogonadism

The receptor for the pituitary gonadotropin, lutropin (luteinizing hormone, LH), LHR, plays a critical role in reproductive physiology (Ascoli et al., 2002). In the gonads, LHR are expressed in Leydig cells in the testis and theca and granulosa cells in the ovary. In males, LH stimulates the production of testosterone in Leydig cells. Testosterone is essential for the development of both internal and external genitalia and the descent of the testis during the fetal period, and for the development of secondary sexual characteristics at puberty. Human chorionic gonadotropin (hCG), which also binds to LHR, stimulates the fetal Leydig cells to produce androgen that is essential for male development. During the ovarian cycle of the postpubertal females, LH stimulates androgen synthesis by theca cells during the follicular phase, induces ovulation of Graffian follicles at the middle of the ovarian cycle, and stimulates progesterone synthesis by luteal cells during the luteal phase.

Because of the critical roles of testosterone in the development of internal and external genitalia during embryogenesis, loss-of-function mutations in LHR leads to Leydig cell hypoplasia, a rare autosomal recessive form of male pseudohermaphroditism with undescended testis when the mutations result in severe defect, or micropenis when the mutations result in milder defect. In females, LHR inactivation results in hypergonadotropic hypogonadism and primary amenorrhea (Latronico et al., 1996; Toledo et al., 1996). So far, 22 distinct mutations have been described, including a 33-bp insertion in exon 1 (Wu et al., 1998a), R124Q (Richter-Unruh et al., 2002), C133R (Misrahi et al., 1997), V144F (Richter-Unruh et al., 2005), F194V (Gromoll et al., 2002), N291S (Laue et al., 1996), C343S (Martens et al., 2002), E354K (Stavrou et al., 1998) (all in ECD), W491X (Richter-Unruh et al., 2002) and L502P in TM4 (Leung et al., 2004), C543R (Martens et al., 2002), R544X (Laue et al., 1995), and C545X (Laue et al., 1995) (all in TM5), R554X in IL3 (Latronico et al., 1996), A593P in TM6 (Kremer et al., 1995), in-frame deletion of L608/V609 (Latronico et al., 1998), Y612X (Salameh et al., 2005), S616Y (Latronico et al., 1996; Laue et al., 1996), and I625K (Martens et al., 1998) (the latter four mutations in TM7), deletions of exon 8 (Laue et al., 1996) or 10 (Gromoll et al., 2000), and a frame-shift mutation by a single nucleotide insertion in exon 11 (codon A589fs) resulting in premature termination (Richter-Unruh et al., 2005). The pattern of inheritance is autosomal recessive. The patients are either homozygous or compound heterozygous.

Functional studies showed that the signaling of most of these mutants was either absent or impaired. There is a correlation between the residual functional activities of the mutant receptors with the phenotypes of the patients. Those mutations that result in total loss of signaling (such as V144F and A593P) are associated with male pseudohermaphroditism whereas those that have residual signaling (such as S616Y and I625K) are associated with male phenotype with micropenis. A number of mutants, such as the 33-bp insertion in exon 1 (Wu et al., 1998a), V144F (Richter-Unruh et al., 2005), F194V (Gromoll et al., 2002), C343S and C543R (Martens et al., 2002), A593P and S616Y (Tao et al., 2004), have impaired trafficking to the plasma membrane.

Functional analysis of the exon 10 deletion mutant showed that the mutant receptor binds to LH and hCG with normal affinities, and signals to hCG stimulation normally, but has impaired signaling in response to LH stimulation (Muller et al., 2003; Zhang et al., 1998a). This is consistent with in vivo hormone stimulation data as well as the phenotypes of the patient (Gromoll et al., 2000). Unlike the other male patients with LHR inactivating mutations, the patient with exon 10 deletion present with normal male development during the fetal period (sustained by hCG stimulation of fetal LHR) but delayed pubertal development and hypogonadism (defective LH action) as an adult. With the mutant receptor, whereas hCG can stimulate testosterone production, LH cannot (Gromoll et al., 2000).

Another interesting observation from the functional characterization of the inactivating mutations in LHR is that a number of mutants have multiple defects. For example, A593P, I625K, and the microdeletion in TM7 (ΔL608/V609), all have defects in both cell surface expression and G protein coupling (Latronico et al., 1998; Martens et al., 1998).

2.5. FSHR mutations and hypergonadotropic hypogonadism

The receptor for the other pituitary gonadotropin, follitropin (follicle stimulating hormone, FSH), FSHR, also plays a critical role in reproductive function (Simoni et al., 1997). In the males, FSH is important for spermatogenesis, whereas in females, FSH is absolutely required for follicle growth. Mutations in the FSHR result in ovarian dysgenesis with amenorrhea and infertility in women. The first mutation reported is A189V in the ECD (Aittomaki et al., 1995). This mutation is very frequent in the Finnish population (Aittomaki et al., 1996) but was not found in patients from Germany (Simoni et al., 1997), England and France (Beau et al., 1998), and North America (Layman et al., 1998a). Since the original report of Aittomaki et al., 8 additional FSHR mutations have been reported, including I160T (Beau et al., 1998), N191I (Gromoll et al., 1996), D224V (Touraine et al., 1999), and P348R (Allen et al., 2003) (all in the ECD), A419T in TM2 (Doherty et al., 2002), P519T in EL2 (Meduri et al., 2003), R573C in IL3 (Beau et al., 1998), and L601V in EL3 (Touraine et al., 1999). It is interesting to point out that the analysis of the male patients harboring A189V showed that in contrast to the dogma that FSH is essential for the pubertal initiation and maintenance of spermatogenesis, male fertility is relatively independent of FSH action (Tapanainen et al., 1997). Therefore FSH is essential for female but not male fertility. Similar observation was made in FSHβ gene knockout mice (Kumar et al., 1997).

Functional analysis showed that A189V results in a total loss-of-signaling (Aittomaki et al., 1995). Although the mutant receptor binds with normal affinity, binding capacity was dramatically reduced, with a corresponding reduction in signaling. Subsequent studies showed that the mutant receptor is retained intracellularly (Rannikko et al., 2002). When expressed in COS-7 cells, I160T and D224V are retained intracellularly, whereas R573C and L601V showed impaired signaling, with maximal responses at 30% and 12% of wild type (wt) FSHR, respectively (Beau et al., 1998; Touraine et al., 1999). A correlation between the residual activity and the severity of clinical phenotype was observed (Touraine et al., 1999). P348R has a complete absence of binding and signaling (Allen et al., 2003). However, it is not known whether the mutant receptor is expressed on the cell surface. Therefore the mutant might be defective in trafficking onto the cell surface or ligand binding per se. P519T is retained intracellularly resulting in minimal binding and absent signaling (Meduri et al., 2003). A419T has normal ligand binding affinity and capacity but defective in signaling (Doherty et al., 2002). A419 lies at the extracellular end of TM2. The mechanism for its effect on signaling remains to be elucidated.

2.6. TSHR mutations and hypothyroidism

TSH (thyroid-stimulating hormone or thyrotropin) is another glycoprotein hormone produced by the anterior pituitary gland (by thyrotropes). Like LH and FSH, TSH 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 cell proliferation and differentiation in the thyroid gland (Rapoport et al., 1998). TSH exerts its effects by interacting with cell surface TSHR. TSHR is primarily coupled to Gs, therefore receptor activation raises intracellular cAMP level. At higher TSH concentrations, TSHR activation also increases phosphoinositols and calcium by coupling to Gq/11 (Kopp, 2001).

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 (Kopp, 2001)). Refetoff and colleagues were the first to report inactivating mutations in the TSHR causing TSH resistance (Sunthornthepvarakul et al., 1995). Three patients were compound heterozygous for P162A and I167N. Since then, more than 30 additional inactivating mutations, including P27T (Camilot et al., 2005), E34K (Camilot et al., 2005), C41S (de Roux et al., 1996), R46P (Camilot et al., 2005), R109Q (Clifton-Bligh et al., 1997), L252P (Tonacchera et al., 2004), R310C (Russo et al., 2000), Q324X (de Roux et al., 1996), C390W (de Roux et al., 1996), D403N (Camilot et al., 2005), and D410N (de Roux et al., 1996) (all in the long ECD), R450H (Nagashima et al., 2001) and L467P (Alberti et al., 2002) in TM2, T477I (Tonacchera et al., 2000) and W488R (Camilot et al., 2005) in EL1, G498S (Nagashima et al., 2001) in TM3, F525L (de Roux et al., 1996) and M527T (Camilot et al., 2005) in IL2, W546X (de Roux et al., 1996) and A553T (Abramowicz et al., 1997) in TM4, C600R (Alberti et al., 2002) in TM5, R609X (Tiosano et al., 1999) in IL3, T655X (Gagne et al., 1998) in EL3, as well as frameshift (fs) mutations: 123-124insTGCA (Camilot et al., 2005), 18 bp (nucleotides 1217-1234) deleted and 4 novel bp inserted (Biebermann et al., 1997) and 555-561delTATTCTT (Camilot et al., 2005), and splice site mutation (Gagne et al., 1998), have been reported. Depending on the severity of the loss of function, patients present with either euthyroid hyperthyrotropinemia (partial loss of function) or overt hypothyrodism (severe loss of functions). There is a clear correlation between the genotype and phenotype (reviewed in (Kopp, 2001)). The mode of inheritance is autosomal recessive. Patients are either homozygous or compound heterozygous.

