



# Constitutive activity of the melanocortin-4 receptor is maintained by its N-terminal domain and plays a role in energy homeostasis in humans

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**The melanocortin-4 receptor (MC4R), a centrally expressed G protein-coupled receptor (GPCR), is essential for the maintenance of long-term energy balance in humans. Mutations in MC4R are the most common genetic cause of obesity. Since activation of this receptor leads to a decrease in food intake, MC4R is also a major therapeutic target for the treatment of obesity. Control of MC4R activity in vivo is modulated by the opposing effects of the anorexigenic agonist  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) and the orexigenic antagonist *agouti*-related protein (AGRP). In addition, experiments in vitro have demonstrated that the human MC4R has an intrinsic constitutive activity on which AGRP also acts as an inverse agonist. The physiological role of this constitutive activity in the control of energy balance as well as the domain of the protein implicated in its maintenance are unknown. By systematically studying functional defects in naturally occurring MC4R mutations from obese patients, we defined a cluster of N-terminal mutations that selectively impair the constitutive activity of the receptor. Further characterization of this domain demonstrated that it functions as a tethered intramolecular ligand that maintains the constitutive activity of MC4R and may provide novel avenues for the design of drugs targeting this receptor. Our results also suggest that the tonic satiety signal provided by the constitutive activity of MC4R may be required for maintaining long-term energy homeostasis in humans.**

## Introduction

Prevention of obesity in mammals is dependent on a number of systems, including the activity of an anorexigenic pathway made up of the adipocyte-secreted hormone leptin, its neuropeptide effector pro-opiomelanocortin (POMC), and the melanocortin-4 receptor (MC4R). In the basal state, leptin maintains the expression of POMC in the arcuate nucleus of the hypothalamus (1), and the POMC-derived peptide ligand  $\alpha$ -melanocyte-stimulating hormone ( $\alpha$ -MSH) activates MC4R in the paraventricular nucleus of the hypothalamus, ultimately resulting in the suppression of food intake. During an orexigenic anabolic response to negative energy balance, decreased leptin levels downregulate POMC expression, which in turn reduces the level of  $\alpha$ -MSH. In parallel, a reduction in leptin levels stimulates the expression of *agouti*-related protein (AGRP) in the orexigenic neurons of the arcuate nucleus (1). Since AGRP is an antagonist at the MC4R (2), the coordinate decrease in  $\alpha$ -MSH and increase in AGRP leads to the sustained repression of MC4R, resulting in increased food intake. Thus through the opposing effects of  $\alpha$ -MSH and AGRP, MC4R serves as a central integrator of peripheral signals that modulate food intake (3).

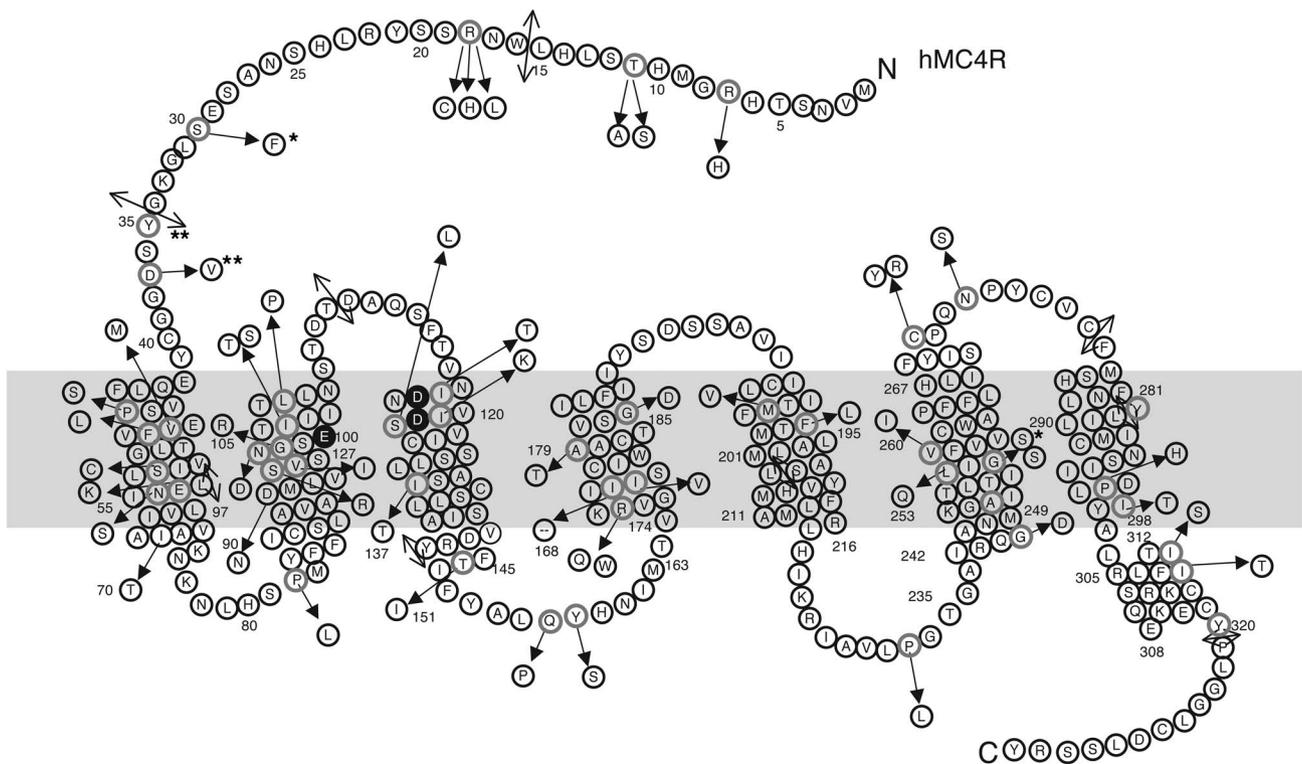
Key for establishing such a model was the discovery that loss-of-function mutations in leptin (4–6), the leptin receptor (7, 8), POMC (9–11), and MC4R (12), all lead to hyperphagia and severe obesity in mice and humans. In particular, heterozygous mutations in *MC4R* account for 1–6% of severe cases of human obesity. Over 50 different obesity-associated mutations have been described, most of which are missense mutations (13–17). Most of these mutations reduce the ability of the receptor to be activated by its POMC-derived agonist  $\alpha$ -MSH. Since loss of MC4R activation is linked to weight gain, the development of an MC4R agonist has been proposed as a therapeutic option for the treatment of obesity (18).

