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Mechanism of N-terminal modulation of activity at the melanocortin-4 receptor GPCR

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Abstract

Most of our understanding of G protein–coupled receptor (GPCR) activation has been focused on the direct interaction between diffusible ligands and their seven-transmembrane domains. However, a number of these receptors depend on their extracellular N-terminal domain for ligand recognition and activation. To dissect the molecular interactions underlying both modes of activation at a single receptor, we used the unique properties of the melanocortin-4 receptor (MC4R), a GPCR that shows constitutive activity maintained by its N-terminal domain and is physiologically activated by the peptide α -melanocyte stimulating hormone (α MSH). We find that activation by the N-terminal domain and α MSH relies on different key residues in the transmembrane region. We also demonstrate that agouti-related protein, a physiological antagonist of MC4R, acts as an inverse agonist by inhibiting N terminus–mediated activation, leading to the speculation that a number of constitutively active orphan GPCRs could have physiological inverse agonists as sole regulators.

GPCRs are characterized by a transmembrane core of seven α -helices (TM1 to TM7) connected by extracellular and cytoplasmic loops and an extracellular N-terminal domain.

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Author contributions

B.A.E. contributed to the hypothesis, designed and performed experiments, analyzed data and wrote the manuscript. L.P. designed and performed computational experiments, analyzed data and wrote the manuscript. S.Z. performed experiments. D.A.T. contributed to the hypothesis, and designed and performed experiments. G.M. contributed to the hypothesis, and provided reagents and expertise. C.G. contributed to the hypothesis, designed experiments and wrote the manuscript, and C.V. contributed to the hypothesis, directed the work, designed experiments and wrote the manuscript.

Competing financial interests

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Additional information

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This superfamily is divided into different subgroups on the basis of phylogenetic criteria and conserved residues within the helices and also according to the size and characteristics of the N-terminal domain of its members, which has an essential role in the activation of most classes of GPCRs (Supplementary Results, Supplementary Fig. 1). For instance, class B secretin receptors have a conserved N-terminal cysteine network that stabilizes their structure, the alteration of which impairs ligand interactions. Similarly, the N-terminal domain of class B adhesion receptors has a diverse variety of N-terminal domain motifs that determine ligand specificity. Class C glutamate receptors have a conserved N-terminal Venus flytrap domain, and Frizzled/Smoothed receptors have N-terminal Wnt-binding domains that regulate ligand binding and receptor activation^{1–3}. In class A rhodopsin-like receptors, the largest family of GPCRs, the N-terminal domain is important for activation of protease-activated receptors (PARs), which is dependent on proteolytic cleavage and unmasking of an N-terminal domain that acts as a tethered ligand⁴, as well as for the activation of glycoprotein hormone receptors (GpHRs)⁵.

Despite this recognized role of the N-terminal domain in GPCR activation, only a limited number of studies have attempted to understand the functional molecular interactions between this domain and the core of the receptor. This is due to the historic emphasis on rhodopsin, the prototypical class A GPCR whose ligand is covalently linked to the core of the receptor, and on GPCRs activated by high-affinity diffusible pharmacological ligands that interact directly with transmembrane helices (for example, adrenergic receptors).

Here we define and compare the relative molecular interactions underlying activation by the N-terminal domain and a high-affinity physiological agonist at a single receptor, the MC4R⁶. MC4R is one of five melanocortin receptors, a subfamily of the α group of class A G_s protein-coupled receptors⁶. MC4R is expressed in the central nervous system and is essential for the maintenance of long-term energy balance in humans. Heterozygous mutations in MC4R are the most common genetic cause of severe human obesity, and over 80 naturally occurring pathogenic mutations in this receptor have been described⁷. A number of studies have mapped the interaction of the endogenous physiological agonist of MC4R, α MSH, to acidic amino acids in TM2 and TM3 of the receptor. In addition to activity induced by this high-affinity ligand, MC4R also shows constitutive activity, as observed by the receptor expression-dependent G protein signaling in cell culture systems⁸. A basal, agonist-independent, G protein-coupling activity is a common characteristic of many GPCRs and is generally associated with the intrinsic ability of the transmembrane domain of the receptor to undergo a spontaneous conformational transition from the inactive to the active state (or states) in the absence of a ligand⁹. In contrast with this classical view of constitutive GPCR activity, we have previously shown that, at the MC4R, the constitutive activity is largely established by the agonistic effect of its own N-terminal domain and that this constitutive activity is physiologically relevant^{8,10,11}. In addition, MC4R and two other members of the melanocortin receptor subfamily are also the only known GPCRs to physiologically respond to an endogenous antagonist^{12–15}. Agouti-related protein (AgRP), which is released by an independent population of neurons, inhibits α MSH activation of MC4R. However, unlike neutral antagonists that do not show phenotypic behavior in the absence of a competitive ligand^{16,17}, AgRP also acts as an inverse agonist as it has negative efficacy on the constitutive activity of MC4R^{14,15}.

Using a peptide mimetic of the MC4R N-terminal domain as a pharmacological agent, we find here that the inverse agonism by the endogenous ligand AgRP at this receptor can be attributed in large part to its specific inhibition of the N terminus-induced self-activation. Comparing the activation of the receptor by its N-terminal domain and by α MSH through systematic alanine-scanning mutagenesis, we found that the signaling promoted by these agonists occurs through distinct molecular mechanisms. Notably, the N-terminal domain

activates the receptor through a set of amino acids that is crucial for a conserved mode of activation among class A GPCRs, leading to the speculation that inverse agonism may be the original mechanism for modulation of the activity of this receptor.

