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Review

Obesity-associated mutations in the melanocortin 4 receptor provide novel insights into its function

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Abstract

Mutations in the Melanocortin 4 receptor are implicated in 1–6% of early onset or severe adult obesity cases. Most of the patients carry heterozygous missense mutations. Arguments for the pathogenicity of these mutations are based on the frequency of rare functionally relevant non-synonymous mutations in severely obese children and adults versus non-obese controls, the segregation of mutations with obesity in the family of the probands (although with incomplete penetrance) and the relevant functional defects described for these mutations. We have developed new assays to study the functional characteristics of these obesity-associated MC4R mutations. Systematic and comparative functional study of over 50 different obesity-associated mutations suggests that multiple functional alterations contribute to their pathogenicity. These studies also lead to new insights into the structure–function relationship of MC4R, provide novel hypotheses for the genetic predisposition to common obesity in humans and allow the development of new molecular tools for studying the physiological role of GPCRs.

Keywords: Obesity; Genetics; Melanocortin receptors

Contents

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1. Introduction

1.1. Mutagenesis and structure function studies of GPCRs

G protein-coupled receptors (GPCRs) are all composed of seven transmembrane α-helices (TM) and transmit a remarkably wide spectrum of extracellular signals by activating hetero-trimeric G proteins. The major pharmacological role of GPCRs has motivated numerous structure–function studies aiming at deciphering the molecular mechanisms of receptor function. Although the crystal structure of the inactive form of rhodopsin has been known since 2000 [29], in the absence of structural details of the active state and of the GPCR ↔ G protein complex, a thorough understanding of the activation process remains elusive. Therefore, structure–function investigations have relied on surrogate approaches, mainly site-directed mutagenesis combined with either biological assays or biophysical measurements. Either exhaustive (e.g. alanine scanning) or hypothesis-driven (e.g. guided by modeling), these mutagenesis studies are complicated by the overwhelming combinatorics involved, as each position in the receptor can be changed into the 19 other amino acids, and each of these mutations can be combined with one or several other changes, many of which may not be informative.

In contrast, clinical studies have identified a number of natural mutations in several families of GPCRs. Linked to a specific phenotype, these mutations have, in most cases, an effect on the function of the receptor when tested in vitro. Natural mutations therefore offer a set of amino acid substitutions with both functional and clinical relevance.

1.2. The melanocortin 4 receptor

The MC4 receptor is a rhodopsin-like GPCR (i.e. a member of family A [13], or "rhodopsin" family [11]) coupled to hetero-trimeric Gs protein and tranduces signal by activating adenylyl cyclase. The expression of MC4R is restricted to the nervous system and is found in hypothalamic nuclei involved in the regulation of energy balance. MC4R regulates food intake by integrating an agonist (satiety) signal provided by alpha-melanocyte stimulating hormone (α-MSH) and an antagonist (orexigenic) signal provided by the agouti-related protein (AGRP). In addition, a number of studies suggest that MC4R exhibits a constitutive, food intake inhibiting activity on which AGRP acts as an inverse agonist [17,27].

1.3. MC4R mutations are associated with human obesity

The human MC4R is a 332-amino acid protein encoded by a single exon gene localized on chromosome 18q22 [12,35]. Systematic screening of the MC4R gene in cohorts of patients demonstrates that heterozygous mutations in MC4R account for 1–6% of severe cases of human obesity. Over 50 different obesity-associated mutations have been described in adults with morbid obesity or children with early onset obesity, most of which are heterozygous missense mutations [8,10,19,21–25,33,38–40]. Arguments for the pathogenicity of these mutations are based on their absence in non-obese control populations, segregation with obesity in the family of the probands (although with incomplete penetrance) and the relevant functional defects described for these mutations.

2. Multiple functional alterations caused by obesity-associated MC4R mutations: implications for a functional classification

One of the arguments for the implication of rare mutations in a common disease is the demonstration of a pathogenic effect of these mutations. Over 50 obesity-associated MC4R mutations have been described, most of which are missense mutations. Three major functional defects have been found to be caused by these mutations

2.1. Alteration of agonist activation of mutated MC4Rs

The function most commonly assayed in MC4R obesity-associated mutants is their response to melanocortin agonists. Since MC4R is a Gs-coupled GPCR, activation of the receptor is assayed in transfected heterologous cell lines by measuring cAMP production in response to the agonist, either directly or indirectly, using a cAMP-inducible reporter gene such as firefly luciferase (Fig. 1). These assays use pharmacological agonists such as NDP-α-MSH or more physiological agonists such as α- or β-MSH. In these assays, most obesity-associated MC4R mutants have an impaired response to melanocortins ranging form a limited increase in EC_{50} to a complete lack of response (Fig. 1). While this finding of a decreased agonist response provides

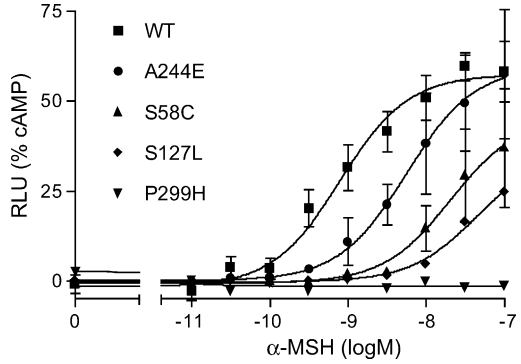

Fig. 1. Obesity-associated MC4R mutants impair melanocortin activation of the receptor. Activity of the receptors is here assayed by analyzing their ability to activate the expression of a cAMP-inducible luciferase reporter gene. Cells stably expressing the luciferase reporter and transiently expressing the wild-type or mutant MC4R were stimulated for 6 h with increasing amount of α-MSH after which luciferase was measured. Data point represent means of three determination divided by maximal level of luciferase activity achieved by 1mM of 8-Br-cAMP. Error bars indicate SD.

an argument for the pathogenic effect of these obesity-associated mutated MC4Rs, they provide only little mechanistic information. Indeed, decrease of agonist response in these assays could result from either reduced membrane expression of the receptor, a decreased affinity for the ligand, a decrease in signal transduction or a combination of these defects.

