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At the Cutting Edge

# Molecular mechanisms of the neural melanocortin receptor dysfunction in severe early onset obesity

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**Abstract**

The neural melanocortin receptors, melanocortin-3 and -4 receptors (MC3R and MC4R), have been shown to regulate different aspects of energy homeostasis in rodents. Human genetic studies showed that mutations in the MC4R gene are the most common monogenic form of obesity. Functional analyses of the mutant receptors revealed multiple defects. A classification scheme is presented for cataloguing the ever-increasing array of MC4R mutations. Functional analysis of the only inactivating MC3R mutation is also summarized. Insights from the analyses of the naturally occurring mutations in the MC3R and MC4R on the structure and function of these receptors are highlighted.


*Keywords:* Melanocortin-4 receptor; Melanocortin-3 receptor; Obesity; Mutation; G protein-coupled receptor

## Contents



## 1. Introduction

Obesity is rapidly becoming one of the foremost health problems in our times, especially in developed countries like the US. Obesity has profound effects on lipid and glucose homeostases, therefore it is usually associated with

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dyslipidemia, hypertension, insulin resistance and hyperglycemia, leading to increased incidences of diabetes mellitus, atherosclerosis, and cardiovascular disease. Obesity and its associated disorders are at epidemic levels in the US and other developed countries (Cummings and Schwartz, 2003), with staggering economic and social costs. Hence there is enormous interest in understanding the molecular mechanisms underlying the regulation of energy intake, accumulation, and expenditure, and the defects of these regulations that result in obesity.

During the past decade, with the discovery of leptin (Zhang et al., 1994), a polypeptide hormone produced by the adipocyte, tremendous progress has been made in elucidating the neural pathways regulating energy homeostasis. A number of peptides, including neuropeptide Y (NPY) (Naveilhan et al., 1999), ghrelin (Nakazato et al., 2001; Tschop et al., 2000), glucagon-like peptides (Tang-Christensen et al., 2000; Turton et al., 1996), melanin-concentrating hormone (Ludwig et al., 2001; Qu et al., 1996; Shimada et al., 1998), orexins (also called hypocretins) (de Lecea et al., 1998; Sakurai et al., 1998), and melanocortins, have been identified to be involved in regulating energy balance (for a review, see Schwartz et al., 2000). Of particular interest to the present article is the leptin-regulated melanocortin circuit. In this circuit, leptin, after its production by the adipocyte, can cross the blood–brain barrier, bind to its receptors in two subsets of neurons in the arcuate nucleus of the hypothalalmus. One subset of neurons expresses NPY and Agouti-related protein (AgRP), another subset of neurons expresses proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript. POMC is processed post-translationally in a tissue-specific manner to melanocortins, including α-, β-, and γ-melanocyte stimulating hormone (MSH) and adrenocorticotropin (ACTH) by prohormone convertases (Smith and Funder, 1988).

Melanocortins exert their diverse functions by activating cell surface integral membrane proteins called melanocortin receptors (MCRs). Five subtypes of MCRs have been cloned and they are named MC1R to MC5R according to the sequence of their cloning (reviewed in Gantz and Fong (2003)). MC1R is the classical MSH receptor expressed in the skin that control pigmentation. MC2R is the classical ACTH receptor that is expressed in the adrenal gland regulating adrenal steroidogenesis and growth. MC3R and MC4R are called neural MCRs because they are expressed primarily in the brain. They regulate different aspects of energy homeostasis (see below). MC5R is expressed in exocrine glands and regulates secretion of these glands (Chen et al., 1997). All five MCRs are G protein-coupled receptors (GPCRs) that activate Gs, resulting in increased cAMP production in the cell. Like other GPCRs, MCRs consist of seven transmembrane α-helices (TMs) connected by alternating intracellular and extracellular loops, with the N-terminus extracellular, and the C-terminus intracellular.

MCRs are intriguing members of the GPCR superfamily in that they are the only GPCRs that are known to have endogenous antagonists, agouti and AgRP. Agouti, usually expressed in the melanocyte, is a natural antagonist for MC1R expressed in the skin. However, when ectopically expressed such as in agouti ($A^y$) mice, it can also antagonize the MC4R (Lu et al., 1994). AgRP, a peptide of 132 amino acids (in humans), is expressed in the hypothalamus. It is an antagonist for the neural MCRs (Ollmann et al., 1997). It has recently been shown that a C-terminal fragment of AgRP, AgRP (83-132), acts as an inverse agonist in that it abolishes the basal activity of the MC4R (Haskell-Luevano and Monck, 2001; Nijenhuis et al., 2001).