RESULTS

N-terminal domain of MC4R can act as a diffusible agonist

We had previously demonstrated that the N-terminal domain of MC4R is a tethered ligand and is essential for the overall maintenance of the receptor's constitutive activity⁸. Specifically, a receptor lacking the first 24 N-terminal amino acids (MC4R Δ 1–24; Supplementary Fig. 2) responds normally to a panel of agonists and to AgRP antagonism of α MSH (Supplementary Fig. 3 and Supplementary Table 1) but has a low constitutive activity that can be rescued by coexpression of the MC4R N-terminal domain chimerically linked to the transmembrane domain of cluster of differentiation 8 (CD-8) (ref. 8).

Receptor titration experiments demonstrate that this N-terminal domain-dependent constitutive activity of MC4R Δ 1–24 represents over 80% of the total MC4R constitutive activity (Supplementary Fig. 3a,b). The activation of MC4R by its N-terminal domain resembles that of PAR1, but the latter requires unmasking of the active site through proteolytic cleavage by thrombin⁴. In the case of PAR1, it had also been shown that, in the absence of thrombin, exogenous addition of a synthetic peptide mimicking the unmasked active N-terminal domain leads to the activation of the receptor⁴. Therefore, we tested whether a peptide mimicking amino acids 2–26 of the MC4R (MC4R 2–26; Supplementary Methods) could act as a partial agonist for MC4R Δ 1–24. The receptor activity was measured in receptor-transfected HEK293 cells using a luciferase reporter system. Indeed, at 100 μ M, MC4R 2–26 but not MC4R 20–39 increased the activity of MC4R Δ 1–24 to the level of the constitutive activity of wild-type MC4R (Fig. 1a). The low potency of the N-terminal domain activation (half-maximum effective concentration (EC_{50}) = 72 μ M; Supplementary Fig. 4a) is compatible with the high local concentration in the tethered form and is similar to that observed in the case of PAR1 (ref. 4).

Indeed, in the presence of 100 μ M MC4R 2–26, activation of MC4R Δ 1–24 by α MSH mimics that of the wild-type MC4R (Fig. 1b,c and Supplementary Fig. 4b). At higher concentrations, as expected for a partial agonist, MC4R 2–26 lowered the potency of α MSH at MC4R Δ 1–24 (Fig. 1b,c and Supplementary Fig. 4c). Finally, MC4R 2–26 displaces radiolabeled NDP- α MSH from MC4R Δ 1–24 (Fig. 1d), suggesting that the N-terminal domain of MC4R and α MSH have overlapping binding sites.

AgRP antagonizes the diffusible N-terminal domain

AgRP is of sufficient size and molecular weight to cover the MC4R extracellular binding pocket, acting as an inverse agonist of the MC4R^{14,15,18}. In addition, AgRP inhibits α MSH-induced receptor activation independent of the absence or presence of the N-terminal domain (Supplementary Fig. 3e). The availability of an active diffusible N-terminal peptide allowed us to test whether the inverse agonist activity of AgRP could be attributed to the antagonism of the N-terminal domain (Fig. 1e). AgRP inhibits the activation of MC4R Δ 1–24 by MC4R 2–26 with similar potency as its inverse agonism of the wild-type receptor (Fig. 1f). It is notable that AgRP also inhibits the residual N terminal-independent constitutive activity of MC4R Δ 1–24 (Supplementary Fig. 3a). In addition, MC4R 2–26 displaced radiolabeled AgRP, with similar affinities for wild-type and Δ 1–24 MC4R (Fig. 1g). Together, these data indicate that the inverse agonist activity of AgRP on the MC4R can be attributed mainly to the inhibition of the N terminal-mediated activation of the receptor.

MC4R 2–26 activation does not mimic α MSH activation

Three acidic residues in the second and third transmembrane domains of melanocortin receptors (Glu100^{2.60}, Asp122^{3.25} and Asp126^{3.29} in MC4R; superscripts are the generic numbering scheme of Ballesteros and Weinstein, which allows direct comparison among residues in the seven-transmembrane segments of different receptors¹⁹; Supplementary Fig. 2) are essential for their activation by melanocortins. Alanine substitution of these residues impairs α MSH binding and activation but does not prevent constitutive activity of the MC4R⁸ (Supplementary Fig. 5a). We tested whether these mutations also affected activation by the N-terminal domain. When introduced into MC4R Δ 1–24, the E100^{2.60}A, D122^{3.25}A and D126^{3.29}A mutations impaired activation of the truncated receptor by α MSH but not by MC4R 2–26 (Supplementary Fig. 5b), demonstrating that these three residues are not implicated in receptor activation by the N-terminal domain.