Upon activation by  $\alpha$ -MSH, MC4R transduces signal by coupling to the heterotrimeric Gs protein and activating adenylyl cyclase (19). In vitro, MC4R also exhibits constitutive activity in the absence of ligand (20). In addition to its antagonistic effects, AGRP also acts as an inverse agonist in vitro to suppress the constitutive activity of MC4R (21). Although specific domains required for the  $\alpha$ -MSH-mediated activation of MC4R have been described (22–24), the structural determinants required for its constitutive activity have never been studied. In addition, the physiological contribution of the MC4R constitutive activity to the maintenance of the anorexigenic catabolic state is unknown. In this report we demonstrate that the constitutive activity of MC4R is provided by its N-terminal domain, which acts as a tethered intramolecular ligand for the receptor. Obesity-associated mutations in the N-terminal domain of MC4R decrease its constitutive activity. This suggests that in addition to the agonist-mediated activation of MC4R, this constitutive

**Nonstandard abbreviations used:** AGRP, *agouti*-related protein; GPCR, G protein-coupled receptor; MC4R, melanocortin-4 receptor;  $\alpha$ -MSH,  $\alpha$ -melanocyte-stimulating hormone; POMC, pro-opiomelanocortin.

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**Figure 1**

Obesity-associated mutations in the human MC4R (hMC4R) are shown in gray circles, and corresponding amino acid changes are indicated with an arrow. The 6 mutations in the extracellular N-terminal domain R7H, T11A,S, and R18C,H,L, have been found in 6 different obese patients (13–15, 28). The key acidic residues E100, D122, and D126 required for activation or repression of the receptor (22, 23, 34) are shown in black. \*S30F/G252S and \*\*Y35X/D37V are double mutants. Double-sided arrows indicate deletions.

activity is also required for the maintenance of the anorexigenic catabolic state and the prevention of obesity in humans.

## Results

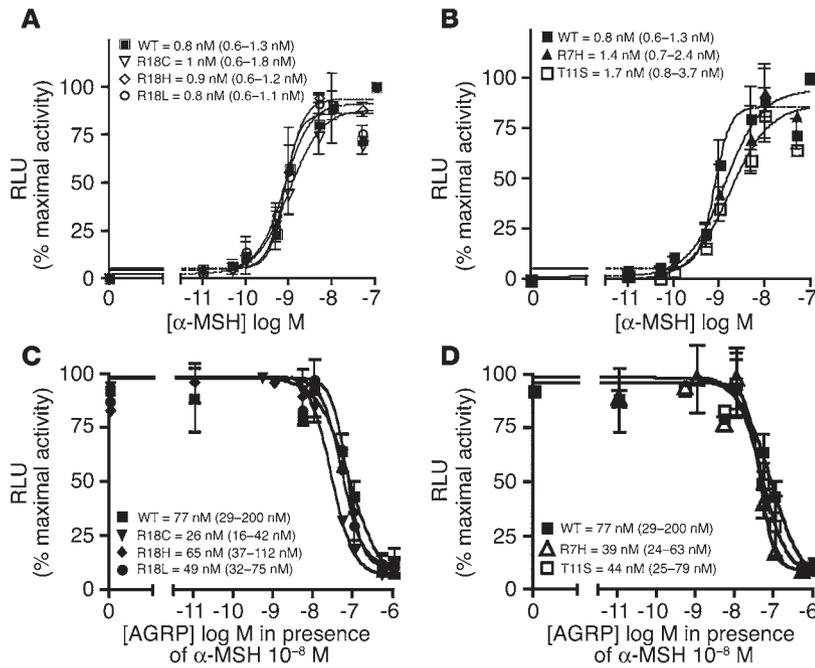
*N-terminal MC4R mutations do not impair ligand action.* To define the molecular basis of the regulation of long-term energy homeostasis by MC4R, we and others have systematically studied the functional alterations of naturally occurring obesity-causing MC4R mutations (13–17). Arguments for the pathogenicity of these mutations is based on the frequency of rare, functionally relevant, nonsynonymous mutations in severely obese children and adults (BMI  $\geq 35$  kg/m<sup>2</sup>) versus nonobese 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. Most of the identified point mutations in MC4R lie in the extracellular and intracellular loops and transmembrane segments (Figure 1). These mutated receptors have impaired ligand binding, internalization, and cellular localization, and all the mutations described to date ultimately reduce the ability of the receptor to respond to  $\alpha$ -MSH (16, 25–27).