2.2. Many obesity-associated MC4R mutations have an impaired membrane expression

Intracellular retention of mutated proteins results in an impaired receptor response to agonists and is a common disease-causing defect [22]. Different assays can be used to test for membrane expression of a mutated receptor including binding assays and immunohistochemistry in transfected cells. As binding of a ligand is not only dependent on membrane expression of a receptor but also on its affinity for the receptor (which might be modified by mutations), the latter method has generally been preferred in the case of MC4R. However, as no reliable antibodies against MC4R are currently available, these immunohistochemistry experiments require the engineering of receptors carrying an N-terminal extracellular antigenic epitope. Using such constructs, lack of cell-surface expression can be easily demonstrated for all frame shift or non-sense mutations and for some missense obesity-associated mutant MC4Rs (Fig. 2).

When assessing more subtle quantitative changes in membrane expression this method is limited by its sensitivity and is not well adapted for high throughput studies. To address this, we developed a method based on immunostaining and fluorescence detection by flow cytometry of chimeric MC4R containing a C-terminal intracellular green fluorescence protein (GFP) and an N-terminal extracellular FLAG epitope-tag (Fig. 2). Using this method we have demonstrated that 80% of early onset obesity-associated MC4R mutations are partially or totally intracellularly retained [22].

2.3. Decreased constitutive activity is a novel defect caused by obesity-associated MC4R mutations

In vitro data show that MC4R exhibits a constitutive activity on which AGRP acts as an inverse agonist [17,27]. These data suggest that, in the absence of ligand, MC4R can exert a food intake inhibiting activity. We recently demonstrated that obesity-associated N-terminal mutant MC4Rs selectively impair the constitutive activity of the receptor but do not affect the membrane expression of the receptor, its response to α-MSH nor the antagonist effects of AGRP [34] (Fig. 3). Deletion and trans-rescue experiments demonstrate that the N-terminal domain of MC4R functions as a tethered intra-molecular ligand that maintains the constitutive activity of this receptor (Fig. 3). The constitutive activation by the N-terminal domain is independent of the full activation provided by α-MSH, since the N-terminal domain is not required for the α-MSH-mediated effects.

These results suggest that the tonic satiety signal provided by the constitutive activity of MC4R is required for maintaining long-term energy homeostasis in humans. Interestingly, by systematically assessing the basal activity of all the other obesity-associated MC4R variants (independently from membrane expression) we found that decreased constitutive activity was the most common defect observed for obesity-associated MC4R mutations (Lubrano-Berthelier et al. submitted).

2.4. Towards a functional classification of obesity-associated MC4R mutations

To determine the respective roles of the biochemical characteristics of the MC4R mutations in the onset and severity of the obesity in the patients, we systematically compared these alterations in a large group of well phenotyped severely obese adult MC4R mutation carriers (Lubrano-Berthelier et al. submitted). When considered independently, we found a positive relationship between the presence of any of the functional defects and both the onset and the severity of the obesity in the carriers.

This relationship was strongest and most significant for the intracellular retention, making this the best predictor of both the onset and the severity of the obesity in MC4R mutation carriers. Based on these findings we propose the following classification of MC4R mutations. Class 1 mutations are those that are largely intracellularly retained resulting in a major loss of MC4R signaling. Class 2 MC4R mutants are expressed at the cell membrane, but display either a decreased constitutive activity (Class 2B), a decreased response to the agonist (Class 2C), or both (class 2A). The pathogenicity of these mutations can be linked to the decreased anorexigenic activity of the receptor.