HLWNRS is the minimal N-terminal activating sequence

To outline the molecular interactions underlying the N-terminal activation of MC4R, we first delineated the minimal activating region of the N-terminal domain. We tested a range of smaller overlapping peptides spanning this domain for their efficacy at MC4R Δ 1–24 (Supplementary Methods). Although MC4R 2–26 showed the greatest activity, some of its segments failed to activate MC4R Δ 1–24 (Fig. 2a). All activating peptides shared the common sequence¹⁴ HLWNRS¹⁹ (MC4R 14–19). Notably, this minimal activating sequence encompasses the most conserved portion of the N-terminal region throughout several species (Supplementary Fig. 6) and includes Arg18, a previously described genetic ‘hot spot’ for obesity-causing mutations in the MC4R^{8,20}. In addition, a monoclonal antibody targeting the overlapping MC4R 11–25 region in rats has been reported to function as an inverse agonist *in vitro* and lead to increased food intake and body weight *in vivo*¹¹.

We further determined the relative role of specific amino acids within this minimal activating sequence. Further truncation of the hexapeptide resulted in complete loss of activation (Supplementary Fig. 7), and H14A, W16A or R18L substitutions markedly disrupted MC4R 14–19 efficacy (Fig. 2a).

TM residues involved in N terminus–mediated activation

To search for potential candidate amino acids interacting with the N-terminal domain, we first surveyed naturally occurring obesity-causing mutations for decreased constitutive activity with preserved activation of the receptor by α MSH (Supplementary Fig. 2)^{7,20,21}. Out of 71 mutations examined, 8 had resulted in decreased constitutive activity and a conserved response to α MSH. Five of these mutations were in the N-terminal domain of the receptor (R7H, R18H, R18C, R18L and T11A)^{8,20}. Three were in the intracellular domains of the receptor (V95^{2.55}I, A154^{3.57}D and G231S)²⁰. None were located in the extracellular portions of transmembrane domains or the extracellular loops that would be accessible to the N-terminal domain for interactions.

Considering that His14, Trp16 and Arg18 are the most mutation-sensitive residues of the N-terminal domain (Fig. 2a), we systematically screened all 22 charged and/or aromatic residues that could be accessible for N-terminal domain interaction (Supplementary Fig. 2) for their role in the activation of the receptor by alanine-scanning mutagenesis.

None of the five acidic residue substitutions decreased the constitutive activity of the receptor (Supplementary Fig. 8a). In contrast, substitution of one out of two basic (His264^{6.54}) and eight other aromatic (Trp174^{4.50}, Tyr187, Phe201^{5.47}, Phe202^{5.48}, Trp258^{6.48}, Phe261^{6.51}, Phe262^{6.52} and Phe284^{7.35}) residues with alanine led to significantly ($P < 0.05$) reduced constitutive activity compared to the wild-type receptor

(Supplementary Fig. 8b,c). From these nine mutant receptors, six (carrying mutations W174^{4.50}A, Y187A, F202^{5.48}A, F261^{6.51}A, H264^{6.54}A or F284^{7.35}A) had considerably altered responses to α MSH (Fig. 2b,c). Three mutant receptors (carrying mutations F201^{5.47}A, W258^{6.48}A or F262^{6.52}A) showed a normal response to the agonist α MSH, a functional profile similar to that of MC4R Δ 1–24, consistent with the possibility that these three residues are involved in the activation of the receptor by its N-terminal domain (Fig. 2d). When introduced into MC4R Δ 1–24, mutations of the residues Phe201^{5.47}, Trp258^{6.48} and Phe262^{6.52} to alanine impaired activation of the receptors by exogenously added N-terminal peptide, confirming their specific involvement in the partial agonism of the N-terminal domain of the receptor (Fig. 2e).

Models of MC4R in complex with the N terminus or α MSH

To provide a structural framework for the interpretation of the experimental data, we developed molecular models of the receptor–peptide complexes on the basis of the crystal structures of the β_2 -adrenergic receptor and of the CXCR4 receptor in complex with a peptide (Supplementary Methods). Figure 3a compares the computationally obtained molecular models of the HLWNRS hexapeptide of the N-terminal domain of MC4R and the ⁴MEHFRWGK¹¹ octapeptide from the core of α MSH, in a complex with a molecular model of MC4R. Both peptides are in the main binding site crevice located between the extracellular segments of TMs 3–7. In these models, Arg8 of α MSH forms a network of ionic interactions with Glu100^{2.60}, Asp122^{3.25} and Asp126^{3.29} (Fig. 3b), in agreement with previous proposals²² and our experimental results (Supplementary Fig. 5a), whereas the N-terminal hexapeptide (Fig. 3c) does not form these interactions, in agreement with what was suggested by our experimental results (Supplementary Fig. 5b). This putative additional network of ionic interactions is supported by the higher affinity of α MSH for the receptor. In addition, these models also provide a viable rational explanation to the experimentally determined involvement of Tyr187 at the extracellular end of TM4, His264^{6.54} in TM6 and Phe284^{7.35} in TM7 (Supplementary Fig. 8b,c) in receptor activation by both the N-terminal hexapeptide (via His14 and Arg18; Fig. 3c) and α MSH (via His6 and Lys11; Fig. 3b) by proposing putative direct interactions between these side chains. Our model suggests that the shorter Phe7 side chain of α MSH cannot interact with Trp258^{6.48} and the associated Phe201^{5.47}-Trp258^{6.48}-Phe262^{6.52} aromatic cluster (Fig. 3d). Accordingly, alanine substitutions of these amino acids do not impair α MSH receptor activation (Fig. 2d). In contrast, the proposed mode of binding of the N-terminal hexapeptide to MC4R positions the key Trp16 deep inside the receptor bundle, where it interacts with the highly conserved Trp258^{6.48} (Fig. 3e). Trp258^{6.48}, together with Phe201^{5.47} in TM5 and Phe261^{6.51} and Phe262^{6.52} in TM6, form a previously reported aromatic cluster involved in the activation of some GPCRs^{23–25}. This provides a possible explanation for the decreased constitutive activity of receptors with alanine substitutions at Trp16 (Fig. 2a), Phe201^{5.47}, Trp258^{6.48}, Phe261^{6.51} or Phe262^{6.52} (Fig. 2c–e and Supplementary Fig. 8c). In addition, in our model, Phe202^{5.48} is also part of this aromatic cluster, in line with the decreased constitutive activity of the F202^{5.48} A mutant (Fig. 2c and Supplementary Fig. 8c).