Functional studies showed that most of the mutations in the ECD, including C41S, R109Q, P162A, R310C, and C390W, impair ligand binding (Wadsworth et al., 1992; de Roux et al., 1996; Costagliola et al., 1999), suggesting that these residues are either directly involved in ligand binding or important for maintaining conformation competent for ligand binding. However, D410N, also located in the ECD, has impaired signaling with normal ligand binding. These results suggest that some amino acids in the ECD are also important for signal transduction (de Roux et al., 1996). Similar findings were also obtained in LHR through laboratory-generated mutants (Huang & Puett, 1995). I167N, L252P, T477I, and the fs mutation that deletes 18 bp and inserts 4 bp, have no or decreased specific TSH binding and signaling in response to

TSH stimulation because of intracellular retention of the mutant receptors (Biebermann et al., 1997; Costagliola et al., 1999; Tonacchera et al., 2000; Tonacchera et al., 2004). A553T was expressed at very low levels on the cell surface. However, residual binding and signaling could be measured (Abramowicz et al., 1997). F525L, located in IL2, bound to TSH normally, but had decreased cAMP production, highlighting the importance of IL2 in Gs coupling and activation (de Roux et al., 1996).

TSHR has a significant level of constitutive activity when expressed heterologously. Some mutations decrease the constitutive activity, whereas others do not. There is no apparent correlation with ligand binding or TSH-stimulated signaling. For example, of the three mutants characterized by de Roux et al, C390W (no ligand binding) has normal constitutive activity, whereas D410N and F525L (with normal ligand binding) have decreased constitutive activities (de Roux et al., 1996). Similarly, R310C has no TSH binding and signaling in response to TSH stimulation but increased constitutive activity (Russo et al., 2000).

TSHR mutation has also been identified from *hyt/hyt* mouse that has autosomal recessive congenital hypothyrodism (Stein et al., 1994). The mutant, P556L in TM4, is expressed on the cell surface but defective in ligand binding leading to absent signaling (Gu et al., 1995).

2.7. V2R mutations and nephrogenic diabetes insipidus

The kidney regulates water retention in response to the antidiuretic hormone, arginine vasopressin, by increasing the permeability of the collecting-duct epithelium to water (Morello & Bichet, 2001). Nephrogenic diabetes insipidus (NDI) is a rare disease characterized by polyuria, polydipsia, and hyposthenuria. The kidney fails to concentrate urine despite normal or high concentrations of vasopressin. Most cases of NDI have an X-linked recessive pattern of inheritance. Soon after the cloning and chromosomal localization of V2 vasopressin receptor (V2R) in Xq28, several groups reported V2R mutations associated with X-linked NDI (Pan et al., 1992; Rosenthal et al., 1992; van den Ouweland et al., 1992). Since then, more than 190 V2R mutations have been identified from patients with X-linked NDI. The website for V2R mutation database is at www.medicine.mcgill.ca/nephros.

Extensive functional studies were performed on many of these mutations. Intracellular retention is the most common defect, accounting for more than 70% of mutants functionally characterized (Lu et al., 2003). For example, in a recent study, all nine mutants were found to be intracellularly retained (Robben et al., 2005). Some mutants are defective in receptor protein production either due to defective biosynthesis or accelerated degradation. A typical example is 804insG, which insert a single G at nucleotide 804-809 that causes a frame shift at codons 247-257 and premature stop codon in IL3 (Tsukaguchi et al., 1995). Some mutants, such as Y128S, R181C, ΔR202, R202C, and P286R, are defective in ligand binding (Pan et al., 1994; Tsukaguchi et al., 1995; Ala et al., 1998). Some mutant receptors might affect multiple aspects of receptor function. For example, R113W was found to affect cell surface expression (20% of wt), ligand binding (20-fold decrease in affinity) and signaling (60-fold increase in EC_{50}) (Birnbaumer et al., 1994). D85N, although expressed normally on the cell surface, binds to arginine vasopressin with 6-fold lower affinity, which together with a 20-fold decrease in coupling efficiency, resulted in a 50-fold increase in EC_{50} (Sadeghi et al., 1997). Some of the mutant receptors, such as G12E, A61V and ΔR247-G250, were found to have normal ligand binding and signaling suggesting that they might not be the cause of NDI in these patients (Pan et al., 1994; Wenkert et al., 1996). They are Class V mutants (see below).

2.8. PTHR1 mutations and Blomstrand 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. Inactivating mutations of PTHR1 result in Blomstrand's chondrodysplasia, typified by short limbs, hypoplasia of the mandibles, advancement of skeletal maturation, and hyoid cartilages. It is also associated with abnormal breast development and tooth impaction (Wysolmerski et al., 2001). The pattern of inheritance is autosomal recessive.

In 1998, three papers reported inactivating mutations of PTHR1 from infants with Blomstrand lethal chondrodysplasia (Jobert et al., 1998; Karaplis et al., 1998; Zhang et al., 1998b). Homozygous P132L mutation was reported in two studies (Karaplis et al., 1998; Zhang et al., 1998b), whereas an in-frame deletion of 11 amino acids in TM5 was detected in the other study (the normal paternal allele was not expressed) (Jobert et al., 1998). Two more inactivating mutations, a homozygous frameshift mutation that deletes a G at nucleotide 1122 changing coding sequence after codon 364 in TM5 (Karperien et al., 1999) and a truncation mutation at the C terminus resulting in the deletion of the last 108 amino acids (Duchatelet et al., 2005) that results in Eiken syndrome, have been reported.

Functional studies of P132L showed that the mutant, although expressed well on the cell surface, has little or no binding and signaling in response to ligand stimulation (Karaplis et al., 1998; Zhang et al., 1998b). These results suggest that P132 is either involved in direct contact with the ligand or critical for maintaining the conformation necessary for high affinity agonist binding. The mutant with in-frame deletion of 11 amino acids in TM5 is expressed but could not bind to the ligand or generate second messengers (cAMP and inositol phosphate) (Jobert et al., 1998). Since the PTHR1 construct was tagged at the C terminus, and immunofloures-cence experiments had to be done with permeabilized cells, it is unknown whether the mutant is defective in cell surface expression or ligand binding per se. Functional studies with both dermal fibroblasts from the fetus and cells transiently transfected with the mutant receptor showed that the frameshift mutation that deleted G at 1122 inactivates the receptor (Karperien et al., 1999).

2.9. CaR mutations and familial hypocalciuric hypercalcemia or neonatal severe hyperparathyroidism

Familial hypocalciuric hypercalcemia (FHH) is a rare disease inherited in an autosomal dominant manner. It is characterized by lifelong mild to moderate hypercalcemia and lower than expected calcium excretion in urine (hypocalciuria). The patients usually do not have the characteristic complications of hypercalcemia such as gastroinstestinal, renal, and mental abnormalities. Their circulating PTH levels are usually within the normal range; however, the regulation of PTH secretion in these patients is at a higher set point, i.e. higher calcium concentration is required to suppress PTH secretion. Neonatal severe hyperparathyroidism (NSHPT), characterized by marked elevation in serum calcium and PTH levels, usually present with severe hypercalcemia symptoms within the first 6 months of life (therefore called neonatal). It is usually lethal unless total parathyroidectomy is performed early. Genetic analyses in FHH families with parental consanguinity showed that NSHPT is the homozygous form of FHH (Pollak et al., 1994).