A novel class of point mutations in MC4R lie within the extracellular N-terminal domain (13–15, 28) (Figure 1). Mutations at these amino acids (or at any position in the N-terminal domain of the molecule) have never been detected in nonobese control individuals (15, 29). Position T11 is one of the rare MC4R amino acids at which two different obesity-associated mutations have been found (T11S and T11A) (13, 14), and R18 is the only

MC4R amino acid at which three different obesity-associated mutations have been detected (R18C, R18H, R18L).

Unlike most MC4R point mutations associated with human obesity, mutations in the N-terminal domain show no defect in the response to  $\alpha$ -MSH (Figure 2A). Greater repression of MC4R activity by AGRP could also result in increased food intake. AGRP competed against  $\alpha$ -MSH at all mutant receptors at the same concentration as the WT receptor (Figure 2B), however, indicating that the N-terminal mutations do not affect AGRP antagonism.

*N-terminal MC4R mutations have lowered constitutive activity.* Decreased constitutive signaling by MC4R could reduce tonic inhibition of feeding by the melanocortin system. We therefore compared the constitutive activities of WT and N-terminal mutant receptors in transiently transfected HEK293 cells. All of the mutants exhibited a significant decrease in constitutive activity (Figure 3A). This effect was independent of membrane expression, as judged by measuring constitutive activity as a ratio of cell-surface expression (Figure 3B), which had been previously demonstrated for other G protein-coupled receptors (GPCRs) (30–32). The decrease in the ratio of constitutive activity to membrane expression in the N-terminal domain mutants was also observed in stably transfected cell lines for R7H, R18C, and T11S (data not shown). Using the N-terminal R18C mutation, we further characterized the decrease in constitutive signaling. The basal activity of R18C was significantly reduced upon overnight accumulation of cAMP, indicating that this effect is preserved even under chronic conditions (Figure 3C). Since MC4R expression in the hypothalamus may be relatively low



**Figure 2**

N-terminal MC4R mutation does not impair ligand action. WT and mutant MC4Rs were transiently transfected into HEK293 cells stably expressing luciferase under the control of a cAMP response element promoter. Cells were stimulated with increasing amounts of  $\alpha$ -MSH (A and B) or with  $\alpha$ -MSH  $10^{-8}$  M and increasing concentrations of AGRP (C and D), and luciferase activity was measured to generate dose response curves. Data were normalized to maximal  $\alpha$ -MSH stimulation after subtraction of basal activity and were fitted by nonlinear regression. Each point represents the mean  $\pm$  SEM of at least two independent experiments. The best-fit estimate of the EC<sub>50</sub> (A and B) or the IC<sub>50</sub> (C and D) and their 95% confidence intervals are indicated for each data set. There was no statistical difference between the WT EC<sub>50</sub> and EC<sub>50</sub> for each of the mutants (A and B) nor between the WT IC<sub>50</sub> and IC<sub>50</sub> for each of the mutants (C and D; for all in A–D,  $P > 0.05$  in an F test under the null hypothesis “log EC<sub>50</sub>/IC<sub>50</sub> same for WT and mutants”). RLU, relative luminescence units.