The recently obtained crystal structures of agonist-bound, active conformations of several GPCRs might help to explain this different process of MC4R activation by the N-terminal domain and α MSH. The structures of metarhodopsin II²⁶, the constitutively active rhodopsin²⁷ and the A_{2A} adenosine receptor in complex with the agonist UK-432097 (ref. 28) have shown that Trp^{6.48} moves toward TM5 relative to the inactive structures, facilitating the rotation and tilt of the intracellular part of TM6. Thus, we hypothesize that Trp16 of the N-terminal domain of MC4R triggers and stabilizes this shift of Trp258^{6.48} in a similar manner. In contrast, the structures of β_1 -adrenergic²⁹ and β_2 -adrenergic^{30,31} receptors bound to agonists do not contain this shift of Trp^{6.48}. In these cases, the major

difference between the binding of full agonists compared to partial agonists or antagonists is the hydrogen bond between full agonists and Ser^{5.46} in TM5. This illustrates that different mechanisms of signal propagation exist among GPCRs and, as a corollary, that different ligands might also trigger different activation mechanisms at one receptor, as shown here for the receptor N-terminal domain and α MSH.

DISCUSSION

Historically, class A GPCRs have been identified and studied on the basis of their ability to be activated by specific high-affinity diffusible pharmacological ligands interacting directly with the core transmembrane region of the receptor. Notable exceptions include PARs and GpHRs (Supplementary Fig. 1). For PARs, activation of the receptor is dependent on proteolytic cleavage and unmasking of an N-terminal domain that acts as a tethered ligand⁴. For GpHRs, binding of the glycoprotein hormone to the N-terminal domain leads to receptor activation. Notably, the N-terminal domain of GpHRs arose from a gene fusion event that linked a leucine-rich repeat domain to a GPCR sequence. In the absence of the hormone, the N-terminal domain is inhibitory, and its removal leads to increased activation of the receptor⁵. Here we find that antagonism by a physiological ligand is a third mode of modulation of an activating N-terminal domain (Supplementary Fig. 1). Activation by the N-terminal domain of MC4R requires the conserved Phe201^{5.47}-Trp258^{6.48}-Phe262^{6.52} aromatic cluster (the so-called 'toggle switch'^{32,33}) that is found in most class A GPCRs, where it is mandatory for activation by the various cognate agonists. Remarkably, these key residues are not necessary for receptor activation by the endogenous diffusible agonist α MSH, suggesting that hormone-induced activation might have arisen second in the evolution of the MC4R, which would therefore represent an intermediate situation between N terminus-dependent GPCRs and receptors activated solely by soluble agonists. One can speculate that the latter may have lost N-terminal activation after preferential adaptation to their endogenous ligand, whether it is diffusible as in the β_2 -adrenergic receptor or covalently bound as in rhodopsin (Supplementary Fig. 1). One can also speculate that such evolutionary separation between receptors activated by soluble and tethered agonists occurred very early in the evolution of class A GPCRs. According to this hypothetical scenario, most current receptor families have evolved to exclusively rely on diffusible agonists, whereas the receptors activated by their N terminus have evolved separately, with some members eventually developing activation by diffusible ligands.

The finding that modulation of MC4R activity by its physiological inverse agonist is independent of the modulation by its endogenous diffusible agonist also suggests that inverse agonism could be the sole mode of activity modulation for some GPCRs (Supplementary Fig. 1, dotted arrow). Indeed, from nearly 400 GPCRs identified within the human genome on the basis of sequence homology, more than 30% are yet to be associated with a physiological ligand³⁴. In addition, although most GPCRs have constitutive activity⁹, a number of these orphan GPCRs have a high level of such activity³⁵. This high constitutive activity, observed after expression *ex vivo* in heterologous cell systems, is compatible with physiological modulation by an inverse agonist *in vivo* and could provide an explanation for the failure to find a physiological ligand using high-throughput reverse pharmacology assays, mostly limited to the detection of agonism. The use of GPCR constitutive activity and the detection of its inhibition by inverse agonism has been proposed as a new approach to GPCR drug discovery and as a tool to study constitutively active orphan GPCRs^{36,37}. Notably, GPR61 is a recent example of an orphan GPCR with a high constitutive activity mediated by the agonistic effect of its own N-terminal domain³⁸.

Systematically testing whether agonism of the N-terminal domain is essential for the constitutive activity of orphan GPCRs could be a first step in the design of assays aiming at finding physiological ligands for these receptors.