## 2. Melanocortin system and rodent energy homeostasis

Several lines of investigations, including genetic and pharmacological studies, demonstrated convincingly the critical importance of the MC4R in regulating food intake and energy homeostasis in rodents. Mice lacking the POMC-derived peptides suffer from obesity in addition to defective adrenal development (due to lack of MC2R signaling) and altered pigmentation (due to lack of MC1R signaling) (Yaswen et al., 1999), recapitulating the phenotype of POMC-deficient patients (see below). The agouti ($A^y$) mice, which overexpress agouti ectopically in many tissues including the hypothalamus, in addition to the phenotype of yellow coat color, are also obese, because agouti is a competitive antagonist of the MC4R (Lu et al., 1994). Over-expression of AgRP also results in obesity (Graham et al., 1997; Ollmann et al., 1997). AgRP expression is elevated in the hypothalamus of *obese* and *diabetic* mice (Ollmann et al., 1997; Shutter et al., 1997), again supporting a role for AgRP and MC4R in the regulation of feeding. Administration of the MC3/4R agonist melanotan II (a cyclic heptapeptide) into brain ventricles of rodents suppressed food intake and decreased body weight (Fan et al., 1997; Thiele et al., 1998), while the MC3/4R antagonist SHU 9119 stimulated feeding and reversed the suppressive effects of melanotan II on food intake (Fan et al., 1997). In addition to the arcuate and paraventricular nuclei as the possible sites of action for these melanocortin analogs, the brainstem, which has the highest expression of MC4R in the brain (Mountjoy et al., 1994), may also be involved in the control of feeding (Grill et al., 1998). Finally, mice lacking MC4R had increased food intake and body weight, increased linear growth, and hyperinsulinemia (Huszar et al., 1997).

## 3. Naturally occurring mutations in the MC4R gene and human obesity

The critical importance of the melanocortin system in regulating energy balance in humans was demonstrated by the fact that mutations in multiple molecules of the circuit, including leptin (Montague et al., 1997; Strobel et al., 1998), leptin receptor (Clement et al., 1998), POMC (Krude et al.,

1998), prohormone convertase 1 (Jackson et al., 1997), and MC4R (see below), all result in severe early onset obesity (reviewed in O'Rahilly et al. (2003)). Essentially human genetic studies replicated the rodent genetic studies. For example, mutations in the POMC gene in humans result in severe early onset obesity in addition to adrenal insufficiency (lack of MC2R signaling) and red hair pigmentation (lack of MC1R signaling), similar to POMC gene knockout mice.

In 1998, O'Rahilly and Vaisse were the first to report that frameshift mutations in the MC4R gene were associated with severe early onset obesity (Vaisse et al., 1998; Yeo et al., 1998). Since then, more than 70 mutations have been reported in various patient cohorts (Biebermann et al., 2003; Donohoue et al., 2003; Dubern et al., 2001; Farooqi et al., 2000, 2003; Gu et al., 1999; Hinney et al., 1999, 2003; Jacobson et al., 2002; Kobayashi et al., 2002; Larsen et al., 2005; Lubrano-Berthelier et al., 2003b, 2004; Ma et al., 2004; Marti et al., 2003; Mergen et al., 2001; Miraglia Del Giudice et al., 2002; Santini et al., 2004; Tarnow et al., 2003; Vaisse et al., 2000; Valli-Jaakola et al., 2004; Yeo et al., 2003), including frameshift, inframe deletion, nonsense and missense mutations, scattered throughout the MC4R (Fig. 1). Up to 6% of early onset morbidly obese patients in some cohorts harbor MC4R mutations (Farooqi et al., 2003), therefore mutations in the MC4R gene are the most common monogenic form of early onset severe obesity. In most of the earlier reports, functional analyses of the mutant receptors were not performed, therefore only an association rather than a causal relationship could be demonstrated. Recently, several groups performed detailed functional studies on some of the mutant MC4Rs to address two questions: (1) Do these mutations result in loss-of-function? (2) For those mutants that result in loss-of-function, what are the molecular defects? These studies are summarized below.

### *3.1. Do the MC4R mutations identified from obese patients cause loss-of-function?*

Various assays have been used to study whether the naturally occurring mutations identified from obese patients cause loss-of-function. MC4R is known to activate Gs after ligand binding, therefore resulting in increased intracellular cAMP levels. Direct measurement of cAMP levels in cells transfected with wild-type or mutant MC4R or indirect measurement of increased reporter gene expression resulting from increased cAMP levels have been used to measure mutant MC4R signaling. We used the direct measurement of cAMP levels. In cells transfected with wild-type MC4R, the superpotent agonist, [Nle$^4$,D-Phe$^7$]-α-MSH (NDP-MSH), increased intracellular cAMP levels in a dose-dependent manner, with an $EC_{50}$ of about 0.24 nM (Tao and Segaloff, 2003). Some mutant receptors have either decreased or absent signaling in response to NDP-MSH stimulation. Further studies showed that many of these mutants have decreased or absent binding to iodinated NDP-MSH. To investigate whether these mutant receptors are expressed on