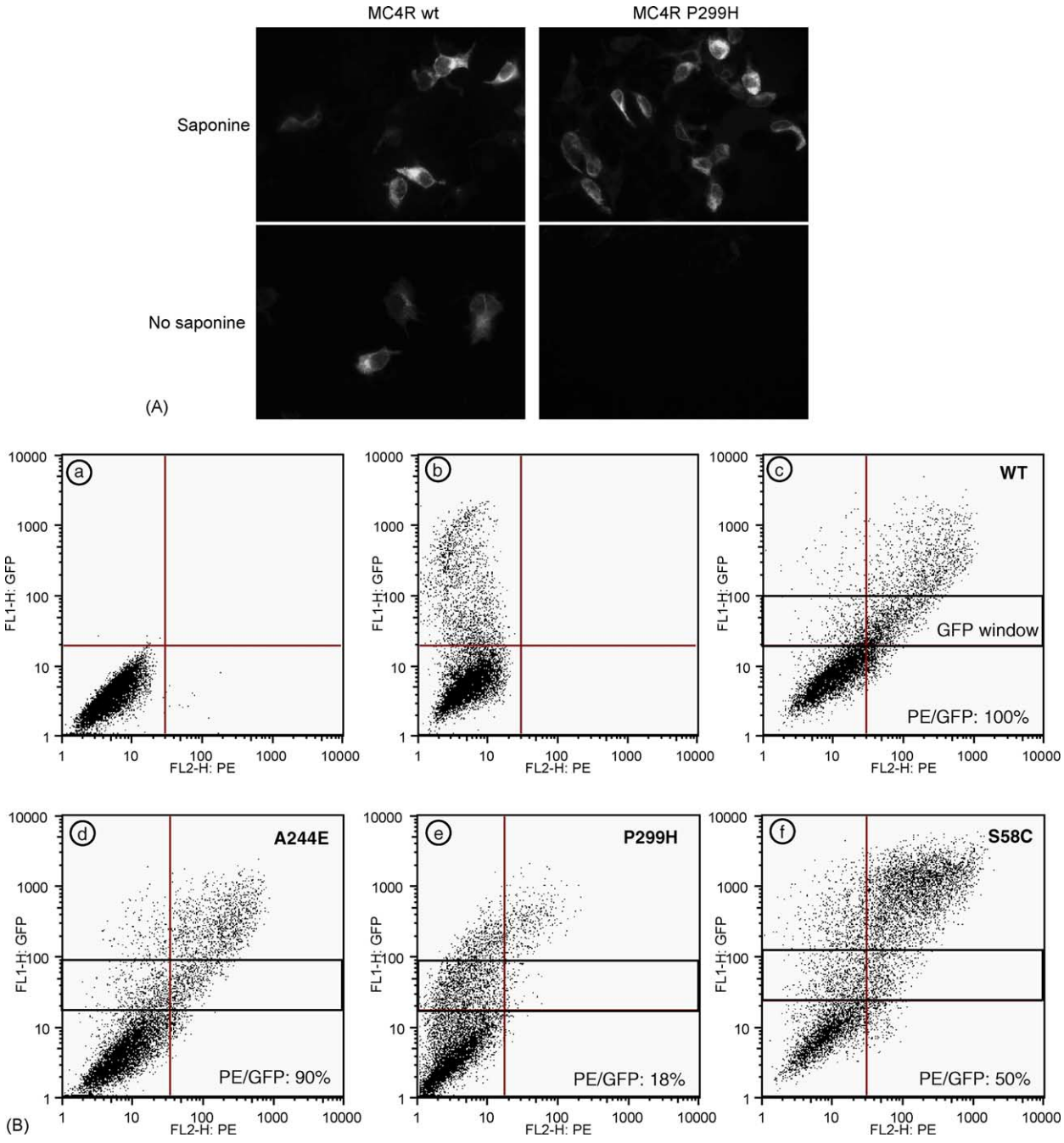

Fig. 2. Assaying membrane expression of obesity-associated mutant MC4Rs. (A) Qualitative assessment of the membrane expression of wild-type and Pro299His MC4-Rs. HEK 293 cells are transiently transfected with the MC4R wild-type or mutants on which was added a N-terminal flag epitope. After 24 h transfection, cells are splitted and grown on coverslips for another 24 h before immunofluorescence. Cells are rinsed, fixed in 3.7% paraformaldehyde (PFA), stained with a monoclonal antiflag antibody M2 and FITC-conjugated anti-mouse IgG in the presence or absence of a membrane permeabilizing agent (Saponine). Cells are mounted on slides and observed by fluorescent microscopy. In absence of membrane permeabilizing agent, only cell surface expressed receptors are stained. (B) Quantitative assessment of MC4R membrane expression by flow cytometry. HEK 293 cells are transiently transfected with a chimeric MC4R containing an extracellular N-terminal flag epitope and an intracellular C-terminal green fluorescent protein (GFP). After 24 h transfection HEK 293 cells are immunostained at 4 °C with an antiflag antibody and a PE-conjugated anti-mouse in the absence of detergent. GFP and PE emissions are analyzed in each individual cell by flow cytometry and results analyzed using the software CellQuest are shown as dot plot. (a) Untransfected HEK 293 cells; (b) HEK 293 cells transfected with WT chimeric MC4R and immunostained without antiflag antibody; (c) HEK 293 cells transfected with WT chimeric MC4R and immunostained with both antibodies. GFP emission represents MC4R total expression and PE emission represents cell surface MC4R expression. To limit artifacts caused by high receptor overexpression, we limit our analysis to cells expressing low levels of the receptor as defined by the GFP window shown. Representative dot plots are shown for three missense mutations; (d) A244E; (e) P299H; (f) S58C. The ratio PE/GFP, representing relative membrane expression, is calculated for each individual cell using the FlowJo software and is expressed as a percentage of the value obtained in the same experiment for the wild-type MC4R. This ratio is indicated on the right bottom corner of dot plot graphs.