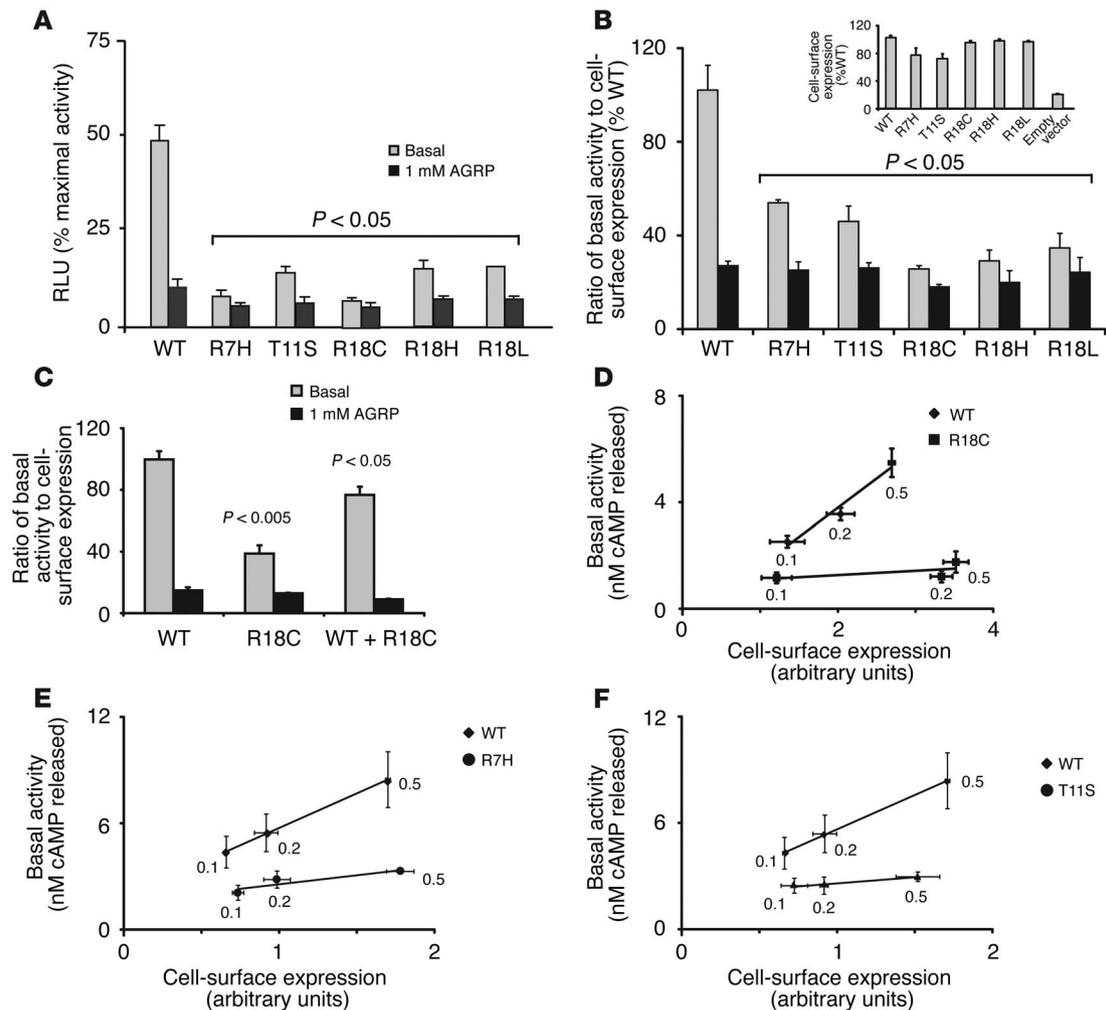
(33), we compared the basal activities of the two receptors at various cell-surface expression levels. As expected, the constitutive activity of the WT receptor increased linearly with cell-surface expression, whereas the R18C receptor showed low constitutive activity regardless of the expression level (Figure 3D). These observations were also found to be true for the R7H and T11S mutants (Figure 3, E and F). Finally, the R18C mutation does not exert a dominant negative effect on the WT receptor as judged by coexpressing the R18C and WT receptors (not shown).

*The N-terminal domain of MC4R is required for its constitutive activity.* Since obesity-associated mutations in the N-terminal domain of MC4R decrease the constitutive activity of the receptor, we next tested the requirement of the N-terminal domain for the maintenance of this tonic food intake–inhibiting constitutive activity. To this end, we generated a deletion mutant that lacks the first 24 amino acids of the receptor (ATG24-MC4R) and studied its effect on basal activity. Since this deletion impaired membrane localization (not shown), we added a signal peptide that promotes cell-surface expression of ATG24-MC4R and compared its basal activity to that of a similarly engineered WT receptor. Both receptors were expressed equally well at the cell membrane (Figure 4A). The N-terminal deletion abolished basal activity (Figure 4B), but did not affect the response to  $\alpha$ -MSH stimulation (Figure 4C). Thus, the N-terminal domain is required for the constitutive activity of MC4R.

*The WT N-terminal domain can transactivate the constitutive activity of MC4R.* The above experiments suggested that the N-terminal domain may act as an intramolecular agonist that maintains the constitutive activity of MC4R. We therefore tested the capacity of the N-terminal domain of MC4R to rescue the low constitutive activity of the ATG24-MC4R in a transactivation experiment. We constructed two fusion proteins containing a C-terminal CD8 single transmembrane domain and either the WT or R18C N-terminal domain and ensured that they were expressed at the cell-surface (Figure 4D, inset). Coexpression of the WT fusion protein and ATG24-MC4R gave a robust rescue of constitutive activity

(Figure 4D) to the level seen with the WT receptor. Coexpression of the R18C fusion protein and ATG24-MC4R, or of the N-terminal domain fusion proteins and WT MC4R, however, had no appreciable effect on basal activity (Figure 4D). Thus, the N-terminal domain of MC4R functions as a tethered partial agonist that generates the constitutive activity of MC4R, and this activity is lost in the obesity-associated R18C mutation.