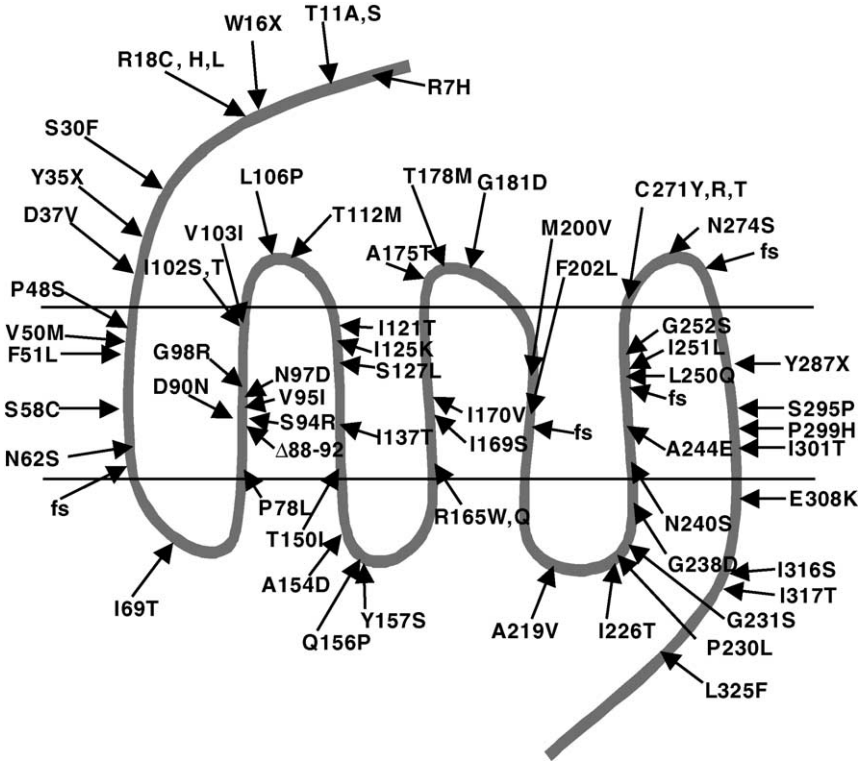


Fig. 1. Naturally occurring mutations of the MC4R identified from various patient cohorts. Shown are the approximate locations of the mutations. See the text for references.

the cell surface, confocal microscopy studies were performed on cells stably transfected with wild-type or mutant MC4Rs. It is important to use stably transfected cells, because transient transfection can result in an artificial over-loading of the cell's quality control system, therefore resulting in intracellular retention of the receptors (see Tao et al., 2004 for an example). With stably transfected cells, we showed that wild-type MC4R is expressed on the cell surface, although there is a portion of the receptor inside the cell. The mutant receptors that have decreased or absent binding were shown to have decreased or absent cell surface expression. When these cells were permeabilized, intracellular staining was observed suggesting that mutant receptors were expressed but retained intracellularly.

Of the naturally occurring mutations in GPCRs that cause human diseases, intracellular retention is the most common defect for loss-of-function phenotype. This has been observed in rhodopsin (mutations result in retinitis pigmentosa) (Sung et al., 1991, 1993), the V2 vasopressin receptor (mutations result in nephrogenic diabetes insipidus) (reviewed in Morello and Bichet (2001)), the endothelin B receptor (mutations result in Hirschsprung's disease) (Tanaka et al., 1998), the calcium-sensing receptor (mutations result in familial hypocalciuric hypercalcemia or neonatal severe hyperparathyroidism; Bai et al., 1996), the gonadotropin-releasing hormone receptor (mutations result in idiopathic hypogonadotropic hypogonadism) (Leanos-Miranda et al., 2002), the lutropin and follitropin receptors (mutations result in infertility) (reviewed in Themmen and Huhtaniemi (2000)). The folding of GPCRs, integral membrane proteins that transverse the plasma membrane seven times, are believed to be complex. Any perturbations in this process will result in misfolded receptor that is being detected by the cell's quality control system and prevented from exiting the endoplasmic reticulum (Ellgaard and Helenius, 2003). Of the only receptor that has been studied in detail, that of δ-opioid receptor, exiting the ER is the rate-limiting step in the receptor's maturation and expression at the cell surface (Petaja-Repo et al., 2000).

### 3.2. *Molecular classification of the MC4R mutations identified from obese patients*

The recent studies about the functional defects of MC4R variants associated with childhood obesity revealed multiple defects. We proposed the following scheme, modeled after the classification of mutations in LDL receptor and cystic fibrosis transmembrane conductance regulator (Hobbs et al., 1990; Welsh and Smith, 1993), for classifying MC4R mutations (Fig. 2):

- Class I: Null mutations. Due to defective protein synthesis and/or accelerated protein degradation, no receptor proteins are present in the cell. Mutants such as W16X (Marti et al., 2003), Y35X (Hebebrand et al., 2002; Hinney et al., 1999) and L64X (Jacobson et al., 2002) likely belong to this class, although expression studies are needed to verify this prediction.