METHODS

MC4R plasmid constructs

Genes encoding wild-type and E42K human MC4R were cloned from genomic DNA into the vector pcDNA 3.1 as previously described³⁹. MC4R Δ 1–24 was made as described, and the prolactin signal peptide and a Flag epitope tag (DYKDDDD) were added to both the wild-type MC4R and MC4R Δ 1–24 for identical membrane localization⁸.

Ligand binding assay

Ligand binding was carried out as previously described⁴⁰. Briefly, HEK293 cells were stably transfected with wild-type or mutant MC4R. Competitive binding was measured as a function of radiolabeled MC4R ligand displacement ($[^{125}\text{I}]\text{NDP-}\alpha\text{MSH}$, $[^{125}\text{I}]\text{AgRP}$; PerkinElmer) in the presence of increasing concentrations of a competitive ligand. Gamma counter readings were normalized for nonspecific binding and plotted as a percentage of maximum radioligand binding.

MC4R activity

MC4R activity was measured as previously described^{39,40}. Briefly, cAMP accumulation was measured in HEK293 cells stably expressing or transiently transfected to express a firefly luciferase reporter driven by a cyclic AMP-responsive element. Cells were transiently transfected with wild-type or mutant MC4R plasmid and a *Renilla* luciferase plasmid. After stimulation with a choice of ligand, luciferase activity was measured using the Steady-Glo Luciferase Assay System (Promega) and a microplate luminescence counter (Packard Instrument). Firefly luciferase activity upon MC4R activation was normalized over the transfection efficiency by dividing the firefly luciferase activity by the *Renilla* luciferase activity. The results were normalized to the corresponding membrane expression for each receptor as determined by a complementary assay (Methods, MC4R membrane expression). The data are represented as percentages in the figures and in the Supplementary Results.

MC4R membrane expression

MC4R membrane expression was measured by ELISA as previously described⁸. HEK293 cells were transiently transfected with N-terminally Flag-tagged MC4R constructs. Cells were fixed with 4% (v/v) paraformaldehyde (Sigma-Aldrich) for 10 min at 4 °C following 48 h of transfection. After three washes with PBS, cells were incubated with 1 $\mu\text{g ml}^{-1}$ M2 Flag-specific antibody (Sigma-Aldrich, cat. no: F1804, 1:1,000) for 2 h at 20 °C. Cells were then washed three times with PBS and incubated with horseradish peroxidase-conjugated goat mouse-specific antibody (Santa Cruz Biotechnology, cat. no: sc-2005, 1:3,000) for 1 h at 20 °C. Following three washes with PBS, 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma-Aldrich) was added to the cells, and the attenuation at 405 nm was measured by a spectrophotometer.

Peptide synthesis and MC4R ligands

Peptide synthesis and commercially available MC4R ligands are described in detail in the Supplementary Methods.

Molecular modeling

Generation of the MC4R model in complex with its N-terminal hexapeptide and α MSH are described in detail in the Supplementary Methods section.

For the competitive ligand binding assay, best-fit estimates of the half-maximum inhibitory concentration (IC_{50}) and its 95% confidence intervals were obtained by nonlinear regression fitting of the one-site competition curves using GraphPad Prism 4. For MC4R activity dose-response curves, best-fit estimates of the EC_{50} , IC_{50} and their 95% confidence intervals were obtained by nonlinear regression fitting of the sigmoidal dose response (variable slope) curves using GraphPad Prism 4. The statistical significance of constitutive activity between the wild-type and mutant receptors was determined using one-way analysis of variance plus a posteriori one-sided Dunnett's *t*-test.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Kristiansen K. Molecular mechanisms of ligand binding, signaling, and regulation within the superfamily of G-protein-coupled receptors: molecular modeling and mutagenesis approaches to receptor structure and function. *Pharmacol Ther.* 2004; 103:21–80. [PubMed: 15251227]
2. Kobilka BK. G protein-coupled receptor structure and activation. *Biochim Biophys Acta.* 2007; 1768:794–807. [PubMed: 17188232]
3. Lagerström MC, Schiöth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. *Nat Rev Drug Discov.* 2008; 7:339–357. [PubMed: 18382464]
4. Scarborough RM, et al. Tethered ligand agonist peptides. Structural requirements for thrombin receptor activation reveal mechanism of proteolytic unmasking of agonist function. *J Biol Chem.* 1992; 267:13146–13149. [PubMed: 1320011]
5. Vassart G, Pardo L, Costagliola S. A molecular dissection of the glycoprotein hormone receptors. *Trends Biochem Sci.* 2004; 29:119–126. [PubMed: 15003269]
6. Breit A, et al. Alternative G protein coupling and biased agonism: new insights into melanocortin-4 receptor signalling. *Mol Cell Endocrinol.* 2011; 331:232–240. [PubMed: 20674667]
7. Bromberg Y, Overton J, Vaisse C, Leibel RL, Rost B. *In silico* mutagenesis: a case study of the melanocortin 4 receptor. *FASEB J.* 2009; 23:3059–3069. [PubMed: 19417090]
8. Srinivasan S, et al. Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans. *J Clin Invest.* 2004; 114:1158–1164. [PubMed: 15489963]
9. Seifert R, Wenzel-Seifert K. Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn Schmiedeberg's Arch Pharmacol.* 2002; 366:381–416. [PubMed: 12382069]
10. Tolle V, Low MJ. *In vivo* evidence for inverse agonism of Agouti-related peptide in the central nervous system of proopiomelanocortin-deficient mice. *Diabetes.* 2008; 57:86–94. [PubMed: 17909095]
11. Peter JC, et al. Antibodies against the melanocortin-4 receptor act as inverse agonists *in vitro* and *in vivo*. *Am J Physiol Regul Integr Comp Physiol.* 2007; 292:R2151–R2158. [PubMed: 17322114]