Human genetic studies identified loss-of-function mutations in calcium-sensing receptor (CaR) as one of the causes of FHH and NSHPT (Pollak et al., 1993). CaR is a large GPCR, with a long N-terminal ECD of more than 600 amino acids, followed by a 250-amino acid serpentine region. The C-terminal cytoplasmic tail is about 200 amino acids. The long ECD is involved in the binding to polycations. Since the pioneering report of Pollak et al. on inactivating CaR mutations and FHH and NSHPT (Pollak et al., 1993), more than 50 inactivating mutations have been identified. Most of the mutations are missense mutations, including L11S (Pidasheva et al., 2005), L13P (Miyashiro et al., 2004), T14A (Pidasheva et al., 2005), Q27R (Chikatsu et al., 1999), P39A (Aida et al., 1995), I40F, S53P (Health et al., 1996), P55L (Pearce et al., 1995), R62M (Chou et al., 1995), R66C (Chou et al., 1995), S137P, T138M (Chou et al., 1995), G143E (Chou et al., 1995), L159P, Y161C, L173P (Felderbauer et al., 2005), L174R (Ward et al., 1997), N178D (Pearce et al., 1996b), R185Q (Pollak et al., 1993), I212T (Marcocci et al., 2003), D215G (Health et al., 1996), Y218C (Cetani et al., 2003), R220W (D'Souza-Li et al., 2002), R220Q (Pearce et al., 1996b), P221S (Pearce et al., 1996b), R227L (Pearce et al., 1995), R227Q (Chou et al., 1995), E250K , E297K (Pollak et al., 1993), C395R (Vigouroux et al., 2000), G549R (D'Souza-Li et al., 2002), G553R, and C582Y (all in the long ECD), S657Y (Health et al., 1996), G670E (Kobayashi et al., 1997), and G670R (Pearce et al., 1995) in TM2, R680C (Pearce et al., 1995) in EL1, P748R (Health et al., 1996) in EL2, R795W (Pollak et al., 1993), P798T (Lam et al., 2005), and A804D (Miyashiro et al., 2004) in IL3, V817I (Pearce et al., 1995) in TM6, F881L (Carling et al., 2000) in the C terminus. Other mutations include nonsense mutations G94X (Ward et al., 2004), R185X (Kobayashi et al., 1997), and S607X (Pearce et al., 1995) in ECD and R648X (Yamauchi et al., 2002) in IL1, frameshift mutations C7f-2X47 in the ECD and Pvfs-2X981 in TM7 (both reported in (D'Souza-Li et al., 2002)) and P747fs (Pearce et al., 1995), Alu element insertion (Janicic et al., 1995), and an acceptor splice site mutation (D'Souza-Li et al., 2001). Most of the inactivating mutations occur in the first two segments of the receptor (with a significant portion of the mutations clustering between residues 13 and 297 in the ECD (Hu & Spiegel, 2003)), whereas variants in the cytoplasmic tail are mostly polymorphisms present in normocalcemic individuals (Health et al., 1996).

Functional studies showed that some mutants such as R62M and T138M decrease the affinity slightly without affecting the maximal response whereas other mutants such as R185Q and R795W significantly decrease affinity and/or maximal response (Bai et al., 1996, 1997). Western blotting analysis showed that R185Q and R795W have decreased levels of mature CaR. G549R and C850^851 ins/fs are retained intracellularly resulting in loss of signaling (D'Souza-Li et al., 2002). Some mutants, such as R220W, R227L, and R227Q, are expressed normally on the cell surface but are defective in binding as suggested by the right shift of dose response curves (D'Souza-Li et al., 2002; Wystrychowski et al., 2005). Indeed, the majority of mutants that clusters between residues 13 and 297 in the ECD are presumably defective in binding to agonists with dose-response curves shifted to the right (Bai et al., 1996; Pearce et al., 1996a). Nonsense mutations such as G94X and Alu element insertion are retained intracellularly whereas R648X and A887X are expressed on the cell surface but devoid of signaling (Bai et al., 1997; Ward et al., 2004).

Functional characterization of three mutations in the signal peptide, L11S, L13P, and T14A, showed that L11S and L13P failed to be inserted into the microsomes representing ER, whereas wt and T14A were inserted into the microsomes. The defect in cotranslational processing resulted in reduced intracellular and membrane expression of L11S and L13P mutants (Pidasheva et al., 2005).

A unique observation in the CaR is that null mutations that were not expressed cause milder phenotypes than mutations that are expressed but are impaired in signaling, presumably because null mutations cannot exert dominant negative activity on the wt receptor. This dominant negative activity, postulated to be due to heterodimerization of the mutant and wt receptor, can be observed in co-transfection experiments (Pearce et al., 1996a; Bai et al., 1997; Hu & Spiegel, 2003; Wystrychowski et al., 2005). In contrast to findings in other GPCRs (see below), intracellularly retained mutant CaRs seem to be unable to retain wt CaR in the ER and exert dominant negative activity (Bai et al., 1997).

2.10. MC1R mutations and pigmentation defect

Skin and hair colors are determined by the ratio of the brown/black eumelanin and the red/yellow pheomelanin. Of all the genes identified to control skin and hair pigmentation variations between individuals, the melanocortin-1 receptor (MC1R) is the major determinant. MC1R, originally called melanocyte-stimulating hormone (MSH) receptor, is primarily coupled to Gs. Therefore activation of the MC1R increases intracellular cAMP levels which in turn results in increased transcription of the genes encoding enzymes involved in eumelanin production, especially that of tyrosinase, resulting in increased production

of eumelanin hence darkening of skin or hair (Rouzaud et al., 2005). The ligand for MC1R, α-MSH, is derived from post-translational processing of the pro-hormone pro-opiomelanocortin (POMC).

The RHC (red hair color) phenotype comprises of red hair, fair skin, and poor ability to tan. Valverde and colleagues first identified variants of the MC1R associated with RHC phenotype (Valverde et al., 1995). Since then, more than 60 variants have been identified in MC1R with various skin and hair abnormalities, including RHC as well as increased susceptibility to melanoma and other skin cancers. These variants include P18A (Bastiaens et al., 2001) and C35Y (Fargnoli et al., 2003) in the ECD, V38M (Fargnoli et al., 2003), I40T (Jimenez-Cervantes et al., 2001a), L44V (Fargnoli et al., 2003), F45L (Pastorino et al., 2004), S47I (John et al., 2003), V60L (Box et al., 1997), A64S (Valverde et al., 1995), and K65N (Box et al., 1997) in TM1, R67Q (Rana et al., 1999) in IL1, F76Y (Valverde et al., 1995), A81P (Bastiaens et al., 2001), S83L (Pastorino et al., 2004), S83P (John & Ramsay, 2002), D84E (Valverde et al., 1996), N91D (Valverde et al., 1995), V92L (Box et al., 1997), V92M (Valverde et al., 1995), L93R (Sanchez Mas et al., 2002), T95M (Valverde et al., 1995), V97I (Valverde et al., 1995), and L99I (John et al., 2003) in TM2, A103V (Valverde et al., 1995), G104S (Bastiaens et al., 2001), L106Q (Valverde et al., 1995), and A111V (Pastorino et al., 2004) in EL2, I120T (Fargnoli et al., 2003), V122M (Jimenez-Cervantes et al., 2001a), R142C (Matichard et al., 2004), and R142H (Box et al., 1997) in TM3, R151C (Box et al., 1997), Y152X (John & Ramsay, 2002), I155T (Box et al., 1997), R160Q (Pastorino et al., 2004), and R160W (Box et al., 1997) in IL2, R162P (Jimenez-Cervantes et al., 2001b), R163Q (Box et al., 1997), A164R (Chhajlani & Wikberg, 1992), A171D (John & Ramsay, 2002), ΔV173 (Bastiaens et al., 2001), ΔV174 (Pastorino et al., 2004), and V174I (Bastiaens et al., 2001) in TM4, F196L (John et al., 2003) and R213W (Pastorino et al., 2004) in TM5, P230L (Bastiaens et al., 2001) in IL3, P256S (John & Ramsay, 2002), H260P (Bastiaens et al., 2001), and V265I (Pastorino et al., 2004) in TM6, K278E (Bastiaens et al., 2001), N279S (Bastiaens et al., 2001), N279K (Bastiaens et al., 2001), I287M (Harding et al., 2000), D294H (Valverde et al., 1995), Y298H (Matichard et al., 2004), A299T (Box et al., 1997), and A299V (Peng et al., 2001) in TM7, T308M (Pastorino et al., 2004), and C315R (Pastorino et al., 2004), all in helix 8 at the C terminus. Two frameshift mutations, N29insA (Harding et al., 2000) in the ECD and F179insC (Palmer et al., 2000) in TM4, were identified.

Original functional studies suggested that many of the variants associated with RHC phenotype, such as V60L, R142H, R151C, R160W, and D294H, result in impaired G protein coupling (Frandberg et al., 1998; Schioth et al., 1999; Sanchez Mas et al., 2002). However, some of these studies did not perform ligand binding and receptor localization experiments. Therefore it was not possible to identify the molecular mechanism of reduced or diminished signaling. Recently, Beaumont and colleagues revisited this question. They showed that whereas D294H consistently have good cell surface expression, even better than wt MC1R, R151C and R160W have greatly decreased cell surface expression (Beaumont et al., 2005). This is due to a defect in trafficking because the total receptor protein levels are similar between the different variants and the wt MC1R. Further experiments showed that V92M and R142H have good cell surface expression, D84E and I155T have greatly decreased cell surface expression, and V60L and R163Q have a smaller decrease in cell surface expression. Ligand binding results showed parallel trends as the immunofluorescence experiments. Indeed, a good correlation was observed with the alleles strongly associated with RHC phenotypes having significant decrease in cell surface expression, whereas alleles weakly associated with RHC phenotypes (such as V92M) having normal or a small decrease in cell surface expression (Beaumont et al., 2005).

In other studies, L93R in TM2 was found to have a defect in ligand binding (Jimenez-Cervantes et al., 2001b). This is consistent with a previous mutagenesis study suggesting that TM2 is important for ligand binding (Yang et al., 1997a). Some variants cause only a partial defect in binding and signaling (Jimenez-Cervantes et al., 2001a).