*The MC4R N-terminal domain does not mimic  $\alpha$ -MSH but does require the conserved D126 residue to activate constitutive signaling of the receptor.* We determined whether the constitutive activity mediated by the N-terminal domain and the full activation of the receptor by  $\alpha$ -MSH used the same conformational pathway (i.e., whether the N-terminal domain mimics the molecular action of the natural agonist). Three conserved acidic residues (E100, D122, and D126) in transmembrane helices 2 and 3 of MC4R interact with the positively charged side chains of  $\alpha$ -MSH (22, 23, 34). Notably, four obesity-associated N-terminal mutations affect the positively charged R7 and R18 residues in the MC4R. Alanine substitution of E100, D122, and D126 abolished  $\alpha$ -MSH activation of the receptor (22, 23, 34) (Figure 5A) but either increased (E100A and D122A) or slightly decreased (D126A) constitutive activity (Figure 5B). The constitutive activities of the E100A and D122A receptors were suppressed by AGRP or by the presence of the R18C mutation, as observed for the WT receptor. Thus, unlike full activation of the receptor mediated by  $\alpha$ -MSH, the partial agonism mediated by the N-terminal domain and the inverse agonism mediated by AGRP do not require residues E100 and D122. These data indicate that the N-terminal domain does not mimic the action of  $\alpha$ -MSH when activating constitutive signaling from the receptor. The R18C mutation was unable to suppress the basal activity of the D126A receptor (Figure 5B), however, suggesting that the D126 residue is required for N-terminal domain-mediated basal activity. Since it is required both for full agonism and constitutive activity of MC4R, D126 appears to be a point of convergence in the constitutive activation by the N-terminal domain and the full activation by the natural agonist  $\alpha$ -MSH.

**Figure 3**

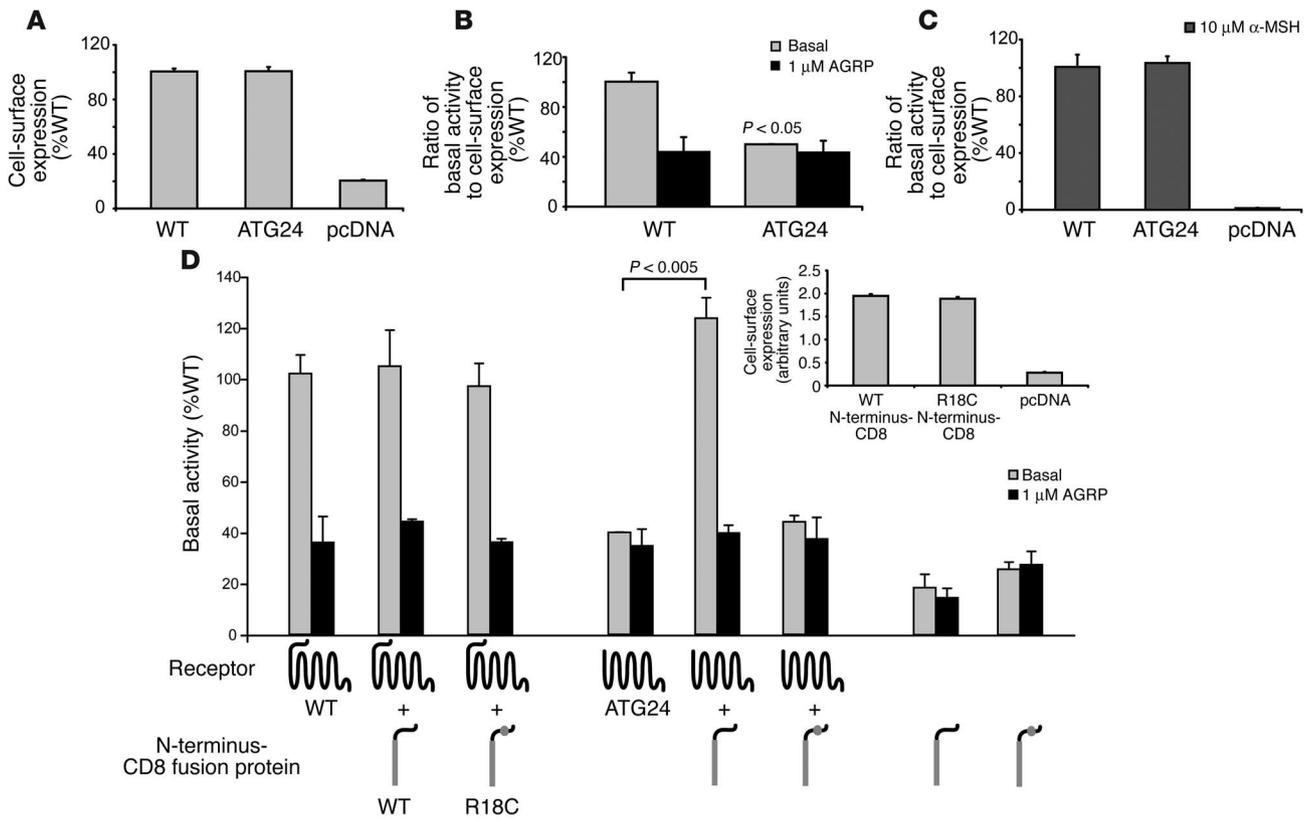
Mutations in the N-terminal domain of MC4R result in reduced constitutive activity. (A) Basal activities of WT and mutated MC4R were assayed by analyzing their ability to activate the expression of a cAMP-induced luciferase reporter gene under basal unstimulated conditions or in response to AGRP. The data are normalized to maximal stimulation obtained in presence of 8Br-cAMP (1  $\mu$ M) and to *Renilla* luciferase activity for assessment of transfection efficiency. (B) Ratios of cAMP accumulation (measured by CatchPoint assays) to membrane expression (measured by ELISA) were determined on the same batch of transiently transfected cells for WT and mutant receptors. Data are expressed as percentage of WT activity as the means of quadruplicate determinations ( $\pm$  SEM) and are the averages of 3 independent experiments. Inset: There were no differences in membrane expression between the WT and mutant receptors. The ratio of basal activity to membrane expression was significantly reduced for all N-terminal domain mutants ( $P < 0.05$ ). (C) The ratio of basal activity to cell-surface expression was measured in the WT and R18C receptors under acute (2-hour incubation) and chronic (overnight incubation) basal conditions. (D–F) Basal activity relative to cell-surface expression of WT and R18C in transiently transfected HEK293 cells. Increasing cell-surface expression was obtained by transfecting 0.1, 0.2, and 0.5  $\mu$ g DNA in 6-well tissue culture dishes. WT MC4R-transfected cells showed a linear increase in basal activity with increasing membrane expression (slope = 2.2;  $r^2 = 0.98$ ). In contrast, R18C, R7H, and T11S show a lower increase in basal activity despite an increase in cell-surface expression similar to the WT receptor.