12. Ollmann MM, et al. Antagonism of central melanocortin receptors *in vitro* and *in vivo* by agouti-related protein. *Science*. 1997; 278:135–138. [PubMed: 9311920]
13. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature*. 1997; 385:165–168. [PubMed: 8990120]
14. Nijenhuis WA, Oosterom J, Adan RA. AgRP(83–132) acts as an inverse agonist on the human-melanocortin-4 receptor. *Mol Endocrinol*. 2001; 15:164–171. [PubMed: 11145747]
15. Chai BX, et al. Inverse agonist activity of agouti and agouti-related protein. *Peptides*. 2003; 24:603–609. [PubMed: 12860205]
16. Costa T, Herz A. Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci USA*. 1989; 86:7321–7325. [PubMed: 2552439]
17. Kenakin T. Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol Pharmacol*. 2004; 65:2–11. [PubMed: 14722230]
18. Patel MP, et al. Loop-swapped chimeras of the agouti-related protein and the agouti signaling protein identify contacts required for melanocortin 1 receptor selectivity and antagonism. *J Mol Biol*. 2010; 404:45–55. [PubMed: 20831872]
19. Ballesteros JA, Weinstein H. Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. *Methods Neurosci*. 1995; 25:366–428.
20. Lubrano-Berthelie C, et al. Melanocortin 4 receptor mutations in a large cohort of severely obese adults: prevalence, functional classification, genotype-phenotype relationship, and lack of association with binge eating. *J Clin Endocrinol Metab*. 2006; 91:1811–1818. [PubMed: 16507637]
21. Calton MA, et al. Association of functionally significant Melanocortin-4 but not Melanocortin-3 receptor mutations with severe adult obesity in a large North American case-control study. *Hum Mol Genet*. 2009; 18:1140–1147. [PubMed: 19091795]
22. Haskell-Luevano C, Cone RD, Monck EK, Wan YP. Structure activity studies of the melanocortin-4 receptor by *in vitro* mutagenesis: identification of agouti-related protein (AGRP), melanocortin agonist and synthetic peptide antagonist interaction determinants. *Biochemistry*. 2001; 40:6164–6179. [PubMed: 11352754]
23. Jongejan A, et al. Linking ligand binding to histamine H₁ receptor activation. *Nat Chem Biol*. 2005; 1:98–103. [PubMed: 16408006]
24. Pellissier LP, et al. Conformational toggle switches implicated in basal constitutive and agonist-induced activated states of 5-HT₄ receptors. *Mol Pharmacol*. 2009; 75:982–990. [PubMed: 19168624]
25. Holst B, et al. A conserved aromatic lock for the tryptophan rotameric switch in TM-VI of seven-transmembrane receptors. *J Biol Chem*. 2010; 285:3973–3985. [PubMed: 19920139]
26. Choe HW, et al. Crystal structure of metarhodopsin II. *Nature*. 2011; 471:651–655. [PubMed: 21389988]
27. Standfuss J, et al. The structural basis of agonist-induced activation in constitutively active rhodopsin. *Nature*. 2011; 471:656–660. [PubMed: 21389983]
28. Xu F, et al. Structure of an agonist-bound human A_{2A} adenosine receptor. *Science*. 2011; 332:322–327. [PubMed: 21393508]
29. Warne T, et al. The structural basis for agonist and partial agonist action on a β_1 -adrenergic receptor. *Nature*. 2011; 469:241–244. [PubMed: 21228877]
30. Rasmussen SG, et al. Structure of a nanobody-stabilized active state of the β_2 adrenoceptor. *Nature*. 2011; 469:175–180. [PubMed: 21228869]
31. Rosenbaum DM, et al. Structure and function of an irreversible agonist- β_2 adrenoceptor complex. *Nature*. 2011; 469:236–240. [PubMed: 21228876]
32. Shi L, et al. β_2 adrenergic receptor activation. Modulation of the proline kink in transmembrane 6 by a rotamer toggle switch. *J Biol Chem*. 2002; 277:40989–40996. [PubMed: 12167654]
33. Swaminath G, et al. Probing the 2 adrenoceptor binding site with catechol reveals differences in binding and activation by agonists and partial agonists. *J Biol Chem*. 2005; 280:22165–22171. [PubMed: 15817484]

34. Civelli O. GPCR deorphanizations: the novel, the known and the unexpected transmitters. *Trends Pharmacol Sci.* 2005; 26:15–19. [PubMed: 15629200]
35. Bond RA, Ijzerman AP. Recent developments in constitutive receptor activity and inverse agonism, and their potential for GPCR drug discovery. *Trends Pharmacol Sci.* 2006; 27:92–96. [PubMed: 16406086]
36. Chen G, et al. Use of constitutive G protein–coupled receptor activity for drug discovery. *Mol Pharmacol.* 2000; 57:125–134. [PubMed: 10617687]
37. Behan DP, Chalmers DT. The use of constitutively active receptors for drug discovery at the G protein–coupled receptor gene pool. *Curr Opin Drug Discov Devel.* 2001; 4:548–560.
38. Toyooka M, Tujii T, Takeda S. The N-terminal domain of GPR61, an orphan G-protein–coupled receptor, is essential for its constitutive activity. *J Neurosci Res.* 2009; 87:1329–1333. [PubMed: 19025769]
39. Lubrano-Berthelie C, et al. Intracellular retention is a common characteristic of childhood obesity–associated MC4R mutations. *Hum Mol Genet.* 2003; 12:145–153. [PubMed: 12499395]
40. Vaisse C, et al. Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. *J Clin Invest.* 2000; 106:253–262. [PubMed: 10903341]