2.11. MC2R mutations and familial glucocorticoid deficiency syndrome

Melanocortin-2 receptor (MC2R), originally called adrenocorticotropin (ACTH) receptor, is a critical regulator of adrenal steroidogenesis and growth (Clark & Weber, 1998). ACTH, derived from posttranslational processing of POMC, is secreted by the anterior pituitary gland. After binding to its receptor in the zonae fasciculata and reticularis of the adrenal gland, ACTH stimulates the production of cortisol and adrenal androgens. ACTH also stimulates cell proliferation in these layers. Inactivating mutations in the MC2R gene cause familial glucocorticoid deficiency syndrome (FGD). In these patients, plasma concentrations of cortisol are low or undetectable, whereas the concentrations of ACTH are usually dramatically elevated. Plasma renin and aldosterone concentrations are usually normal (differentiating it from Addison's disease where high renin and low aldosterone levels are observed). This is likely due to the fact that mineralocorticoid secretion is primarily regulated by angiotensin. Since ACTH can also act on MC1R, these patients usually have excessive pigmentation. If the patients are not diagnosed and treated early, they usually fail to thrive and die prematurely.

Since the first reports of MC2R mutations causing FGD (Clark et al., 1993; Tsigos et al., 1993), more than 30 distinct mutations associated with FGD have been reported, including missense mutations D20N and P27R (Weber & Clark, 1994) in the ECD, I44M (Weber & Clark, 1994) and V45I (Naville et al., 1996) in TM1, L55P in IL1, S74I (Clark et al., 1993) in TM2, D103N (Elias et al., 2000; Ishii et al., 2000) in EL1, D107N (Naville et al., 1996), G116V, S120R (Tsigos et al., 1993), R128C (Weber & Clark, 1994), and I130N (all in TM3), R137W (Ishii et al., 2000), H139Y, V142L (Penhoat et al., 2000), and R146H (Weber et al., 1995) (all in IL2), T152K and T159K (Elias et al., 2000) in TM4, H170L in EL2, L198P in TM5, R201X (Tsigos et al., 1993) in IL3, G226R and A233P

(Penhoat et al., 2000) in TM6, C251F (Naville et al., 1996) and Y254C (Tsigos et al., 1995) in EL3, S256F and P273H (Wu et al., 1998b) in TM7, and frameshift mutations 1052delC (Elias et al., 2000) in TM3, 1255delT and 1272delTA in TM5 (Weber & Clark, 1994), and 1347insA in IL3 (Naville et al., 1996) (reviewed in (Clark et al., 2005)). MC2R mutations account for ~25% of FGD (Clark et al., 2005). The mode of inheritance is autosomal recessive. Patients are either homozygous or compound heterozygotes. Pedigree analysis showed that all mutations in the MC2R gene co-segregate with the disease.

Functional studies of the MC2R mutants have been hampered by the fact that MC2R cannot be expressed on the cell surface in cell lines routinely used for expression of GPCRs such as HEK293 or CHO cells. Earlier studies used cell lines that express endogenous melanocortin receptors (MCRs) such as the Cloudman M3 melanoma cells or COS-7 cells. Since high concentrations of ACTH can stimulate endogenous MCRs to produce cAMP, the interpretation of the results is not straightforward. Although some authors subtracted the cAMP levels produced by cells expressing empty vector from the cAMP levels in cells expressing MC2R, they did not take into account the possibility that exogenously transfected receptor could enhance the expression or signaling of endogenous MCRs (Elias et al., 1999). Adrenal cell lines such as Y-6 or OS3, originated from Y-1 mouse adrenal cell line but lack endogenous MC2R expression (Schimmer et al., 1995), have been successfully used to express MC2R (Yang et al., 1997b). The studies using these cell lines identified mutant MC2Rs that are defective in signaling, including reduced maximal response (such as S74I, I44M, and R146H) or increased EC_{50} (such as R128C, D103N, and T159K) (Elias et al., 1999). Using a sensitive reporter gene assay in OS-3 cells transiently transfected with MC2R, Fluck et al. showed that R137W had low activity, and the S74I or Y254C mutants elicited no measurable response (Fluck et al., 2002). However, in most studies, no ligand binding or localization experiments were done. Although Lefkowitz et al. elegantly showed that MC2R binding studies could be performed with low nonspecific binding (Lefkowitz et al., 1970), most investigators encountered high nonspecific binding with various iodinated ACTH analogs. ACTH was found to bind nonspecifically to glass, plastics, and cell particulates as well as undergo rapid proteolysis when in contact with cells or cell membranes (Hofmann et al., 1988). No studies have used microscopy techniques to localize the cellular localization of the mutant MC2Rs that were defective in signaling, therefore no conclusions can be reached as to whether the mutant MC2Rs are defective in cell surface expression or ligand binding or signaling. This could be accomplished by inserting epitope tags into the MC2R constructs and immunostain the epitope tags as routinely done with numerous GPCRs including mutant MC1R (see above), MC3R, and MC4R (see below).

2.12. MC3R mutations and obesity

The melanocortin-3 receptor (MC3R) was originally cloned by degenerate PCR (Gantz et al., 1993; Roselli-Rehfuss et al., 1993). Although melanocortins, including γ-MSH, were known to activate the MC3R, the physiological role of the MC3R remained unknown. In 2000, two groups reported gene-targeting studies of the MC3R. These studies showed that the MC3R does not affect food intake (Butler et al., 2000; Chen et al., 2000). Rather, through a yet incompletely understood mechanism, the MC3R regulates fat deposition. Mutant mice lacking MC3R have twice the amount of fat mass with corresponding decrease in lean mass so the total body weights for the mutant mice are similar to the wild type littermates. Mice lacking both MC3R and MC4R have exacerbated obesity compared with MC3R or MC4R single gene knockout mice, suggesting that MC3R and MC4R serve non-redundant roles in energy balance regulation (Chen et al., 2000). A recent knockout study using a mouse strain that is resistant to obesity (Black Swiss;129) showed that MC3R knockout mice had a comparable degree of increased adiposity as the MC4R knockout mice (Zhang et al., 2005).

Before these gene-targeting studies were published, several studies reported strong linkage of the loci encompassing the MC3R gene on chromosome 20q.13 with obesity and Type 2 diabetes mellitus (Bowden et al., 1997; Ji et al., 1997; Lembertas et al., 1997; Zouali et al., 1997; Ghosh et al., 1999). However, several large scale screening studies failed to identify any mutations in the MC3R gene from patients with obesity and Type 2 diabetes mellitus (Li et al., 2000; Hani et al., 2001; Wong et al., 2002; Schalin-Jantti et al., 2003). Only two polymorphic variants (K6T and I81V) were reported. In 2002, a group in Singapore reported the first potential mutation from two obese patients (father and daughter) (Lee et al., 2002). The mutation, I183N, changes a highly conserved Ile at the cytoplasmic end of TM3 (of the DRYxxI/V motif) to Asn. Very recently, three additional mutations were identified in obese patients from Italy, including A293T in TM6, I335S in TM7, and X361S (which changes the stop codon to Ser resulting in the addition of seven amino acids before the downstream stop codon) (Mencarelli et al., 2004).

Two groups independently reported the functional analysis of I183N (Rached et al., 2004; Tao & Segaloff, 2004). Both studies showed that this mutation indeed results in a complete loss-of-function. Rached and colleagues suggested that the defect of I183N lies in the intracellular retention of the mutant receptor (Rached et al., 2004). However, in intact cell surface binding experiments, we showed that the mutant receptor could bind to the ligand normally, suggesting that the mutant receptor is expressed on the cell surface, since the radiolabeled ligand cannot cross the membrane. The mutant receptor cannot transmit this binding to the activation of the receptor, resulting in increased production of cAMP. Therefore we conclude that the mutant receptor is defective in receptor activation. Indeed, when the corresponding mutation was introduced in the related melanocortin-4 receptor (MC4R) and another Gs-coupled receptor, human LHR, similar observation was obtained in that the mutant MC4R and hLHR can bind to their respective ligands but cannot generate cAMP (Tao & Segaloff, 2004). The reason for the discrepancy between the two studies might be due to the fact that Rached and colleagues used GFP-tagged

receptor. The GFP tag might affect the trafficking of the receptor (Tarasova et al., 1997). As can be seen from their confocal images, GFP-tagged wt MC3R is also mainly retained inside the cell (reviewed in (Tao, 2005)).

I recently characterized the three novel MC3R mutations reported by Mencarelli et al. The results showed that I335S results in total loss of ligand binding and signaling due to intracellular retention of the mutant receptor. A293T and X361S bind to the ligand normally and respond to the ligand similarly as the wt MC3R (unpublished observations).