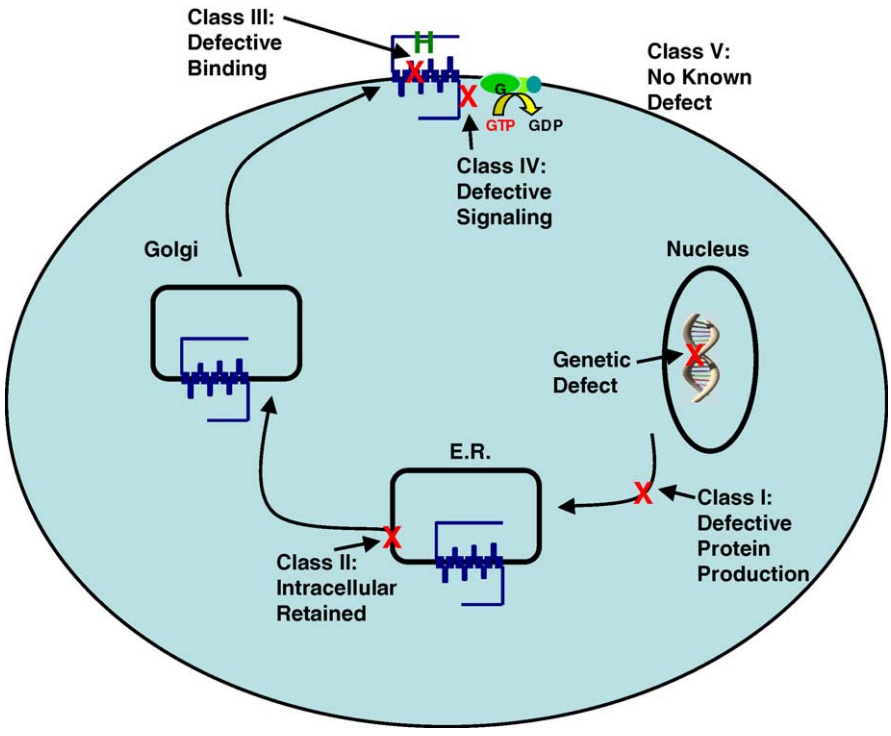


Fig. 2. Molecular classification of naturally occurring MC4R mutations in early onset severe obesity. See the text for detailed description.

- Class II: Intracellularly trapped mutants. The mutant receptors are produced but are retained intracellualrly, most likely in the endoplasmic reticulum due to misfolding being detected by the cell's quality control system. This class comprises the largest set of MC4R mutations reported to date, including the frameshift mutations ΔCTCT at codon 211 (Ho and MacKenzie, 1999), the TGAT insertion at codon 244 (Ho and MacKenzie, 1999), and Δ750-751GA (Lubrano-Berthelier et al., 2004), S58C (Lubrano-Berthelier et al., 2003b; Tao and Segaloff, 2003), N62S (Tao and Segaloff, 2003; Yeo et al., 2003), P78L (Lubrano-Berthelier et al., 2003b; Nijenhuis et al., 2003; Tao and Segaloff, 2003), N97D (Yeo et al., 2003), G98R (Tao and Segaloff, 2003), I102S (Lubrano-Berthelier et al., 2003b), L106P (Yeo et al., 2003), I125K (Yeo et al., 2003), R165Q (Nijenhuis et al., 2003), R165W (Lubrano-Berthelier et al., 2003b; Nijenhuis et al., 2003), N240S (Tao and Segaloff, in press), L250Q (Lubrano-Berthelier et al., 2003b), Y287X (Yeo et al., 2003), C271R (Tarnow et al., 2003), C271Y (Tao and Segaloff, 2003; Yeo et al., 2003), P299H (Lubrano-Berthelier et al., 2003b), I316S (Yeo et al., 2003), I317T (Lubrano-Berthelier et al., 2003b; VanLeeuwen et al., 2003).
- Class III: Binding defective mutants. These mutant MC4Rs are expressed on the cell surface, but are defective in ligand binding per se, with either decreased binding capacity and/or affinity, resulting in impairments in hormone-stimulated signaling. These mutants 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). We recently showed that I102S and I102T have partial defect in NDP-MSH binding (Tao and Segaloff, in press). Since AgRP is the natural antagonist of the MC4R, if the mutants are more sensitive to inhibition by AgRP, the mutants are functionally defective. It is of particular interest that 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 (AgRP) (Yeo et al., 2003). Therefore, this mutant can be classified as a subclass within this class. It is not known how prevalent this defect is; most of the functional studies, including our own, did not include AgRP in ligand binding studies.
- Class IV: Signaling defective mutants. These mutant MC4Rs are expressed on the cell surface, bind ligand with normal affinity, but are defective in agonist-stimulated signaling (decreased efficacy and/or potency). Mutants D90N (Biebermann et al., 2003), I137T (Gu et al., 1999), A175T and V253I (Yeo et al., 2003) may belong to this class, although in one report, V253I was found to have a relatively normal maximal response, albeit the $EC_{50}$ was increased three-fold (Lubrano-Berthelier et al., 2003b). In fact, this variant was found recently in one normal weight control subject (Marti et al., 2003), suggesting that this variant might have relatively normal functions.
- Class V: Variants with apparently normal function. These variants behave similarly as the wild-type MC4R in heterologous expression systems in the parameters studied. Some of the variants, such as T11A, D37V, P48S, V50M, F51L, A154D, I170V, M200V, N274S, and S295P, exhibit normal cell surface expression, ligand binding, and agonist-stimulated cAMP (Tao and Segaloff, 2003, in press). Whether and how these variants cause energy imbalance and therefore obesity is unclear. The difference between these variants and common polymorphisms such as V103I and I251L is that these variants are not assumed to be present in normal subjects. Regarding T112M, whereas it was originally identified in obese subjects (Farooqi et al., 2000; Hinney et al., 1999) and recent studies suggest that it causes a loss-of-function phenotype (Nijenhuis et al., 2003), earlier functional studies showed that it had normal functions (Farooqi et al., 2000; Gu et al., 1999) and it has since been reported in one control subject (Jacobson et al., 2002). Our results suggested that indeed T112M has normal function (Tao and Segaloff, in press).