## Discussion

Genetic studies in mice and humans have established the essential role of the central leptin-melanocortin axis in the maintenance of long-term energy homeostasis (35) and identified MC4R as a prime target for therapeutic intervention in obesity. Our study demonstrates that the N-terminus of MC4R is required for the constitutive activity of the receptor and that loss of this constitutive activity is associated with obesity in humans (Figure 6). Deletion and *trans*-rescue experiments demonstrate that the N-terminal domain of MC4R functions as a tethered intramolecular ligand that maintains the constitutive activity of this receptor. The constitutive activation by the N-terminal domain is indepen-

dent of the full activation provided by  $\alpha$ -MSH, since the N-terminal domain is not required for the  $\alpha$ -MSH-mediated effects.

The role of the N-terminal domain in receptor activation has been clearly established for several GPCRs. For instance, N-termini of the PAR receptor family act as tethered full agonists after protease cleavage by thrombin or other serine/threonine proteases (36). To our knowledge, however, MC4R is the first example of a receptor whose N-terminal domain is required to maintain constitutive signaling and may have therapeutic implications for the pharmacological treatment of obesity (Figure 6). Our studies support the notion that a ligand that elicits a low, sustained level of MC4R activation (mimicking its constitu-



**Figure 4**

The N-terminal domain of MC4R acts as a tethered ligand to maintain constitutive activity of the receptor. (A) Cell-surface expression of WT MC4R and the ATG24 receptor. Empty vector (pcDNA 3.1) was transfected as a control to detect background. (B) The ratio of activity (as measured by cAMP accumulation) to cell-surface expression (as measured by ELISA) was measured in the WT and the ATG24-MC4R. (C) After stimulation with 10 μM α-MSH, cAMP accumulation was measured in cells transfected with WT MC4R, ATG24-MC4R, or empty vector (pcDNA 3.1). (D) HEK293 cells were cotransfected with the WT receptor or the ATG24 receptor and either the WT N-terminus–CD8 fusion protein or the R18C N-terminus–CD8-fusion protein. The ratio of receptor to fusion protein was maintained at 1:10. To measure constitutive activity of the WT and ATG24 receptors alone, pcDNA 3.1 was transfected in place of the fusion proteins to keep the amount of total transfected DNA the same. Inset: Cell-surface expression of WT MC4R and the ATG24 receptor. Empty vector (pcDNA 3.1) was transfected as a control to detect background.