Although the two polymorphisms (K6T and I81V) individually have no effect on ligand binding and signaling (Wong et al., 2002; Tao & Segaloff, 2004; Feng et al., 2005), co-occurrence of the two polymorphisms causes partial inactivation and is associated with increased obesity in African Americans (Feng et al., 2005), suggesting interaction of genotype and race. Whether MC3R mutations are also important in obesity in other ethnic populations such as Pima Indians and Asians remains to be investigated. The aforementioned studies that fail to identify any mutations in the MC3R were mostly done in Caucasians.

2.13. MC4R mutations and obesity

During the past few years, several lines of investigations, including mouse genetic and pharmacological studies, demonstrated the critical importance of the MC4R in regulating energy homeostasis in rodents (Cone, 2005; Tao, 2005). Mice lacking POMC or MC4R or over-expression of the antagonist Agouti-related protein are obese (Graham et al., 1997; Huszar et al., 1997; Ollmann et al., 1997; Yaswen et al., 1999). Administration of the MC3/4R agonist melanotan II into brain ventricles of rodents decreased food intake and body weight which was inhibited by the MC3/4R antagonist SHU 9119 (Fan et al., 1997; Thiele et al., 1998).

Human genetic studies quickly confirmed that the MC4R is also important for maintaining energy homeostasis in humans. In 1998, two groups independently reported that frameshift mutations in the MC4R gene were associated with early-onset morbid obesity (Vaisse et al., 1998; Yeo et al., 1998). About 80 mutations have been reported since then, including frameshift, inframe deletion, nonsense and missense mutations, scattered throughout the MC4R in various patient cohorts (Gu et al., 1999; Hinney et al., 1999; Farooqi et al., 2000; Vaisse et al., 2000; Dubern et al., 2001; Mergen et al., 2001; Jacobson et al., 2002; Kobayashi et al., 2002; Miraglia Del Giudice et al., 2002; Biebermann et al., 2003; Donohoue et al., 2003; Farooqi et al., 2003; Hinney et al., 2003; Lubrano-Berthelier et al., 2003; Marti et al., 2003; Tarnow et al., 2003; Yeo et al., 2003; Lubrano-Berthelier et al., 2004; Ma et al., 2004; Santini et al., 2004; Valli-Jaakola et al., 2004; Larsen et al., 2005).

Extensive functional studies have been done for some of these mutant receptors. Different defects were identified. Intracellular retention is the most common defect, including the frameshift mutations ΔCTCT at codon 211 (Ho & MacKenzie, 1999), the TGAT insertion at codon 244 (Ho & MacKenzie, 1999), and Δ750-751GA (Lubrano-Berthelier et al., 2004), the missense and nonsense mutations S58C (Lubrano-Berthelier et al., 2003; Tao & Segaloff, 2003), N62S (Tao & Segaloff, 2003; Yeo et al., 2003), P78L (Lubrano-Berthelier et al., 2003; Nijenhuis et al., 2003; Tao & Segaloff, 2003), N97D (Yeo et al., 2003), G98R (Tao & Segaloff, 2003), I102S (Lubrano-Berthelier et al., 2003), L106P (Yeo et al., 2003), I125K (Yeo et al., 2003), R165Q (Nijenhuis et al., 2003), R165W (Lubrano-Berthelier et al., 2003; Nijenhuis et al., 2003), N240S (Tao & Segaloff, 2005), L250Q (Lubrano-Berthelier et al., 2003), Y287X (Yeo et al., 2003), C271R (Tarnow et al., 2003), C271Y (Tao & Segaloff, 2003; Yeo et al., 2003), P299H (Lubrano-Berthelier et al., 2003), I316S (Yeo et al., 2003), I317T (Lubrano-Berthelier et al., 2003; VanLeeuwen et al., 2003). A few mutations are defective in ligand binding per se, including N97D, L106P, I125K (Yeo et al., 2003), I102S and I102T (Tao & Segaloff, 2005), I137T (Gu et al., 1999), I316S (Yeo et al., 2003) and Δ88-92 (Donohoue et al., 2003). Only a few mutants are defective in G protein coupling/activation, including D90N (Biebermann et al., 2003), I137T (Gu et al., 1999), A175T and V253I (Yeo et al., 2003). Some of the mutant receptors do not have obvious defects, including T11A, D37V, P48S, V50M, F51L, A154D, I170V, M200V, N274S, and S295P. They exhibit normal cell surface expression, ligand binding, and agonist-stimulated cAMP production (Tao & Segaloff, 2003, 2005). Whether these variants cause the phenotypes (obesity or binge eating disorder) is questionable.

2.14. ET_BR mutations and Hirschsprung disease

In 1994, Puffenberger and colleagues first identified a mutation in endothelin-B receptor (ET_BR) that changes a highly conserved Trp in TM5 to Cys (W276C) from a large inbred Mennonite kindred with a high incidence of Hirschsprung's disease or aganglionic megacolon (Puffenberger et al., 1994). Since then, 14 additional mutations have been reported, including G57S (Amiel et al., 1996) at the ECD, N104I and C109R in TM1 (Tanaka et al., 1998), A183G (Attie et al., 1995) and S196N (Sangkhathat et al., 2005) in TM3, W226C (Svensson et al., 1999) and R253X (Syrris et al., 1999) in EL2, W275X and Y293fs in TM5 (Kusafuka et al., 1996), S305N (Auricchio et al., 1996) and R319W (Amiel et al., 1996) in IL3, N378fs (Auricchio et al., 1996) and P383L (Amiel et al., 1996) in TM7, and S390R (Tanaka et al., 1998) in the proximal C terminus adjacent to TM7.

Functional analysis of W276C showed that the mutant receptor is expressed on the cell surface and binds to endothelin with normal affinity, but signaling is reduced (decreased maximal response compared with wt receptor) (Puffenberger et al., 1994; Imamura et al., 2000). Tanaka and coworkers showed that C109R is retained intracellularly near the nucleus and S390R binds to the ligand with normal affinity but decreased signaling (Tanaka et al., 1998). N104I has normal binding and signaling properties, suggesting that it might be a polymorphism (Tanaka et al., 1998), belonging to Class V as described below. Fuchs et al. showed that P383L, which changes a highly conserved Pro in TM7 (of the N/DPxxY

motif), results in intracellular retention and lack of ligand binding. However, Abe et al. showed that P383L has normal localization but the total protein level is decreased resulting in decreased binding capacity (Abe et al., 2000). Some mutations affect the G protein binding specificity. The wt ET_BR couples to both Gq and Gi. G57S and R319W bind to the ligand and activate Gq normally. However, G57S couples to Gs and R319W cannot activate Gi (Fuchs et al., 2001). Indeed, detailed studies on W275 and W276 showed that mutations at these two loci also change the G protein coupling profile (Imamura et al., 2000).

Mutations in ET_BR have also been found in animals with disease etiologies similar to Hirschsprung's disease, including horse (Metallinos et al., 1998; Yang et al., 1998), rat (Ceccherini et al., 1995; Gariepy et al., 1996), and mice (Hosoda et al., 1994; Matsushima et al., 2002) highlighting the importance of this signaling pathway in the development of myenteric ganglion neurons in mammals.

2.15. CCR5 mutations and susceptibility to HIV infection

CCR5 is the main coreceptor used by macrophage (M)-tropic nonsyncytium-inducing strains of HIV-1 (human immunodeficiency virus type 1) (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996). These strains of HIV-1, recovered during the first years of seroconversion, are responsible for disease transmission. Therefore CCR5 plays an essential role in AIDS pathogenesis. This is demonstrated by the discovery that inactivating mutations in CCR5 is associated with strong yet incomplete resistance to HIV infection after exposure. The first mutation identified was a 32 bp deletion in EL2 (designated CCR5Δ32) (Liu et al., 1996; Samson et al., 1996). This frameshift mutation results in a receptor with only four TMs that cannot function as a chemokine receptor. Subsequently, Carrington and colleagues identified 11 missense mutations, including I12T, C20S and A29S in the ECD, I42F and L55Q in TM1, R60S in IL1, A73V in TM2, R223Q in IL2, G301V in TM7, A335V, and Y339F in the C terminus, one nonsense mutation C101X in EL1, and one inframe deletion of K228 in IL3 (Carrington et al., 1997). A few additional mutations, including S63C in IL1, C178R in EL2, S125L in TM5, frameshift at Y299 (of the N/DPxxY motif), and R319H in the C terminus, have been reported since then (reviewed in (Blanpain et al., 2002)).

CCR5Δ32 is frequent in most European populations with a north to south downhill gradient with the highest frequencies found in northern Russia, Finland and Sweden and the lowest in southern countries like Turkey and Portugal (Blanpain et al., 2002). Epidemiological studies showed that patients homozygous for the mutant allele are strongly (but not completely) protected from HIV infection. Heterozygotes are partially protected from HIV infection with a slower progression of AIDS. The other variants are relatively rare with no homozygotes reported. The relevance of these variants on HIV infection and AIDS progression is unknown.