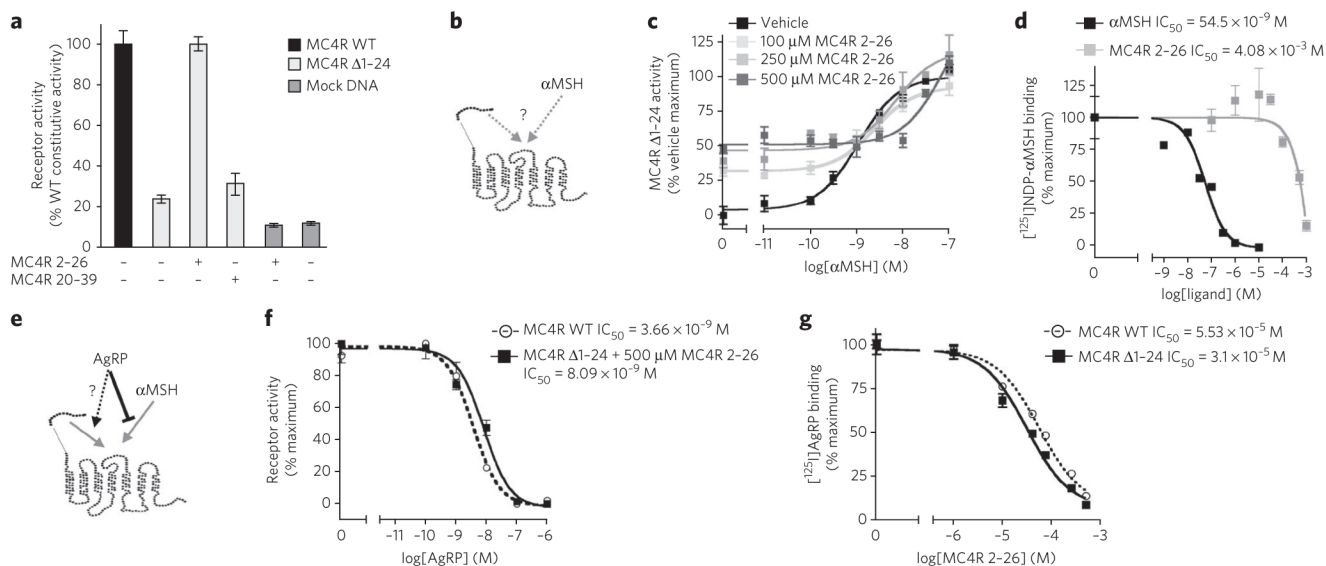


Figure 1. Functional properties of MC4R 2–26

(a) The full sequence of residues 2–39 corresponding to the N-terminal domain of MC4R has been synthesized as two peptides: residues 2–26 (MC4R 2–26) and residues 20–39 (MC4R 20–39). MC4R Δ1–24 was stimulated by either peptide. Cells transfected with the plasmid pcDNA3.1 encoding LacZ (mock DNA) were used to control for nonspecific activation. WT, wild type. (b) Tested model of interaction of MC4R with the N-terminal domain and αMSH. (c) Dose response curve of MC4R Δ1–24 activation by αMSH in the presence of vehicle (BSA; EC₅₀ = 1.1 nM) versus 100 μM (EC₅₀ = 2.4 nM), 250 μM (EC₅₀ = 7.0 nM) or 500 μM (EC₅₀ = 74.5 nM) MC4R 2–26. (d) αMSH and MC4R 2–26 binding to MC4R Δ1–24. [¹²⁵I]NDP-αMSH was used as the competitive radio-labeled ligand. (e) Tested model of AgRP interaction with the N-terminal domain. (f) AgRP antagonism of MC4R 2–26 activation of MC4R Δ1–24 compared to AgRP inverse agonism of wild-type MC4R. (g) MC4R 2–26 binding to MC4R Δ1–24 and wild-type MC4R. [¹²⁵I]AgRP was used as the competitive radio-labeled ligand. Receptor activity is normalized to membrane expression for each receptor. Error bars represent s.e.m. for experiments performed in triplicate.