Recently it was suggested that some of the mutant MC4Rs may have decreased constitutive activity and that this may be the cause of the obesity for some of the mutants (Srinivasan et al., 2004). If confirmed, this would open a new avenue of therapeutic treatment. These mutants would comprise a new class.

There are spare receptors in the transient expression systems that are currently used. This is reflected by the fact that the $EC_{50}$ (the concentration of hormone that is needed to result in 50% maximal response) is smaller than the $IC_{50}$ (the concentration of hormone that is needed to result in 50% inhibition in binding). In our experiments, the $EC_{50}$ is about 0.2 nM, whereas the $IC_{50}$ is about 5 nM for wild-type MC4R. Some mutants such as S58C and I102T, with only 15–20% of wild-type binding capacity, have similar maximal response as wild-type MC4R (Tao and Segaloff, 2003, in press). Mutants such as N62S, I102S, and C271Y, although exhibiting minimal binding (5% of wild-type MC4R), can elicit more than 20% of wild-type maximal response (Tao and Segaloff, 2003, in press). Concomitant with their decreased expression, the mutants have increased $EC_{50}$ values, consistent with the spare receptor model first proposed by Strickland and Loeb (Strickland and Loeb, 1981). Whether there are spare receptors in the arcuate nucleus remains to be studied. Mutations of the MC4R cause obesity by haploinsufficiency would argue against the presence of spare receptors.

The fact that there are spare receptors in heterologous expression system is important in functional studies of the mutant receptors identified from obese patients. Some mutants that are defective in cell surface expression and have decreased binding capacity may have normal maximal response. If only the signaling is measured, these mutants will be erroneously considered as having normal functions. To really reach a conclusion on a mutant's defect, cell sur-

face expression, ligand binding, and signaling all need to be assessed.

In summary, it is important to analyze the functional properties when new variant MC4Rs are identified in obese subjects in order to more accurately determine if the phenotype of the variant is indeed consistent with the clinical phenotype. A classification system such as one summarized here will be beneficial in cataloging the increasingly large array of MC4R mutations associated with severe childhood obesity. It is unfortunate that some of the recent reports did not study the cell surface expression and/or binding of the mutant receptors. Therefore from the data provided it is not possible to classify these mutants. If ever a personalized treatment is to be achieved, it is imperative to identify the exact defects (see below).

Since the level of MC4R gene expression is important for regulating food intake (haploinsufficiency causes obesity), several studies tried to identify mutations or variants in the regulatory sequences, especially promoter region, in obese subjects. Although several variants were identified, none of them segregates with obesity phenotype (Lubrano-Berthelier et al., 2003a; Ma et al., 2004) therefore their relevance to the pathogenesis of obesity is unknown at present. A polymorphism in the promoter of the MC4R gene is related to physical activity phenotype, potentially related to obesity (Loos et al., 2005). Defect in MC4R gene transcription is likely not a major cause of severe early onset obesity.

### 3.3. Do mutant MC4Rs cause obesity by dominant negative activity or haploinsufficiency?

Since most of the patients harboring MC4R mutations are heterozygous, an important question is whether obesity results from haploinsufficiency or dominant negative activity exerted by the mutant receptor. Co-transfection studies showed that most of the mutants do not have dominant negative activity (Farooqi et al., 2000; Ho and MacKenzie, 1999; Yeo et al., 2003). The only mutation that has been shown to have dominant negative activity is the D90N mutation in which the most conserved Asp in TM2 was mutated to Asn (Biebermann et al., 2003).

Biebermann and colleagues, using fluorescence resonance energy transfer, demonstrated that D90N MC4R heterodimerize with wild-type MC4R and suggested that this is why D90N MC4R has dominant negative activity (Biebermann et al., 2003). However, this cannot explain why the other MC4R mutants do not exert dominant negative activity, especially since all the co-transfection studies were done in transient transfections, and the majority of the mutants are intracellularly retained. Extensive studies in other GPCRs have shown that in transient transfections, intracellularly retained mutant receptors decrease cell surface expression of co-transfected wild-type receptors (see Benkirane et al., 1997; Colley et al., 1995; Overton and Blumer, 2000; Tao et al., 2004 for examples). Dimerization of GPCRs is thought to occur in almost all GPCRs (Bulenger et al., 2005), including MCRs (Mandrika et al., 2005) and an important role for homo- and hetero-dimerization in GPCR biosynthesis and maturation has been recently reviewed (Bulenger et al., 2005). It is difficult to imagine that D90N MC4R is the only mutant MC4R (that has been studied for dominant negative activity) that can dimerize with wild-type MC4R. Certainly this is an area that needs clarification.

### 3.4. Therapeutic implications

Since several of the loss-of-function MC4R mutants have residual activity in terms of hormone-stimulated cAMP generation, approaches that result in increased expression of the mutant receptor on the cell surface could potentially be of therapeutic value. Studies in other protein trafficking diseases, such as cystic fibrosis, have identified some methods of increasing cell surface expression in vitro. For example, decreasing the temperature of the cultured cells (Denning et al., 1992) or treating the cells with chemical chaperones such as glycerol and DMSO (Brown et al., 1996; Sato et al., 1996) increases the cell surface expression of the most common mutation ($\Delta$F508) in the cystic fibrosis transmembrane conductance regulator gene that cause cystic fibrosis (see Gelman and Kopito, 2002 for a review). As mentioned before, some of the mutations in V2 vasopressin receptor that cause nephrogenic diabetes insipidus also result in the mutant receptor trapped intracellularly (Morello and Bichet, 2001). Bouvier and colleagues identified small molecules of vasopressin analogues that can cross the cell membrane and act as pharmacological chaperones, increasing the cell surface expression of the mutant V2 vasopressin receptors (Morello et al., 2000). Similar results were achieved recently in δ-opioid receptor (Petaja-Repo et al., 2002), gonadotropin-releasing hormone receptor (Janovick et al., 2002) and the prototypical GPCR, rhodopsin (Noorwez et al., 2003).