Functional studies showed that CCR5Δ32 is retained in the ER (Rana et al., 1997). Howard and colleagues characterized 6 of the 13 mutants identified by Carrington et al, including I12T, C20S, A29S, I42F, L55Q, and A73V. They showed that all 6 mutants are expressed on the cell surface, although at lower levels than wt CCR5. Mutations in the ECD (such as I12T, C20S, and A29T) result in absent or severely impaired ligand binding. Mutations in the TMs (such as I42F, L55Q, and A73V) do not impair ligand binding (Howard et al., 1999). In a comprehensive study characterizing all 16 CCR5 mutants known at that time, Blanpain and colleagues showed that 10 variants have normal cell surface expression, ligand binding and signaling as well as functioning as HIV coreceptors. Six mutations had major defects including intracellular trapping and poor expression at the cell surface (C101X, C178R, frameshift at Y299), ligand binding (C20S, A29S), or receptor activation (L55Q) (Blanpain et al., 2000). A29S displayed an altered pharmacological profile, with normal binding and signaling to MCP-2 but severely impaired binding and signaling to other agonists such as macrophage inhibitory protein (MIP)-1α, MIP-1β, and RANTES (regulated on activation, normal T-cell expressed and secreted) (Blanpain et al., 2000). R60S was also found to cause reduced cell surface expression (60% of wt) and signaling (also 60% of wt CCR5) (Tamasauskas et al., 2001). However, this mutation seems to be affecting protein stability rather than intracellular trafficking. The total protein level was also similarly decreased (Tamasauskas et al., 2001).

Interestingly, mutations in CCR5 in several species of monkeys have also been reported. Negative selection pressure, likely to avoid simian immunodeficiency virus infection, was suggested to favor the mutant alleles (similar selection pressure might be undergoing in humans). A deletion allele with a frequency of 4% was identified in sooty mangabey monkeys (Palacios et al., 1998). The mutant, a 24 bp deletion resulting in deletion of 8 amino acids in TM4, is retained intracellularly (Palacios et al., 1998). Peripheral blood mononuclear cells from monkeys heterozygous for the mutant allele also showed decreased cell surface expression of CCR5 as detected using antibody against human CCR5 (Palacios et al., 1998), similar to humans heterozygous for CCR5Δ32 (Wu et al., 1997). Frequent substitutions in CCR5 that inhibit simian immunodeficiency virus infection have also been reported in African green monkeys (Kuhmann et al., 2001). These variants may (such as Y14N) or may not (such as Q93R) affect signaling in response to chemokines (Kuhmann et al., 2001).

3. Towards a molecular classification of inactivating GPCR mutations

GPCRs begin their life with the transcription of their mRNAs in the nucleus and the synthesis of the polypeptides on the ribosome. These polypeptides are inserted into the ER membrane co-translationaly. Most of the GPCRs are glycoproteins. Therefore carbohydrates are added onto the polypeptides in the ER, specifically carbohydrates of the immature type containing high mannose. After initial folding, with the assistance of molecular chaperones such as calnexin and calreticulin, the receptors are exported to the Golgi apparatus (where the carbohydrates are modified into the mature type, the

mannoses are trimmed, and sialic acids are added) and further transported onto the cell surface. Ligands bind to the receptors on the cell surface. This binding results in conformational change in the receptor. The active receptors bind to the cognate G proteins, catalyzing the conversion of GDP for GTP on Gα, and the disassociation of Gα and Gβγ. Both Gα and Gβγ can activate downstream effectors. Activated GPCRs are usually quickly desensitized by phosphorylation and internalization. Internalized GPCRs can either recycle back to the cell surface where they can undergo another cycle of activation or degraded in the lysosome.

Based on the lifecycle of GPCRs described above, modeled after classification schemes proposed for mutations in low density lipoprotein receptor (Hobbs et al., 1990) and cystic fibrosis transmembrane conductance regulator (CFTR) (Welsh & Smith, 1993), we proposed a classification scheme to classify the MC4R inactivating mutations (Tao & Segaloff, 2003). We suggest that it should apply to most or all GPCR inactivating mutations. Specifically, we propose that inactivating mutations can be classified as follows.

3.1. Class I: Defective receptor biosynthesis

Mutations belong to this class include mostly mutations that terminate the receptor prematurely. The most clearly documented case is the V2R mutation 804insG (Tsukaguchi et al., 1995). This mutation results in frame shift and premature termination. Functional study showed that mRNA is produced at similar level as wt receptor; however, little or no receptor proteins could be detected (Tsukaguchi et al., 1995). Another V2R mutation that results in defective protein synthesis is the deletion of nucleotide 102 (delG102) that results in frameshift and premature truncation (Schoneberg et al., 1998). We speculated that MC4R mutants, such as W16X, Y35X, and L64X, likely belong to this class (Tao & Segaloff, 2003), although expression studies are needed to confirm or refute this prediction. The CaR mutants in the signal peptide sequence (L11S and L13P) that are defective in cotranslational processing (Pidasheva et al., 2005) also belong to this class. The CCR5 mutant R60S might be a milder mutant in this class (the protein level is 60% of wt) (Tamasauskas et al., 2001). Either defective synthesis/processing and/or accelerated degradation can result in this phenotype. Few studies have attempted to differentiate between these two possibilities.

3.2. Class II: Defective trafficking to the cell surface

This class of mutants are synthesized relatively normally but are retained inside the cell. It represents by far the most common defect in all GPCR inactivating mutations. For example, in V2R, 70% of mutants belong to this class (Lu et al., 2003). Mutations in rhodospin are also well-known examples of this class of mutants (Sung et al., 1991, 1993; Kaushal & Khorana, 1994). In the first set of studies characterizing MC4R inactivating mutations, this class was also shown to be the most common (Lubrano-Berthelier et al., 2003; Nijenhuis et al., 2003; Tao & Segaloff, 2003; Yeo et al., 2003). However, of the 13 mutants that we recently characterized, all of them are expressed on the cell surface, although some of them might be expressed at reduced levels ((Tao & Segaloff, 2005) and Rong et al., in press). In the MC1R, D84E, R151C, I155T, and R160W also belong to this class (Beaumont et al., 2005).

3.3. Class III: Defective ligand binding

Mutant receptors belonging to this class are produced and transported onto the cell surface, but are defective in ligand binding. A classic example is the *little* mutation in GHRHR present in the *lit/lit* mice (Gaylinn et al., 1999). V2R mutants such as Y128S, R181C, ΔR202, R202C, and P286R (Pan et al., 1994; Tsukaguchi et al., 1995; Ala et al., 1998) also belong to this class. MC4R mutants that belong to this class include N97D, L106P, I125K (Yeo et al., 2003), I137T (Gu et al., 1999), I316S (Yeo et al., 2003) and Δ88-92 (Donohoue et al., 2003). Our recent data suggest that I102S and I102T MC4R are partially defective in ligand binding (Tao & Segaloff, 2005). In the CaR, mutations in the ECD, such as R62M, R66C, T138M, and R185Q, also change binding affinity for calcium ion without significant effect on the intracellular signaling (Bai et al., 1996). Ligand binding defect can be due to the inability of the receptor to adopt a conformation competent for ligand binding or the mutated residue directly involved in ligand binding.

3.4. Class IV: Defective receptor activation

This class of mutants are produced and transported to the cell surface and can bind to the ligand normally, but are defective in signaling. They showed reduced maximal response and/or decreased sensitivity (increased EC_{50}). A typical example is the MC3R mutation I183N (Tao & Segaloff, 2004). We showed that the mutant receptor can bind to the ligand with similar affinity, but are totally devoid of signaling. Since this mutation affects a highly conserved residue, we performed additional mutagenesis in the MC4R and LHR. These experiments showed that the corresponding Ile is also important for MC4R and LHR signaling (Tao & Segaloff, 2004).

In the MC1R, R142H and D294H are Class IV mutants. They are expressed well on the cell surface, bind to the ligand normally, but are devoid of cAMP production in response to ligand stimulation (Beaumont et al., 2005). R142H mutates an invariant residue in Family A GPCRs (of the DRY motif near the intracellular end of TM3). This Arg has been shown to be critical for G protein-coupling/activation in a number of GPCRs (reviewed in (Gether, 2000)). D294H mutates a highly conserved residue in Family A GPCRs (of the N/DPxxY motif in TM7). A variant in pig MC4R that mutates the Asp to Asn has a similar phenotype (Kim et al., 2004).