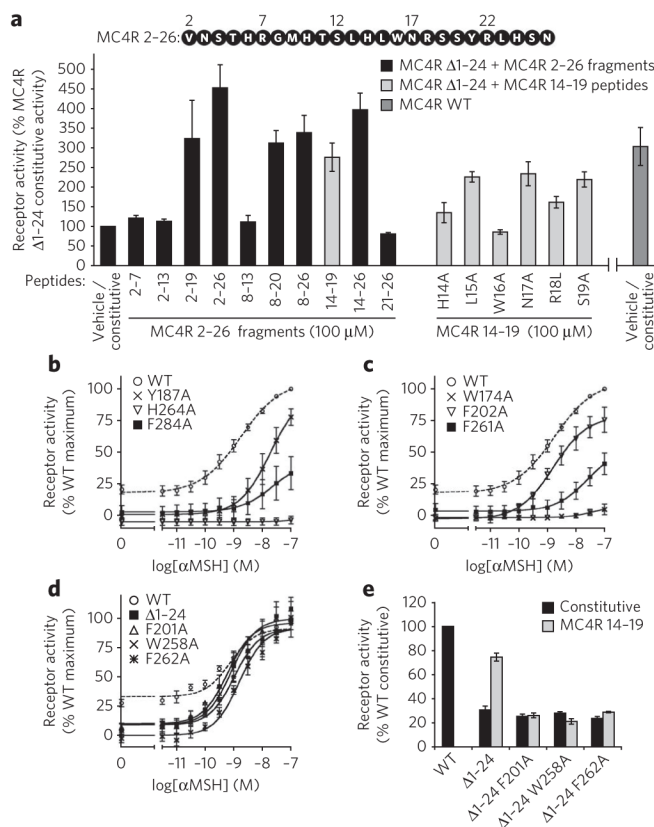


Figure 2. Essential residues in the maintenance of N terminus-mediated receptor activation
(a) Minimal activating sequence and essential amino acids required for MC4R 2–26 efficacy. MC4R 2–26 peptide sequence is shown at the top of the figure. The stimulations of MC4R Δ 1–24 by MC4R 2–19, 2–26, 8–20, 8–26, 14–19, 14–26, 14–19 L15A, 14–19 N17A and 14–19 S19A are not significantly different from the constitutive activity of wild-type MC4R ($P > 0.05$). Receptor activity is normalized to membrane expression for MC4R Δ 1–24 and wild-type MC4R. Error bars represent s.e.m. for experiments performed in triplicate.
(b,c) Activation profiles of Y187A, H264A and F284A **(b)** and W174A, F202A and F261A **(c)** mutants with low constitutive activity and impaired α MSH response. **(d)** Activation profiles for F201A, W258A and F262A mutants with low constitutive activity and wild-type-like α MSH response. **(e)** Activation of MC4R Δ 1–24 mutants by the MC4R 14–19 peptide. Receptor activity is normalized to membrane expression for MC4R Δ 1–24 and wild-type MC4R. Error bars represent s.e.m. for experiments performed in triplicate.

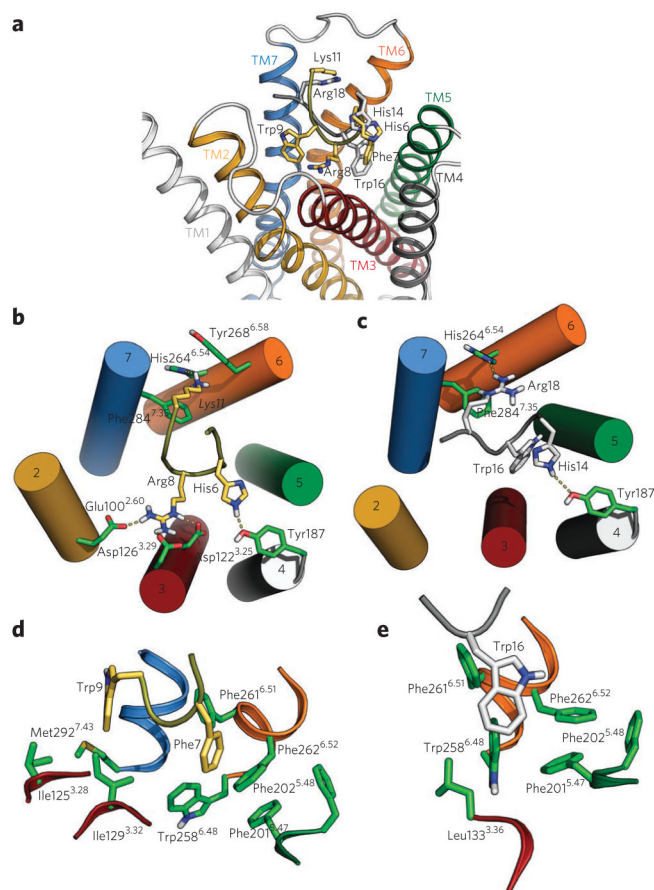


Figure 3. Models of MC4R in complex with the N terminus or α MSH

(a) Comparison of the binding modes of the HLWNRS hexapeptide of the N-terminal domain (gray; His14, Trp16 and Arg18 are shown) and the central portion of α MSH (yellow; His6, Phe7, Arg8, Trp9 and Lys11 are shown) to the β_2 -adrenergic receptor–based model of MC4R. (b,c) View of the complexes between α MSH (b) or the N-terminal hexapeptide (c) and MC4R. Numbered cylinders in b and c represent TMs. (d,e) Detailed view of the complexes between α MSH (d) or the N-terminal hexapeptide (e) and the Phe201^{5.47}-Trp258^{6.48}-Phe262^{6.52} aromatic cluster of MC4R. The side chains of the N-terminal HLWNRS hexapeptide of MC4R are shown in white, the central portion of α MSH (Supplementary Methods) is shown in yellow, and the side chains of the β_2 -adrenergic receptor–based model of MC4R are shown in green.