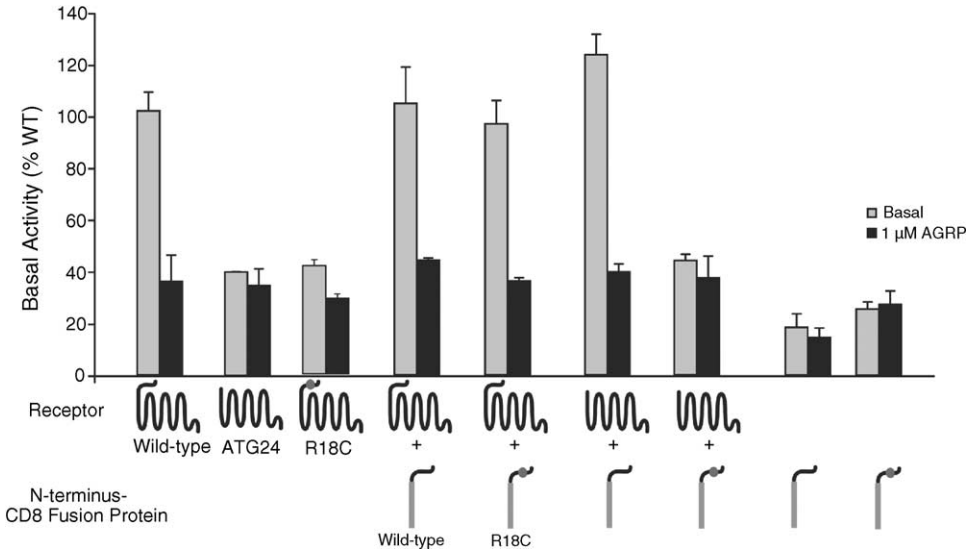

Fig. 3. Impaired basal activity of N-terminal MC4R mutant can be rescued by co-transfection of the normal N-terminus (REF). HEK293 cells were transfected with the plasmids expressing the constructs schematically represented below the bar graph: wild-type MC4R, ATG24MC4R (lacking the 24 first aa of the N-terminal domain), R18C mutant MC4R, wtMC4R + wild-type N-terminus-CD8 fusion protein, wtMC4R + R18C N-terminus-CD8 fusion protein, ATG24MC4R + wild-type N-terminus-CD8 fusion protein, ATG24MC4R + R18C N-terminus-CD8 fusion protein, CD8 fusion proteins alone. All constructs were under the control of the CMV promoter and in fusion with a signal peptide leading to equal expression at the cell membrane. cAMP was measured in the absence or presence of 10^{-6} M AGRP. The wtMC4R displays a basal activity on which AGRP acts as an inverse agonist. This basal activity is decreased by the R18C mutation and by removal of the N-terminal domain of the receptor. In this N-terminally deleted receptor, basal activity can be rescued by co-expression of the wt MC4R N-terminal domain expressed in fusion with the single transmembrane domain CD8 molecule but not by the mutated N-terminal domain.

3. Structural insights from the functional studies of MC4R missense mutations

The diversity of functional effects caused by natural mutations associated with disease provides a remarkable set of data to examine the structure–function relationship in GPCRs.

While, as for most GPCRs, the crystal structure of MC4R is not available, the sequence homology between the melanocortin receptors and bovine rhodopsin allows for construction of models providing a structural framework for the MC4r (Govaerts et al. in preparation). Using this molecular model, one can analyze the molecular environment of the mutated amino acids in order to rationalize the functional effect in a structural framework. It is mandatory to not just consider the amino acid change on its own but to place it in its structural local environment, also called structural or functional microdomain.

Most of the mutations tested appear to affect (impair) to some extent the ability of the receptor to activate Gs. However, the profile of the impairment varies considerably throughout the set of mutations, providing valuable information's on the molecular requirement for receptor activation. As events leading to intracellular retention can often occur during folding or transport to the membrane, mutants with impaired membrane expression (Class 1 mutants) provide little information concerning the molecular mechanism of activation and will not be considered here.

Since mutations examined here are all single point mutations, the structure of the mutant receptor should be highly similar to that of the wt (no significant structural changes). Note that overall structure conservation does not prevent modification of functional features, and dynamic properties in particular.

In this section we analyze several natural mutants in order to illustrate the structural analysis mentioned above. As detailed in Sections 2.3 and 4.1, we have demonstrated that the constitutive activity of the MC4r is caused by the N-terminal region that acts as a tethered agonist. Therefore, we use the term "agonism" for both α-MSH-induced and basal activities. We also use the generalized numbering scheme [1] to label residues in the TM domain (in superscript when placed next to the MC4r numbering) so as to allow for comparison with positions in other GPCRs and bovine rhodopsin in particular.

3.1. Mutations affecting all types of agonists (Class 2A)

A number of mutants appeared to be severely impaired for both types of agonism, indicating that the mutation has broad impact on the receptor function (all point mutants are illustrated on Fig. 4).

Some mutations, such as $T150^{3.53}I$ (located in TM3, see [1] for complete description of the generalized numbering), modify structural microdomains whose sequence and function is conserved throughout the superfamily of GPCRs. In class I of GPCRs, position 3.53 is located one turn below the highly conserved $D^{3.49}R^{3.50}Y^{3.51}$ motif (see Fig. 4). This motif is known to be crucial for receptor function,