The defect in receptor activation can be due to the receptor unable to undergo conformational change to generate active receptor or inability to couple to G protein or the receptor is coupled to but cannot activate G protein (catalyze the exchange of GDP for GTP). Defective receptor regulation

can also inactivate the receptor. An elegant example is the R147H mutation in the V2R that mutates the highly conserved Arg in the DRY motif. Original studies suggested that the mutation results in a receptor that can bind to the ligand normally but are defective in signaling (Rosenthal et al., 1993). Further studies by Caron and colleagues showed that the mutant receptor is constitutively internalized and desensitized (Barak et al., 2001). Similarly, in rhodopsin, mutation of the corresponding Arg in ADRP patients (R135L and R135W) was found to be unable to activate transducin despite normal folding and binding to 11-*cis*-retinal (Min et al., 1993). Sung and colleagues showed that these mutant rhodopsins are constitutively phosphorylated and bind to visual arrestin with high affinity, therefore constitutively internalized (Chuang et al., 2004). Whether similar defect in receptor internalization and desensitization is also operational in other GPCRs remains unknown. Since laboratory-designed mutants in other GPCRs such as α_{1B}-adrenergic receptor and angiotensin type 1A receptor that mutate the same Arg have the same cellular phenotype (Wilbanks et al., 2002), the so-called "signaling" defect in other GPCRs that mutate the highly conserved Arg (such as MC1R) might be due to constitutive internalization and desensitization.

3.5. Class V: Mutants with no known defects

Some of the mutant receptors, although identified from patients suspected of carrying inactivating mutations in the said receptors, are expressed on the cell surface, bind to the ligand normally, and generate a normal response to ligand stimulation. Therefore no apparent defect can be discerned. These results would suggest that the mutations are not the cause of the disease. In the V2R, mutations such as A61V and inframe deletion of R247-G250 belong to this class (Pan et al., 1994). The ET_BR mutant N104I functions normally (Tanaka et al., 1998), therefore also belongs to Class V. As mentioned above, many of the CCR5 mutants have normal functions therefore also belong to this class (Blanpain et al., 2000).

In our characterization of the MC4R mutations we have identified several variants (such as T11A, Y35C, D37V, C40R, P48S, V50M, F51L, A154D, I170V, M200V, M218T, N274S, and S295P) that have normal cell surface expression, ligand binding, and signaling (Tao & Segaloff, 2003, 2005; Rong et al., in press). I also showed that the MC3R mutants A293T and X361S have normal ligand binding and signaling (unpublished observations) therefore belong to this class too.

It should be emphasized that a mutant cannot be classified as Class V just because it signals normally. Normal cell surface expression and ligand binding should also be observed. This is due to the fact that in the in vitro expression system used in characterizing the mutants, there are spare receptors (reviewed in (Tao, 2005)). Some mutants might have decreased expression but still have normal maximal response in vitro. These mutants, not apparent from studies just measuring signaling properties of the mutants, are defective.

MacKenzie recently suggested that mutations that disrupt ligand binding or signaling should be classified as a single class (Mackenzie, 2006). The reason for this suggestion is not elaborated. Mutations that disrupt ligand binding or signaling are very distinct. Most of them would also locate in distinct domains of the receptors. They also provide insights into two distinct functions of the receptors. We feel that they should be categorized as different classes. It is unfortunate that many studies did not investigate the ligand binding properties of the mutants therefore a definitive assignment could not be made for these mutants.

Some mutants might have multiple defects. For example, the V2R mutant R113W decreases ligand binding affinity by 20-fold, signal transduction by 3-fold, and receptor maturation by 4-5 fold. These defects combined together result in the mutant receptor being unresponsive to arginine vasopressin stimulation (Birnbaumer et al., 1994). Similarly, a microdeletion in TM7 of LHR decreases both cell surface expression and effector activation of the receptor that does reach cell surface (Latronico et al., 1998). I102S and I102T MC4R are defective in cell surface expression and ligand binding (Tao & Segaloff, 2005).

4. Insights into the structure-function of GPCRs

Careful characterizations of naturally occurring mutations of GPCRs that cause diseases not only provide novel insights into the physiological and pathophysiological roles of the underlying systems, but also shed lights on the structure-function relationships of the GPCRs. Experiments of nature provided us a lot of excellent clues. Below we summarize some of the lessons learned in these respects.

4.1. Cell surface expression

There are multiple mechanisms for misfolding and intracellular retention in the naturally occurring inactivating mutations in GPCRs. The best characterized, pioneered by Khorana, is the rhodopsin, where the disruption of a highly conserved disulfide bond between the cysteines in EL1 and EL2 or the formation of alternate disulfide bond between Cys185 and Cys187 in EL2 accounts for a number of mutations that are misfolded (reviewed in (Stojanovic & Hwa, 2002)).

In the MC4R, it was also suggested that the formation of alternate disulfide bond could also result in intracellular retention (Tarnow et al., 2003). C271 located in EL3 forms an intra-loop disulfide bond with C277. In the mutant C271R, not only this disulfide bond is disrupted, but C277 and C279 forms an improper disulfide bond, detrimental for the receptor's folding. Disruption of the improper disulfide bond by additional mutations or reducing agents can restore the mutant's function (Tarnow et al., 2003). In CCR5, C20R, which disrupts a disulfide bond linking ECD with EL3, and C178R, which disrupts the disulfide bond between EL1 and EL2, also result in misfolding and decreased cell surface expression (Blanpain et al., 2000). It is interesting to note that naturally occurring inactivating mutations that either mutates an existing Cys or introducing a new Cys are very common. Therefore this mechanism might prove to be a common one. In the V2R,

though, two mutations (R181C and R202C) that introduce a new extracellular Cys residue, supposedly disrupting the essential disulfide bond, have normal cell surface expression but are defective in ligand binding (Pan et al., 1994; Tsukaguchi et al., 1995), indicating that correct formation of the disulfide bond in V2R may be important for ligand recognition of receptor.

In rare cases, a mutation might specifically disrupt a motif important for cell surface targeting (Duvernay et al., 2005) therefore resulting in intracellular retention. For example, disruption of a dileucine motif at the proximal C terminus was found to disrupt trafficking of V2R and MC4R (Schulein et al., 1998; VanLeeuwen et al., 2003).

Armed with the crystal structure of rhodopsin, investigators have been taking advantage of the naturally occurring mutations to elucidate the interhelical interactions of residues in TMs. For example, functional characterization of G51V and G89D, two rhodopsin mutations associated with ADRP, revealed that G51V forms chromophore normally, whereas G89D could only form chromophore partially (Hwa et al., 1997). Garriga and colleagues generated additional mutants at these two loci. The results showed that the size of the introduced side chain at G51 is important, whereas the charge is more important at G89 (Bosch et al., 2003). Inspection of the rhodopsin crystal structure suggested that the substitution of G51 with the bulky Leu results in steric clash with V300 in TM7. Similarly, through compensatory mutations and structure analysis, it was shown that in another rhodopsin mutation L125R, the introduced Arg results in steric clash with the neighboring W126 and disrupts a critical salt bridge between E122 in TM3 and H211 in TM5 (Stojanovic et al., 2003). Mutation of A164 to Val, by hindering L119 and I123, also disrupts the E122-H211 salt bridge (Stojanovic et al., 2003). However, such detailed analyses have rarely been done with other GPCRs. Homology modeling using rhodopsin structure as the template can be used for probing the TM interactions in other GPCRs.

4.2. Dominant negative activity

Dimerization of GPCRs is rapidly being recognized as a general phenomenon that occurs to most if not all GPCRs. A well-known observation is that intracellularly retained mutant receptors can retain the co-expressed wt receptors and decrease wt receptor expression and signaling. One of the first examples is the CCR5Δ32 mutation that confers protection of HIV infection (Benkirane et al., 1997). Similar observation has been obtained in both laboratory-designed mutants (such as rhodopsin (Colley et al., 1995) and α-factor receptor (Overton & Blumer, 2000), among many others) and naturally occurring inactivating mutants (such as LHR (Tao et al., 2004) and TSHR (Calebiro et al., 2005), among others). Indeed, this mechanism has been suggested to be responsible for dominant transmission of diseases in heterozygotes (Benkirane et al., 1997; Calebiro et al., 2005). In FHH patients with CaR mutations, the autosomal dominant mode of inheritance can also be explained by dominant negative activity of mutant receptor on wt CaR (Bai et al., 1997; Hu & Spiegel, 2003; Pearce et al., 1996a). Interestingly, in the MC4R, the intracellularly retained mutants do not exert dominant negative activity on the wt receptor in co-transfection experiments (Farooqi et al., 2000; Ho & MacKenzie, 1999; Yeo et al., 2003). The only known MC4R mutant that exerts dominant negative activity on wt MC4R is D90N (Biebermann et al., 2003). This mutant is expressed on the cell surface and bind to ligand normally. The mechanism for the observed dominant negative activity of D90N remains to be further elucidated. In the MC3R, we showed that I183N does not have dominant negative activity (Tao & Segaloff, 2004) whereas Rached et al. showed that it has (Rached et al., 2004). I recently showed that I335S MC3R has some dominant negative activity on co-expressed wt MC3R (unpublished observations). Mutations in the MC3R and MC4R associated with obesity are identified mostly in heterozygotes. It is important to differentiate whether the mutations cause obesity by dominant negative activity or haploinsufficiency. The studies on the MC4R showing lack of dominant negative activity are consistent with results obtained in MC4R knockout mice where heterozygotes have increased body weight (Huszar et al., 1997) implicating haploinsufficiency as the cause of obesity.