It has been reported that the C-terminal peptide of AgRP can increase the cell surface expression of MC4R (Shinyama et al., 2003). It is possible that of the numerous agonists and antagonists developed for MC4R, some might act as pharmacological chaperones, which can potentially be of therapeutic value. The fact that some mutants, such as N62S, I102S, Y157S and C271Y, can respond to NDP-MSH stimulation with increased cAMP production in spite of minimal binding capacity (less than 5% of weight) (Tao and Segaloff, 2003, in press) suggests that these mutants are competent in G protein coupling and effector activation. Increasing their expression could be beneficial for treating obesity in the subjects carrying these mutant MC4R genes.

Although pharmacological chaperones may be used to correct transport defective mutants, they are not of therapeutic value for the mutants that are transported to the plasma membrane but defective in ligand binding or G protein coupling/activation. It remains to be seen whether analogs that can bind and activate the mutant MC4Rs defective in binding the natural ligands can be identified. For the mutants that are defective in G protein coupling/activation, perhaps the most

promising option would be the introduction of a normal gene through gene therapy (Schoneberg et al., 1997).

### 3.5. Lessons learned from the naturally occurring mutations on the structure and function of the MC4R

Functional analyses of naturally occurring mutations in GPCRs not only help us understand the etiologies of the associated diseases, these natural mutagenesis experiments frequently provide important insights into the structure–function relationship of the GPCRs. Constitutively activating mutations can help us understand the mechanism of activation. Based on the role of MC4R in food intake, it is expected that constitutively activating mutations might be involved in the pathogenesis of anorexia nervosa. However, so far, no constitutively activating mutations have been identified from these patients. The only naturally occurring constitutively activating mutation reported thus far, L250Q, paradoxically, was identified from an obese patient (Vaisse et al., 2000). Recent studies suggested that L250Q might result in obesity due to decreased cell surface expression (Lubrano-Berthelier et al., 2003b; Nijenhuis et al., 2003).

MC4R mutations cause obesity due to loss-of-function. Since most of the mutations associated with obesity are intracellularly retained, they cannot provide insights into the roles of the mutated residues in ligand binding and signaling. Any mutation that result in misfolding will result in the receptor being recognized by the cell's quality control system and retained inside the cell, most likely in the endoplasmic reticulum. Therefore, it is not appropriate to conclude from these results that the mutated residue constitutes an important trafficking motif (the di-leucine motif should be considered an exception; VanLeeuwen et al., 2003). Consistent with this is the fact that mutations that are retained intracellularly may occur in any part of the molecule, without any apparent pattern.

The few mutations that do result in a clear-cut defect in either ligand binding or signaling are very interesting. The binding defective mutants N97D, L106P, I125K, I137T and the inframe deletion mutant Δ88-92 cluster around TM2, extracellular loop 1 and TM3, suggesting that this domain is important for ligand binding, consistent with previous mutagenesis experiments (Haskell-Luevano et al., 2001; Yang et al., 2000). The reason for the binding defect of I316S (a mutation located in the C terminus) is not apparent at present.

Of the signaling defective mutants, decreased signaling of I137T (Gu et al., 1999) may be explained by decreased binding affinity. Further studies on A175, a codon at the middle of TM4 that is not conserved in MCRs, are needed to gain a better understanding of A175T phenotype. In most of the GPCRs, TM4 is not critical for signaling.

D90N is clearly defective in signaling (Biebermann et al., 2003). This mutation is very interesting in that the most highly conserved Asp in TM2 is mutated. Extensive mutagenesis studies in numerous GPCRs demonstrated that mutations of this conserved Asp frequently result in a defect in receptor-G protein coupling (Ceresa and Limbird, 1994; Roche et al., 1999; Tao and Abood, 1998).

It is interesting to note that in certain strains of pigs, the highly conserved Asp in MCRs (in the N/DPxxY motif) in TM7 is mutated to Asn (Kim et al., 2000). Functional analyses suggested that pig D298N MC4R binds to NDP-MSH normally, but is devoid of NDP-MSH-stimulated cAMP production (Kim et al., 2004). This is surprising, because MCRs are some of the few Family A (rhodopsin-like) GPCRs that have DPxxY sequence rather than NPxxY sequence. Since the majority of Family A GPCRs has Asn at this position, an Asn in the MC4R should be able to confer normal signaling. Therefore these results would suggest that at least in pig MC4R, this Asp has unique function in signaling. Studies in other GPCRs have shown that the conserved Asn is critical for stabilizing both inactive and active conformations of GPCRs (Claeysen et al., 2002; Whistler et al., 2002).