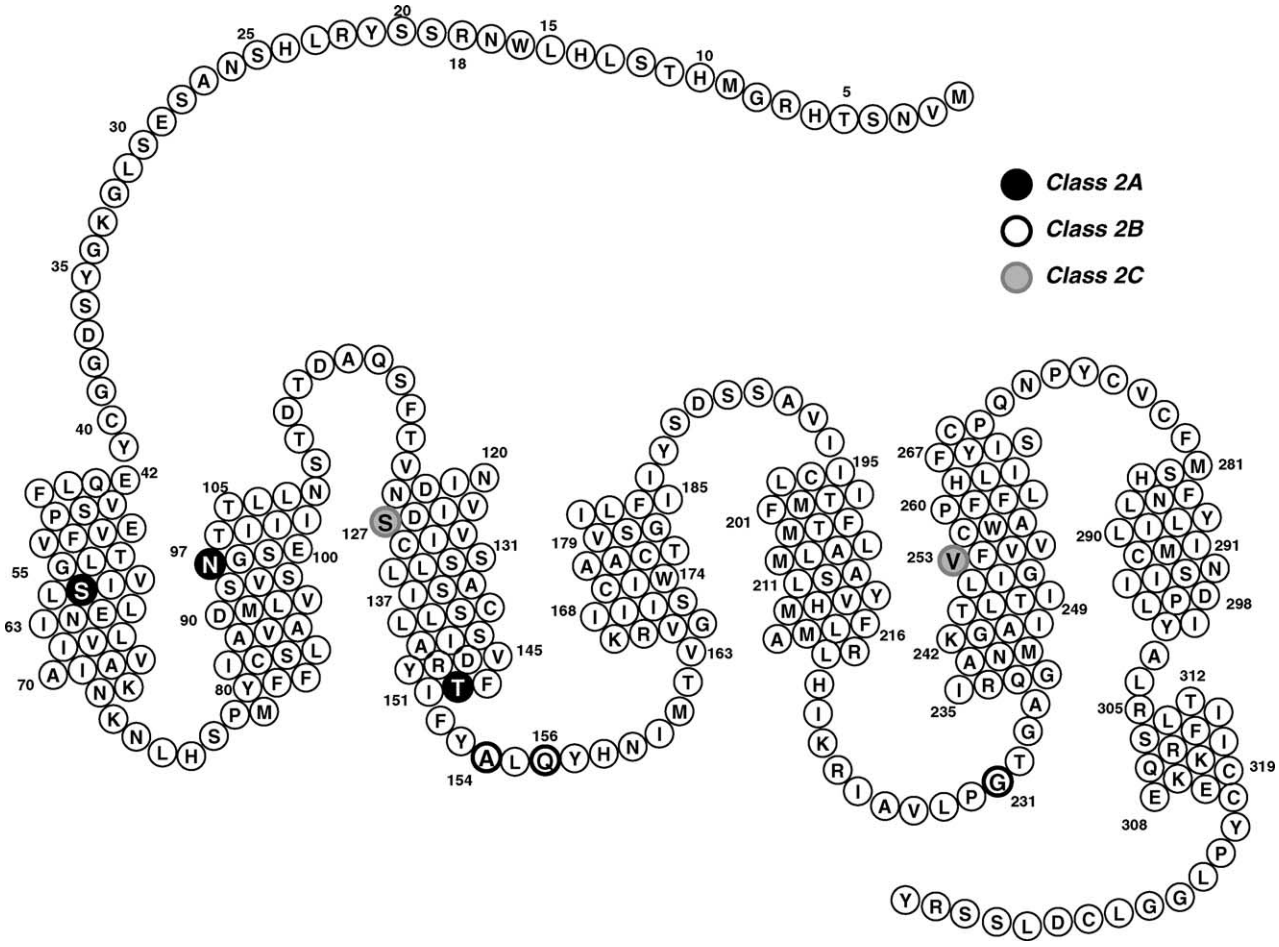

Fig. 4. Helical net representation of the human MC4 receptors. The MC4R numbering is shown. The mutations discussed in Section 3 are highlighted, with black background (Class 2A), black borders (Class 2B) and gray shading (Class 2C).

probably involved directly in coupling the G protein (see [14] for review). Studies on various GPCRs have shown that the residues surrounding the DRY motif are known to be important for regulation of this function as mutations can profoundly affect the ability of the receptor to transduce signal. Position 3.53 is no exception as mutation at this locus have been found to affect receptor signaling in other receptors, including the lutropin receptor [36] and the angiotensin II receptor [26]. Therefore, the T150$^{3.53}$I mutation in the MC4R is expected, as we observed, to affect broadly the ability of the receptor to transduce signal, independently of the agonism source. A similar example is found with the mutant S58$^{1.46}$C in TM1. As shown in Figs. 4 and 5A, this residue is located one helical turn above the conserved N62$^{1.50}$, the most conserved of all residues in Class A of GPCRs [20]. This asparagine is known to play a major role in activation and interacts with other highly conserved polar and charged side chains such as D$^{2.50}$ in TM2 and N$^{7.49}$ in TM7, whose importance in activation has been demonstrated [7,30,31]. Our molecular model shows that Ser58 is likely to participate in the network of interaction involving the conserved residues. While a cysteine could also participate in a hydrogen bond, the presence of a bulky sulfur atom and the relative weakness of such hydrogen bond underline that a Cys is not interchangeable for a Ser. It is therefore likely that the S58$^{1.46}$C change affects the ability of the 1.50–2.50–7.49 polar side chains to interact constructively upon activation and is expected to impair all types of activations.

Other mutants modify microdomains that are unique the receptor subfamily. The MC4R, like the other melanocortin receptors, is characterized by a high number of acidic side chains inside the TM bundle. They include E100$^{2.60}$, D122$^{3.25}$ and D126$^{3.29}$ which form a tight acidic cluster between TM2 and TM3 (see Fig. 5A). These residues are known to be involved in the ligand-induced activation, probably through direct interaction with the hormone [18,43]. Point mutations at these loci have also been shown to affect basal level, either increasing (E100A and D122A) or slightly decreasing (D126A) constitutive activity [34]. Introduction of an additional negative residue in the vicinity of this cluster is likely to destabilize the charge balance at this important site of the receptor. It is hence conceivable that the N97$^{2.57}$D natural mutation (located one helical turn below E100$^{2.60}$, see Fig. 5A) strongly perturbs the ionic equilibrium