4.3. Receptor activation/G-protein coupling

As detailed above, the rhodopsin mutants at R135 and V2R mutants at R142 were the first examples of the naturally occurring mutants that bind to the ligands normally but cannot activate the cognate G proteins. Laboratory-designed mutants confirmed that the Arg was important for receptor activation (see (Franke et al., 1990; Franke et al., 1992) for examples). Studies in MC1R also highlighted the importance of DRY and N/DPxxY motifs in G protein-coupling/receptor activation. Inactivating mutations, including R142H and D294H, were found to have relatively normal binding but defective signaling (see above).

We showed that I183N MC3R (of the highly conserved DRYxxI/V motif at the intracellular end of TM3) is defective in signaling (Tao & Segaloff, 2004). To gain further insight into what characteristics of the amino acids at I183 are required for MC3R activation, we generated additional mutants of I183 including I183A, I183D, I183L, I183R, and I183V. The results revealed a discrete requirement for I183 in mediating ligand-induced signaling. Therefore, although all the mutants have normal ligand binding characteristics, only I183V signals normally, the other mutants have either decreased (I183L and I183A) or no (I183D and I183R) signaling (Tao & Segaloff, 2004).

5. Therapeutic implications

5.1. Class I mutants: Aminoglycosides

Although missense mutations are the most common loss-of-function mutations, a significant portion, which varies among the receptors, are nonsense and frameshift mutations that truncate the receptors prematurely. Previous studies in other

genetic diseases caused by nonsense mutations discovered that aminoglycoside antibiotics, by binding to the decoding site on the ribosome, could decrease the codon-antocodon proofreading efficiency resulting in read-through of the premature stop codon. Using this strategy as a therapeutic approach has been investigated in diseases such as muscular dystrophy (Barton-Davis et al., 1999), hemophilia (James et al., 2005), ataxia-telangiectsia (Lai et al., 2004), and spinal muscular atrophy (Wolstencroft et al., 2005). The most extensively studied is in cystic fibrosis where very promising clinical results were obtained (Wilschanski et al., 2003).

Schoneberg and colleagues investigated the potential of aminoglycosides in treating NDI caused by nonsense V2R mutations (Schulz et al., 2002; Sangkuhl et al., 2004). In vitro, geneticin can rescue nonsense V2R mutations such as W200X, E242X, and R337X as evidenced by increased cell surface expression of full-length receptor and signaling capacity of the read-through receptor. Geneticin can also increase cAMP production in cells from transgenic mice expressing E242X. Dramatically, geneticin treatment in these transgenic mice increased urine osmolality in both normal and challenged conditions, suggesting that geneticin can partially rescue the nonsense mutation in vivo (Sangkuhl et al., 2004). It is expected that with high-throughput screening, aminoglycosides with less side effects could be identified as drug candidates for further animal and clinical studies.

5.2. Class II mutants: Chemical and pharmacological chaperones

Since intracellular retention is the most prevalent defect in inactivating mutations in GPCRs, we review here recent progresses in understanding the maturation of mutant GPCRs and studies exploring the therapeutic potentials of chemical and pharmacological chaperones.

Human diseases caused by intracellular retention of inactivating GPCRs immediately place these diseases as protein conformational diseases, joining well-known examples such as familial hypercholesterolemia and cystic fibrosis. Lessons learned from the studies of those diseases can be used to guide the studies on the mutated GPCRs. For example, the most common mutation in CFTR, ΔF508, was found to be temperature sensitive; incubation at a lower temperature increases the maturation of the mutant CFTR (Denning et al., 1992). Similar manipulation was also found to increase the maturation of mutant LHRs (Jaquette & Segaloff, 1997). However, in a recent study with V2R, growth at 27 C was found unable to increase cell surface expression of nine mutants associated with NDI (Robben et al., 2006).

Mutant CFTRs such as ΔF508 were found to associate with molecular chaperones (Pind et al., 1994). Similarly, mutant GPCRs were also found to associate with molecular chaperones such as calnexin and immunoglobulin heavy chain binding protein (Rozell et al., 1998; Morello et al., 2001; Mizrachi & Segaloff, 2004; Robert et al., 2005). Mutant receptors usually have prolonged association with calnexin (Morello et al., 2001; Robert et al., 2005). This prolonged association results in the retention of the mutants in the ER and finally degradation by the ubiquitin-proteasome pathway (Petaja-Repo et al., 2001). Indeed, different mutations of the same GPCR were found to associate with different chaperones (Mizrachi & Segaloff, 2004).

Low molecular weight compounds, such as glycerol, dimethyl sulfoxide, and trimethylamine N-oxide, known as chemical chaperones, have been reported to improve the maturation of a number of misfolded proteins including CFTR (Brown et al., 1996; Sato et al., 1996). In V2R, it was shown that chemical chaperones are not as effective as pharmacological chaperones in promoting maturation of ER-retained mutants (Robben et al., 2006).

Bouvier and colleagues were the first to explore the potential utility of pharmacological chaperones to increase the cell surface expression of mutant GPCRs (Morello et al., 2000b). They showed that selective, membrane-permeable nonpeptidic V2R antagonists dramatically increase cell surface expression and restore the function of eight mutant V2Rs associated with NDI, presumably by binding to and stabilizing partially folded mutants (Morello et al., 2000b) (reviewed in (Morello et al., 2000a)). Recently, this has been extended to in vivo where treatment of patients with the nonpeptide antagonist SR49059 decreased urine volume and water intake (Bernier et al., 2006).

Retinoids have been used as pharmacological chaperones to improve folding of Class II rhodopsin mutants. In transgenic animals expressing T17M, a Class II mutant, vitamin A supplementation in diet alleviated retinal degeneration; similarly, in vitro, inclusion of 11-*cis*-retinal in the culture media improved the folding of the mutant (Li et al., 1998). The addition of 9-*cis*-retinal, 11-*cis*-retinal, or 11-*cis*-7-ring retinal, to the media increased the amount of P23H that could reach the plasma membrane (Saliba et al., 2002; Noorwez et al., 2003; Noorwez et al., 2004) suggesting that they might be useful therapeutic agents for the rescue of Class II rhodopsin mutants.

Conn and colleagues discovered a membrane-permeable nonpeptide receptor antagonist for the GnRHR, IN3, that can also act as pharmacological chaperone rescuing the functions of the majority of the naturally occurring GnRHR mutations associated with hypogonadotropic hypogonadism (Janovick et al., 2002; Leanos-Miranda et al., 2002).

5.3. Class III and IV mutants: Searching for novel ligands

If the mutant receptor is defective in ligand binding or signaling for the natural or superpotent agonists used in the functional studies or in clinics, it might be possible to design new agonists the can activate the mutant receptors. Therefore it may prove fruitful to test those mutant receptors for binding to other ligands. A mutant receptor may be defective in binding to one ligand but not the others. For example, O'Rahilly and colleagues identified a MC4R mutation (I316S) that alters the relative affinities of the receptor for its endogenous agonist (α-MSH) and antagonist Agouti-related protein (Yeo et al., 2003). Similarly, CCR5 mutant A29S has normal binding and

signaling to MCP-2 but severely impaired binding and signaling to MIP-1α, MIP-1β, and RANTES (Blanpain et al., 2000).

In fact, the first clinical trial of this strategy has already been done with excellent outcome. The LHR mutation that deletes exon 10 has altered signaling for LH vs hCG. Although the mutant receptor cannot respond to LH stimulation (therefore the patient, although having high serum LH level, has very low testosterone level, unable to initiate pubertal development), it responds to hCG normally (see Section 2.4 above). In the clinic, treatment with hCG was found to increase serum testosterone levels and testicular volumes, normalize serum LH level (through negative feedback), and enhance spermatogenesis (Gromoll et al., 2000).

6. Conclusions

During the past 15 years, an ever-expanding list of diseases caused by inactivating mutations in GPCRs has appeared in scientific literature. With a few exceptions (such as rhodopin, V2R, MC3R, and MC4R), the mode of inheritance is autosomal recessive for these diseases. Patients are either homozygous or compound heterozygous. We reviewed the literature where multiple mutations in 15 GPCRs have been identified to cause diseases. In vitro functional studies of these naturally occurring mutations not only advanced our understanding of the pathophysiology of these diverse diseases, but also provided interesting insights into the structure-function of these GPCRs. Investigators have taken advantage of the clues provided by these experiments of nature to gain a deeper understanding of the loci in multiple aspects of receptor function, including cell surface trafficking, ligand binding, and signaling. A generalized classification scheme was proposed for cataloging the ever-increasing list of mutations in these receptors. A definitive classification is helpful for directing scientists interested in different aspects of receptor function to choose the mutants for further investigation. It should also be helpful in further research towards personalized medicine. For Class I mutants, aminoglycoside antibiotics are worth further testing. For Class II mutants, studies exploring pharmacological chaperones as potential therapeutic tools have obtained exciting results in several GPCRs. For Class III/IV mutants, different ligands can be tested to see whether they can bind and activate these mutant receptors, especially those that have altered pharmacological profiles.

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