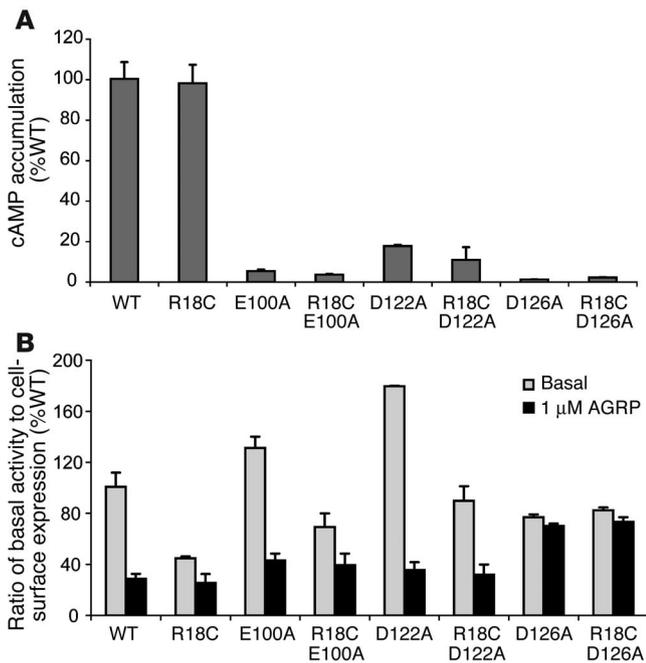
tive activity) would be more effective than a potent ligand that induces receptor internalization and desensitization.

The only MC4R mutational hot spot found to date in severely obese patients (Figure 1) is located at position R18 with three different missense substitutions in the N-terminal domain that decrease the constitutive activity of the receptor. Two different obesity-associated mutations have been identified at position T11 (13, 14). None of these N-terminal domain mutations have been found in nonobese controls (15, 29). Taken together, these findings argue in favor of a physiological role for constitutive activity in the maintenance of the anorexigenic catabolic state required for normal body weight in humans (Figure 6). Point mutations in the N-terminal domain from obese patients reduce MC4R constitutive activity 50% or greater. The physiological significance of the decrease in constitutive activity observed in vitro is strengthened by the demonstration that variations of the same order in the level of constitutive activation of the closely related MC1R lead to drastic changes in coat color in mammals (34). Indeed, the preservation of MC1R signaling in the absence of ligand, as judged by eumelanin synthesis in *Pomc*<sup>-/-</sup> mice, strongly suggests a physiological role for its constitutive activity in rodents (37). In humans, numerous studies have shown the pathological consequences of abnormally

high basal signaling, most notably in the thyroid-stimulating hormone receptor (TSHR) and the luteinizing hormone receptor (LHR) leading to adenomas associated with hyperthyroidism and male precocious puberty, respectively (38). By showing that mutations causing decreased basal signaling in MC4R result in a pathological state, our data provide the first evidence for the essential role of constitutive GPCR signaling in normal human physiology.

Since the constitutive activity of MC4R is inhibited by AGRP in vitro, our findings support a model in which the in vivo effects of AGRP are also mediated through its inverse agonist effects on MC4R. Precise comparisons of the obesity phenotypes of *Pomc*<sup>-/-</sup> and *Mc4r*<sup>-/-</sup> mice in the same background, as well as the study of double-null *Pomc*<sup>-/-</sup>*Agrp*<sup>-/-</sup> mice, expected from our results to be less obese than the *Pomc*<sup>-/-</sup> mice, should provide valuable information to support this model. Interestingly, a recent report indicates that *Pomc*<sup>-/-</sup> mice did not demonstrate elevated neuropeptide Y (NPY) levels in the dorsomedial hypothalamus as seen in *Mc4r*<sup>-/-</sup> mice (11), suggesting that constitutive activity of MC4R might be sufficient to maintain NPY signaling in the dorsomedial hypothalamus.

It should also be noted that while studies in rodents have been key for the discovery and study of the leptin-melanocortin system



**Figure 5** The N-terminal domain uses a subset of the acidic residues used by  $\alpha$ -MSH. (A) The cAMP accumulation was measured in cells transiently transfected with WT and mutated receptors after stimulation with 10  $\mu$ M  $\alpha$ -MSH. (B) The ratio of receptor activation (as measured by cAMP accumulation) to cell-surface expression (as measured by ELISA) was measured in the WT and the mutated receptors.

and genetic studies in humans have been largely confirmatory, subtle differences such as the degree of in vivo constitutive activity of MC4R and its importance for the maintenance of the long-term energy balance might reflect species-specific differences in this regulatory system. The study of the effects of naturally occurring obesity-causing mutations offers a unique approach to unraveling the molecular mechanisms underlying this regulation in humans in whom therapies will ultimately be used.