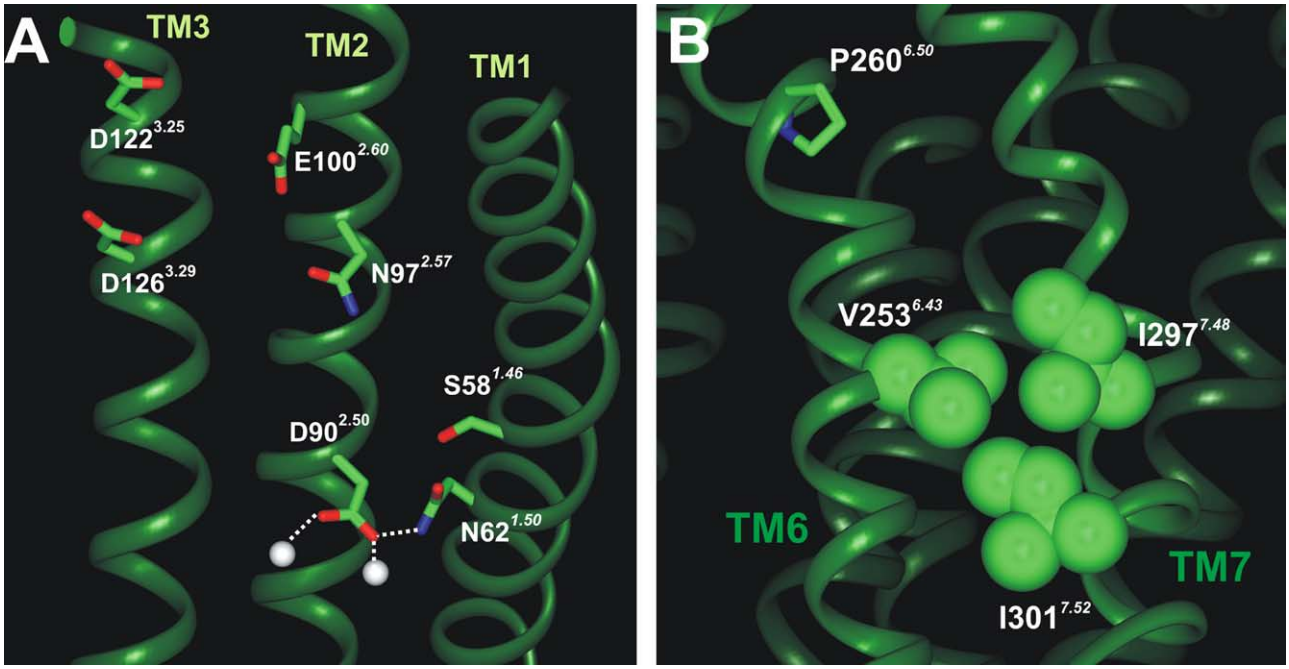

Fig. 5. Key residues of the MC4R in the structural model of the receptor. (A) Negative and polar residues at the TM1-TM2-TM3 locus: the model of the human MC4r was build similarly to that of the CCR5 receptor [15,16]. Briefly, the structure of bovine rhodopsin was used as a template for the transmembrane region. The side chains of the MC4r receptor were placed with the program SCWRL [9]. In bovine rhodopsin, TM2 is tilted due to an opsin-specific GG motif [2,15]. We have therefore remodeled TM2 of the MC4r using molecular dynamics simulation of an isolated helix in apolar environment as described in [15] which removes the rhodopsin bent and places the charged $E100^{2.60}$ inside the TM bundle (Govaerts et al., in preparation). The figure shows the three negative charges in the TM2–TM3 interface. It also shows the conserved $D^{2.50}$–$N^{1.50}$ polar interaction, as seen in rhodopsin (illustrated by dashed white lines), including two coordinated water molecules (in white). The view shows that $S58^{1.46}$ is able to interact directly with $N62^{1.50}$. (B) Hydrophobic interactions at the TM6-TM7 interface: the molecular model allows to identify specific residues involved in the interface. The side chains of residues $V253^{6.43}$, $I297^{7.48}$ and $I301^{7.52}$ are shown in space-filling representation to illustrate show how these hydrophobic residues can interact and modulate the TM6–TM7 interhelical interactions. The structural hinge of TM6 is created by the highly conserved $P^{2.50}$, also highlighted on the figure.

in the TM2–TM3 region and prevents proper activation of the receptor. Note that O'Rahilly and colleagues [44] have described a profound ligand-binding impairment for this mutant leading to the conclusion that the associated phenotype was due to binding defect (no basal activity was measured). Residue $N97^{2.57}$ has not been described as a direct hormone-binding site, in contrast to the residues in acidic cluster ($E100^{2.60}$, $D122^{3.25}$ and $D126^{3.29}$). We suggest here that the functional impairment of the $N97^{2.57}D$ mutation is due to a broad disruption of an important network of hydrogen bonds, conserved in the melanocortin receptors. In the MC4r, this leads on one hand to disorganization of the binding pocket and, on the other hand, of a severe perturbation of the interaction with the N-terminus, muting the constitutive activity. This would be consistent with our previous studies which suggest that acidic cluster is important for the constitutive activity of the receptor, although the precise mechanism remains unclear [34].

3.2. Mutations affecting primarily the basal activity (Class 2B)

We observed a number of natural mutations that affect the basal level of the receptor with little or no effect on α-MSH activity, three of which ($A154^{3.57}D$, $Q156^{3.59}P$ and $G231^{6.21}S$) are located in or close to the intracellular loops (ICL), as represented in Fig. 4. It is important to note that no sequence similarity is detected in the loop regions between bovine rhodopsin and most GPCRs, including the MC4r. As a consequence, the homology models cannot be extended to those regions. Due to the size and complex topology of both the extra and intracellular domains, ab initio modeling techniques cannot be applied model these regions either, preventing any real structural analysis for these domains. However, the mere location of the mutations is highly informative.