## 4. Naturally occurring mutation in the MC3R gene and human adiposity

### 4.1. MC3R in rodent energy homeostasis

After the cloning of the MC1R and MC2R, MC3R was cloned independently from human and rat by Gantz and Cone, respectively, by PCR and low-stringency hybridization techniques (Gantz et al., 1993a; Roselli-Rehfuss et al., 1993). By fluorescence in situ hybridization, the MC3R gene was localized at 20q13.2 (Gantz et al., 1993b; Magenis et al., 1994). In situ hybridization studies showed that the MC3R gene is expressed in several brain regions including the hypothalamus, cortex, thalamus, and hippocampus (Gantz et al., 1993a; Roselli-Rehfuss et al., 1993). MC3R is also expressed in the placenta, heart, and the gut (Gantz et al., 1993a) as well as in immune cells such as macrophages (Getting et al., 2003, 2004). Consistent with their expression in these tissues, MC3R is involved in direct regulation of the cardiovascular system, including the heart and blood pressure (Humphreys, 2004; Ni et al., 2003; Versteeg et al., 1998). Elegant studies from the groups of Humphreys and Cone using genetically modified mice provided convincing evidence that γ-MSH, acting through the MC3R, is involved in the coordinated blood pressure response to low sodium diet (Ni et al., 2003). Melanocortins, through activation of MC3R, have also been found to decrease or prevent myocardial reperfusion injury (Mioni et al., 2003). Together with MC1R, MC3R is also involved in controlling the inflammation process. By inhibiting the nuclear transcription factor NF-κB activation, melanocortins thus have anti-inflammatory effects (Manna and Aggarwal, 1998). MC3R is becoming a promising target for treating inflammatory diseases (Catania et al., 2004).

Mouse genetic studies demonstrated non-redundant roles for MC3R and MC4R in regulating energy homeostasis. Whereas the MC4R primarily regulates food intake, MC3R affects feed efficiency without significant effect on food

intake; increased feed efficiency leads to increased fat mass (Abbott et al., 2000; Butler et al., 2000; Chen et al., 2000; Huszar et al., 1997; Marsh et al., 1999). Double knockout mice lacking both MC3R and MC4R had exacerbated obesity compared with MC3R or MC4R single gene knockout mice (Chen et al., 2000). In addition, MC3R expression is not modulated by food restriction or diet-induced obesity (Harrold et al., 1999).

### 4.2. MC3R in human energy homeostasis

Although it is well established that naturally occurring mutations in the MC4R gene is the most common monogenic form of obesity (see above), the relevance of MC3R mutation in human obesity was not clear. Human genetic studies suggested that loci encompassing the MC3R gene on chromosome 20q.13 are associated with obesity and type 2 diabetes (Bowden et al., 1997; Ghosh et al., 1999; Ji et al., 1997; Lembertas et al., 1997; Zouali et al., 1997). However, several large-scale screening studies failed to identify any mutations in the MC3R gene from patients with morbid obesity and type 2 diabetes except for two variants, K6T and I81V, which were also found in normal control subjects at similar frequencies, and therefore likely represent polymorphisms (Boucher et al., 2002; Hani et al., 2001; Li et al., 2000; Schalin-Jantti et al., 2003; Wong et al., 2002). In 2002, a Singaporean group reported two patients (father and daughter) who each harbor a potential mutation (I183N) in the MC3R gene (Lee et al., 2002). However, no functional data were provided to verify whether this variant was indeed a pathogenic mutation. To test their functional properties, we generated these MC3R variants by site-directed mutagenesis and found that indeed K6T and I81V MC3Rs have normal functions. However, the mutant receptor, I183N, completely lacks signaling in response to NDP-MSH (up to $10^{-6}$ M) stimulation, even though it is expressed at the cell surface and binds NDP-MSH with normal affinity. Since the patients harboring I183N mutation were heterozygous, we asked whether the mutant allele exerts dominant negative activity on wild-type allele. Cells were co-transfected with wild-type and mutant MC3Rs and used for measuring binding and signaling. The results showed that I183N does not exert dominant negative activity on the wild-type allele, suggesting that if I183N is causing obesity in the patients, it does that by haploinsufficiency rather than dominant negative activity (Tao and Segaloff, 2004). Similarly, in MC4R, inactivating mutations usually do not have dominant negative activity, although an exception was recently reported (Biebermann et al., 2003).

It should be mentioned that Penhoat and coworkers also reported the functional analyses of I183N MC3R (Rached et al., 2004). Although they also observed a total loss of signaling in response to NDP-MSH stimulation, using GFP-tagged I183N MC3R, they found that the mutant receptor is trapped inside the cell (Rached et al., 2004). We did not perform localization studies since we observed normal binding to intact cells. The radio-labeled ligand cannot cross the cell membrane therefore by definition the mutant receptor has to be expressed on the cell surface. We generated multiple mutations including changing the hydrophobic Ile to charged residues such as Asp and Arg. All the mutants can bind to NDP-MSH, suggesting that they are all expressed on the cell surface (Tao and Segaloff, 2004). In addition, we mutated the corresponding Ile in MC4R and human lutropin receptor to Asn. These mutant receptors are also expressed on the cell surface as demonstrated by the intact binding to their corresponding ligands (Tao and Segaloff, 2004). One reason for the discrepancy between our results and those of Penhoat is that they used GFP-tagged receptor, whereas we used a non-tagged receptor. It is possible that the GFP tag affected the trafficking of the receptor (Tarasova et al., 1997). From their confocal images, it is obvious that wild-type MC3R is also mainly retained inside the cell. Whether this is a unique property of the MC3R (i.e. the maturation rate of wild-type MC3R is low) or it is due to the GFP tag remains to be investigated. They found that the I183N GFP-MC3R has dominant negative activity; this may again be related to the fact that the tagged mutant receptor is retained intracellularly. As mentioned above, mutant receptors retained intracellularly frequently results in decreased cell surface expression of wild-type receptor.