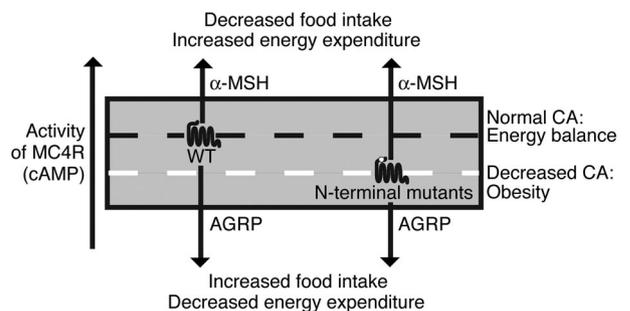
**Methods**

*MC4R cloning and mutagenesis.* Since *MC4R* is a single-exon gene, we initially amplified WT and mutated *MC4R* genes from the genomic DNA of patients with the R7H, T11S, and R18C-H-L mutations as described (26). These experiments were carried out with approval of the institutional review board on human research (UCSF Committee on Human Research). The genes were cloned in the vector pcDNA 3.1 (Invitrogen Corp.). ATG24-MC4R was made by PCR deletion mutagenesis as described (39). The prolactin signal peptide and a FLAG epitope tag were added to the WT and ATG24-MC4R genes by standard recombinant DNA methods. Point mutations in the *MC4R* gene were introduced with a site-directed mutagenesis kit (QuikChange; Stratagene) and sequenced to confirm the substitutions. The ATE-CD8 plasmid was a gift from Shaun Coughlin (UCSF, San Francisco, California, USA) (40). The N-terminal domain CD8 fusion proteins were constructed with the N-terminal 48 amino acids of MC4R and the single transmembrane domain of CD8 by standard recombinant DNA methods and PCR. The prolactin signal sequence and the FLAG tag were added to ensure localization to the plasma membrane and detection by ELISA.

*cAMP assays for MC4R activity.* For dose response curves, HEK293 cells were maintained in  $\alpha$ -MEM supplemented with 10% calf serum

(Hyclone Laboratories) containing L-glutamine and penicillin/streptomycin. WT and mutant *MC4R* were transfected with Effectene (Qiagen Inc.) into HEK293 cells expressing a luciferase reporter gene under the control of the cAMP response element (26). A plasmid containing the *Renilla* luciferase gene was cotransfected for assessment of transfection efficiency. Thirty-six to 48 hours after transfection, cells were incubated in stimulation medium (26) with increasing concentrations of  $\alpha$ -MSH (Sigma-Aldrich) and AGRP (Phoenix Pharmaceuticals Inc.) for 6 hours at 37°C in a 5% CO<sub>2</sub> incubator. Luciferase activity was measured with a luciferase assay system (Steady-Glo; Promega Corp.) in a microplate luminescence counter (Packard Instrument Co.). Three replicates were used for each condition, and the experiments were repeated at least twice. For statistical analysis best-fit estimates of the EC<sub>50</sub>, IC<sub>50</sub>, and their 95% confidence intervals were obtained by nonlinear regression fitting of the sigmoidal dose response curves using Prism 4 software. For statistical comparison of each mutant curve with the WT curve, data are first fitted independently and then globally to find a shared best-fit value for the EC<sub>50</sub>/IC<sub>50</sub>. Results are compared by an F test. For measurement of constitutive activity, cAMP production was measured directly using a cAMP kit (CatchPoint; Molecular Devices). Transiently transfected cells were plated at 10<sup>5</sup> per well into 96-well plates coated with poly-D-lysine (Sigma-Aldrich). Forty-eight hours after transfection, cells were rinsed in Krebs-Ringer bicarbonate buffer containing glucose (KRBG; Sigma-Aldrich). Cells were then incubated in prestimulation medium containing 0.75 mM 3-isobutyl-1-methylxanthine in KRBG for 10 minutes at room temperature. Cells were then stimulated with PBS (basal conditions), 10  $\mu$ M  $\alpha$ -MSH (agonist), or 1  $\mu$ M AGRP (inverse agonist) for 2 hours at 37°C, after which cells were lysed, and cAMP accumulation was assayed according to the CatchPoint protocol. The cAMP generated under the different experimental conditions was interpolated from a cAMP standard curve for each experiment. Four to six replicates were used for each condition, and all experiments were repeated at least twice.

*ELISA.* Cell-surface expression of receptors was measured by an ELISA that detects the extracellular FLAG tag, as previously described (41). Transiently transfected cells were plated at 10<sup>5</sup> per well into 96-well plates coated with poly-D-lysine (Sigma-Aldrich). Forty-eight hours after transfection, cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) for 10 minutes at 4°C. After two washes in PBS, the cells were incubated in 1  $\mu$ g/ml M2 anti-FLAG Ab (Sigma-Aldrich) for 2 hours at room temperature, washed twice in PBS, and



**Figure 6** Model depicting reduced constitutive activity (CA) of N-terminal domain MC4R mutants and its effect on energy balance. The constitutive activity of WT MC4R is responsible for the tonic inhibition of food intake and determines the set point for normal energy balance. Mutations in the N-terminal domain of the receptor have reduced constitutive activity. These mutant receptors are still fully responsive to the satiety signal provided by  $\alpha$ -MSH and the orexigenic signal provided by AGRP, but the reduction in tonic inhibition of food intake is sufficient to cause obesity.



incubated for 1 hour at room temperature with HRP-conjugated goat anti-mouse (1:1,000; Bio-Rad Laboratories). Cells were then washed three times in PBS, and 0.2 ml of 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) liquid substrate (Sigma-Aldrich) was added to each well. After 15–60 minutes, the optical density was read at 405 nm in a spectrophotometer. Each experiment included four replicates per condition and was repeated at least twice.

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