Located in the intracellular region, these residues cannot interact directly with the N-terminus (the tethered ligand responsible for the basal level, see Section 2.3). More specifically $A154^{3.57}$and $Q156^{3.59}$ are in ICL2 or at the border with TM3. This region is known to be involved in G proteins activation [41,42]. For exampled, work on the muscarinic receptors has shown that mutations in this region can either trigger activation or inhibit coupling [4]. It is therefore striking that the two MC4R mutants have strongly impaired constitutive activity but retain wt-like α-MSH stimulation ($A154^{3.57}D$ is slightly affected, see Table 1). One could speculate that different regions of the ICL2 could interact

Table 1
Differential effect of natural point mutations on the expression, basal activity, and α-MSH response of the MC4R

	TM	General Numbering	Expression (wt.%)	Basal (wt.%)	EC50 Fold of WT	Ref.
Class 2A						
S58C	1	1.46	50	11	18.5	[22]
N97D	2	2.57	76	3	No activity	Unpublished data
			"Yes"		No activity	[44]
					No activity	[10]
T150I	3	3.53	113	7	19.6	Unpublished data
					24.0	[39]
Class 2B						
A154D	3	3.57	119	7	3.4	Unpublished data
Q156P	3	3.59	66	12	1.7	Unpublished data
G231S	6	6.21	123	56	0.8	Unpublished data
Class 2C						
S127L	3	3.30	101	73	33.5	[22]
			Normal		Impaired	[40]
				∼200	50.0	[19]
V253I	6	6.43	90	101	4.3	[22]
					"Partially active"	[44]
					"Partially active"	[10]
			76		0.8	[28]

with distinct G proteins but in the MC4R both the constitutive and agonist-induced activities result in the activation of the same G protein, Gs. This is a first indication that the active state could be different upon activation by either the N-terminus (i.e. basal activity) or by the hormone (see Section 4).

3.3. Mutations affecting primarily the α-MSH-induced activity (Class 2C)

A third group of mutations, all located in the TM bundle, result in moderate or no defect in basal activation but very strong impairment in hormone stimulation. An example is the S127$^{3.30}$L mutant where the EC_{50} is shifted by over 30-fold, while the constitutive activity is almost not affected (see Table 1). A simple interpretation would be that S127 is a binding site for α-MSH. Position 3.30 is usually not described as a ligand-binding site in the melanocortin receptor but, admittedly, this could be due to the lack of experimental evidence. Residue S127$^{3.30}$ is predicted to point towards TM4, suggesting that S127$^{3.30}$L change could modify the interactions between the hormone and aromatic side chains in TM4, such as F184. On the other hand, a serine at position 3.30 could theoretically interact directly with the highly conserved D126$^{3.29}$ charge (located one position before) and modulate its implication in the network of polar interactions involving the acidic cluster (E100$^{2.60}$, D122$^{3.25}$ and D126$^{3.29}$) and surrounding polar groups (e.g. N97$^{2.57}$, N108$^{2.68}$ or N123$^{3.26}$). A Ser → Leu change could significantly modify the equilibrium of interactions leading to functional impairment. Considering that the mutant shows a wt-like basal activity, this would be at odds with mutagenesis data on D126$^{3.29}$ which suggest that this Asp is involved in both α-MSH and N-terminal-induced activations [34]. Additional binding data will be required to determine if S127 is involved in binding of the agonist or modulates the conformational change of the receptor.

The V253$^{6.43}$I mutant shows no decrease in basal activity but its hormone stimulation is significantly affected. Two groups have reported wt-like EC_{50}'s but only partially active [10,44], although Nijenhuis et al. [27] describe it as unaffected. In our hands, a 4-fold right-shift in EC_{50} of α-MSH activity is observed [22]. The structure of rhodopsin [29] shows that position 6.43 is located in the middle of the lipid bilayer, on the outside of the bundle (close to the lipids) and can therefore not be a ligand-binding residue (see Fig. 5B). It is a 100% conserved Val in MC4 receptors and highly conserved in other melanocortin receptors, although a number of MC1 receptors (including human, chimpanzee, mouse, dog and fox) have an Ile instead. It is therefore somewhat surprising that a large effect is observed upon such a mild chemical change that, in addition, has been evolved in other closely related sequence. Of interest, Val253$^{6.43}$ is predicted to point towards TM7, along the lipid core of the bilayer.

In our model it is only able to interact with I297$^{7.48}$ (not conserved in MC1 receptors) and I301$^{7.52}$ (conserved), as illustrated in Fig. 5B. As shown, these hydrophobic side chains are part of the interface between TM6 and TM7, and a mutation at any of these positions can potentially affect interhelical interactions. We therefore suggest that the V253$^{6.43}$I introduces a structural modification in this interface that impacts the mechanism of receptor activation (see below).

4. Summary: insights into MC4R and GPCR biology from obesity-associated MC4R mutations

The systematic functional study of obesity-associated MC4R mutations has confirmed their pathogenic role. In addition, it has led to novel findings regarding the importance of the N-terminal domain in MC4R function, the structure–function relationship in the MC4R and allowed the development of new tools to study GPCR signaling.