These pharmacological studies, together with the original clinical report, suggested that inactivating mutation in the MC3R gene might be another genetic factor that predispose to increased adiposity. Although more studies are needed to firmly establish the role of MC3R mutations in human adiposity, three novel mutations have been identified from obese subjects in Italy, with co-segregation within the family (Mencarelli et al., 2004). Detailed report is eagerly awaited. Furthermore, it has been suggested that the common polymorphism may be a genetic factor in the pathogenesis of obesity in African Americans, suggesting an interaction of the polymorphism and race (Feng et al., 2004). Large-scale studies are needed to confirm this correlation. Based on these studies, it is tantalizing to hypothesize that the MC3R might also be important for normal body weight regulation in humans. Previously, Cummings and Schwartz, based on mouse genetic studies, speculated that combined treatments with specific agonists for the MC3R and MC4R might result in greater weight loss by decreasing food intake through activating MC4R and decreasing fat storage by activating MC3R (Cummings and Schwartz, 2000). The studies on human MC3R mutation are supportive of this hypothesis.

### 4.3. Insight from functional analysis of I183N MC3R into structure–function of GPCRs

From a mechanistic point of view, the only inactivating MC3R mutation characterized so far, I183N, belong to a class IV mutation according to the classification scheme outlined above for the MC4R. I183N MC3R is expressed on the cell surface, binds to ligand, but is unable to transform the ligand binding into receptor activation as measured by intracellular

cAMP accumulation. In the MC4R, of the numerous naturally occurring mutants that have been characterized in detail, few are defective in signaling per se (see above). Similarly, in mutations in other GPCRs that are associated with human diseases, defects due to ligand-bound receptor and G protein coupling/activation were observed, for example, in rhodopsin (Min et al., 1993), V2 vasopressin receptor (Rosenthal et al., 1993; Sadeghi et al., 1997), endothelin receptor subtype B (Puffenberger et al., 1994; Tanaka et al., 1998), thromboxane $A_2$ receptor (Hirata et al., 1994), and MC1R (Frandberg et al., 1998; Schioth et al., 1999). But these mutations are rare compared with mutations that result in intracellular retention (Schoneberg et al., 2002).

Upon inspection of the MC3R sequence, we found that this Ile belongs to the highly conserved DRYxxI/V motif. Further studies revealed a discrete requirement for ligand-induced receptor activation. When this Ile in the MC3R was mutated to Ala, Leu, Val, Asp, and Arg, we found that I183D and I183R completely lacks signaling, I183A retains minimal signaling, I183L retains partial signaling, and I183V signals normally (Tao and Segaloff, 2004). We further showed that the corresponding Ile is also important for ligand-induced receptor activation in the MC4R and human lutropin receptor (Tao and Segaloff, 2004). An exhaustive random saturation mutagenesis study did not identify this conserved residue as important for G protein coupling in V2 vasopressin receptor, another Gs-coupled receptor (Erlenbach et al., 2001). It will be interesting to see whether the corresponding Ile (I141) is indeed important for Gs coupling in V2 vasopressin receptor by site-directed mutagenesis.

We speculated that this hydrophobic Ile/Val might interact with the hydrophobic residues at the extreme C termini of Gα subunits (Tao and Segaloff, 2004). The loss-of-signaling mutants described above can be used as powerful tools in these studies.

## 5. Conclusions

It is amazing that during the past few years, more than 70 mutations in the MC4R have been identified from obese as well as normal weight subjects. About 20% of the residues of the MC4R have been found to be mutated naturally at present and new mutations continue to be identified. Detailed functional studies are necessary to bridge the clinical studies identifying an association of the mutation with obesity or other phenotype (such as binge eating disorder) to a causative relationship. In addition, it is imperative to identify the exact defect if we are ever going to use the results of these studies for personalized treatment for mutation-harboring obese patients. Without detailed functional studies, no insight can be gained on the structure–function relationship of the receptor. Unfortunately some of the very recent reports on novel MC4R mutations either did not perform functional studies at all or did not identify the exact defect. It is hoped that more complete reports will be published in the future when new MC4R mutations are reported.

Perhaps more exciting are studies directed at addressing the question of the relevance of the MC3R in human adiposity. Although the exact mechanism of the increased feed efficiency in the MC3R knockout mice is not clear, if the MC3R has the same function in humans, i.e. decreases fat deposit when activated, it is expected that loss-of-function mutations in the MC3R will lead to increased fat mass. Although several earlier screening studies failed to identify any mutation in the MC3R, four mutations have been reported recently. We anticipate that important insights will be gained from endeavors directed at identifying and characterizing novel MC3R mutations from patients with high fat contents.

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