4.1. Lessons from obesity-associated N-terminal MC4R mutations

The finding that a number of pathogenic point mutations in the N-terminus of MC4R lead to a decreased basal activity with no effect on α-MSH activity [34] and that the N-terminal domain is a tethered agonist of the receptor has important implications. First, although increased constitutive GPCR signaling has been shown to be pathological [32], a physiological role for the basal activity levels observed in vitro for numerous GPCRs had never been demonstrated. By identifying an association between reduced constitutive GPCR signaling and disease, this provides the first evidence for a possible physiological role of constitutive GPCR signaling.

Second, while the role of N-terminal domain that acts as full tethered agonists after protease cleavage by thrombin or other serine/threonine proteases in receptor activation had been clearly established in the case of the PAR receptor family [37] this is the first example of a receptor whose N-terminal domain is required to maintain constitutive signaling. Finally, since the MC4R has already two endogenous agonist and antagonist ligands (α-MSH and AGRP) these findings further highlight the role of the MC4R as a molecular sensor matching energy intake to energy expenditure. As MC4R is a lead target for the treatment of both obesity and cachexia, demonstration of an essential role for the N-terminal domain acting as a ligand to maintain the constitutive activity of this receptor could lead to novel strategies for the design of drugs targeting MC4R.

4.2. Differential impairment reveal multiple receptor conformations

Although functional impairment is a negative observation, it becomes functionally interpretable when at least one of the agonism is preserved (mutants described in Sections 3.2 and 3.3) which demonstrates that the receptor is still functional.

A body of evidence [13] indicates that activation of GPCRs does no involve ‘one’ active state but rather ‘multiple’ active states (distributions of active states). We believe that some of the natural mutations described here reveal the existence of multiple active states in the MC4 receptor as well. Mutations such as A154$^{3.57}$, Q156$^{3.59}$ or V253$^{6.43}$I most certainly do not affect the ligand-binding site. Their differential effect over the two types of agonism can therefore not be described in terms of modified ligand affinity. Rather, they must be interpreted in structural terms. Specific changes may modify the ability of the receptor to reach some active states, either favoring or disfavoring them.

For example, the V253$^{6.43}$I mutation affects specifically the response to α-MSH. Based on our modeling, we hypothesize that the V253$^{6.43}$ is at the interface between TM6 and TM7. Activation of GPCRs is known to involve a significant conformational change in the cytoplasmic part of TM6 [13]. This change is predicted to occur below the flexible hinge provide by the highly conserved Pro$^{6.50}$, therefore including residue 6.43 (see Fig. 5B). The helix–helix interactions between TM6 and surrounding helices, including TM7, are likely to modulate the conformational space of TM6 and should therefore play an important role in the activation process. We propose here that a residue change at position 6.43, as chemically minute as a Val → Ile mutation, can affect this interface and modify the ability of the receptor to reach specific active states. As the basal level is not affected by the change, we suggest that constitutive activation involves active conformations of the receptor which tolerate an Ile at position 6.43. In contrast, activation by the hormone would be more sensitive to the change if its corresponding active states would favor/require a Val at position 6.43. Biophysical studies will be required to effectively measure structural or dynamical differences upon activation of the wt MC4R versus these natural mutant receptors.

4.3. Developing novel molecular tools using obesity-associated MC4R mutations

Receptors that are activated solely by synthetic ligands (RASSLs), are genetically engineered receptors that are designed to be insensitive to their natural, endogenous ligand(s) but can still be fully activated by synthetic small molecule drug. Such engineered receptors can be used, following in vivo tissue-specific gene targeting, to activate a G protein pathway of interest rapidly and reversibly, mimicking the speed, localization, regulation and amplification of endogenous GPCR signals. The resulting cellular, biochemical and physiological changes are therefore attributable to the specific G protein pathway activated, and can be studied in detail.

The design of RASSLs usually requires large numbers of preliminary site-directed or saturation mutagenesis studies to define ligand-specific activation interactions. The availability of a large number of functionally relevant mutations in the MC4R has allowed us to bypass these cumbersome biochemical studies by directly assessing the activation of Class 2B an 2C mutants by the pharmacological agonist THIQ. This has led to the engineering of two MC4R-derived RASSLs Rm1 and Rm2. Both receptors do not respond to endogenous melanocortins but respond to THIQ. Rm1 possesses a low basal activity while the basal activity of Rm2 is elevated.

The most immediate application of the MC4R-based RASSLs lies in their use as Gs switches for studies of food-intake and body-weight regulation in mice. A detailed,

mechanistic understanding of the cellular and biochemical changes in the hypothalamus in response to MC4R signaling has been difficult to elucidate because of the presence of the endogenous hormone, α-MSH, and MC3R (also activated by α-MSH), which may also contribute to the maintenance of energy homeostasis [3,5,6]. A targeted gene knock-in approach in mice could be used to replace the genomic copies of the endogenous receptor with the MC4R RASSLs. Administration of THIQ can then be used to separate the effects of ligand-mediated versus basal signaling in the specific MC4R-expressing neurons at different times during pre- and post-natal development to elucidate the contribution of MC4R-mediated Gs signaling in synaptic plasticity and other downstream events required to maintain energy homeostasis.

5. Conclusions

Natural mutations associated with diseases are remarkable from a clinical standpoint as they provide insights into the mechanism of pathogenicity and possible avenues for development of therapeutics. But they also provide grounds for more fundamental studies. They can help us to decipher to complex relationship between protein structure and function by identifying key actors in the molecular machinery. Additionally these mutations can allow for the development of laboratory tools aiming at understanding specific processes. These general principles will certainly apply to other genes in which rare mutations will be found to predispose to obesity in humans.

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