CHAPTER FOURTEEN

Constitutive Activity of Neural Melanocortin Receptors

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Contents

Abstract

The two neural melanocortin receptors (MCRs), melanocortin-3 and -4 receptors (MC3R and MC4R), are G protein-coupled receptors expressed primarily in the brain that regulate different aspects of energy homeostasis. The MCRs are unique in having endogenous antagonists, agouti and agouti-related protein (AgRP). These antagonists were later shown to be inverse agonists. The MC3R has little or no constitutive activity, whereas the MC4R has significant constitutive activity that can easily be detected. We describe herein methods for detecting constitutive activities in these receptors and small molecule ligands as inverse agonists. AgRP is an inverse agonist for both MC3R and MC4R. We also provide models for the constitutively active MC4R mutants.

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Methods in Enzymology, Volume 484
ISSN 0076-6879, DOI: 10.1016/S0076-6879(10)84014-5

1. Introduction

Obesity is an epidemic in the United States and an increasing health problem worldwide. Current therapeutic approaches, including pharmaceutical, surgical, and lifestyle interventions, are not effective or associated with significant side effects. Identifying novel targets for obesity treatment is a very active area of research.

The two neural melanocortin receptors (MCRs), the melanocortin-3 and -4 receptors (MC3R and MC4R), were recently found to regulate different aspects of energy homeostasis (Cone, 2005; Tao, 2005). The primary role of the MC3R is regulation of feed efficiency, the amount of energy ingested that is stored as fat in the body (Butler *et al.*, 2000; Chen *et al.*, 2000). The MC4R regulates both food intake and energy expenditure (Huszar *et al.*, 1997), with the effect of food intake accounting for 60% of the effect on body weight (Balthasar *et al.*, 2005; reviewed in Tao, 2010). Human genetic studies provided further evidence that these two receptors are important in maintaining energy homeostasis in humans. The role of *MC3R* in human obesity pathogenesis is controversial, with some studies supporting (Feng *et al.*, 2005; Lee *et al.*, 2002, 2007; Mencarelli *et al.*, 2008; Tao, 2007; Tao and Segaloff, 2004) and another study refuting (Calton *et al.*, 2009) a causal relationship (earlier studies reviewed in (Tao, 2005)). The role of *MC4R* in human obesity pathogenesis is undisputed. Since the original reports of *MC4R* mutations associated with childhood obesity (Vaisse *et al.*, 1998; Yeo *et al.*, 1998), more than 150 distinct mutations in the *MC4R* have been identified from populations of different ethnic backgrounds (reviewed in Tao, 2009). Mutations in the *MC4R* are the most common monogenic form of obesity (Farooqi *et al.*, 2003).

The MC3R and MC4R are G protein-coupled receptors that are positively coupled to the adenylyl cyclase system. Therefore, receptor activation will lead to increased cyclic AMP (cAMP) production. Receptor activation can be either due to ligand binding or mutation. A mutation that causes the receptor being activated in the absence of ligand is called constitutively active mutation. Indeed, the wild-type (WT) MC4R has some basal activity, whereas the MC3R has little or no basal activity (Tao, 2007). We describe here the methods used for measuring the constitutive activities of WT and mutant receptors.

2. Signaling Assay for the Neural Melanocortin Receptors

2.1. Culture and transfection of HEK293T cells

Human embryonic kidney (HEK) 293T cells, highly transfectable derivative of the HEK293 cell line, constitutively express the simian virus 40 (SV40) large T antigen. These cells, obtained from American Type Culture

Collection, were maintained in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, at 37 °C in humidified air containing 5% CO_2. Although fetal bovine serum was suggested by American Type Culture Collection, we found that newborn calf serum can fully support these cells, resulting in significant savings in cell culture cost. For maintenance of culture, cells were cultured in 75 cm^2 flasks. For transfection, cells were plated into 6-well clusters (Corning, Corning, NY, cat # 3506) coated with 0.1% gelatin.

Transient transfection was routinely performed using the calcium phosphate precipitation method (Chen and Okayama, 1987). Other investigators have used various commercial transfection reagent kits. We have found that in HEK293T cells, the calcium phosphate precipitation method works well with minimal cost compared with commercial kits. For each well in the 6-well cluster, add the following sequentially:

86 μl sterile double-distilled water
10 μl of 2.5 *M* calcium chloride
4 μl of plasmid DNA (0.25 mg/ml in sterile water)

After mixing these components by gentle swirling, 100 μl of 2× BSS is added to the bottom of the tube (2× BSS consists of 280 m*M* sodium chloride, 1.5 m*M* Na_2HPO_4, and 50 m*M* of BES (*N*,*N*-bis[2-hydroxyl]-2-aminoethane sulfonic acid, Sigma cat # B9879), pH adjusted to 6.95 with sodium hydroxide). After these components are mixed, the tube is left in the hood for 15 min. Then, the content is combined with 1.8 ml growth media and added into each well.

Cells can be used for signaling assays 24–96 h after transfection. We have routinely used cells at 48 h after transfection when maximal expression of the receptors is frequently observed.

2.2. Signaling assay for measuring receptor activation

Because the primary signaling pathway of the MC3R and the MC4R is activation of the stimulatory G protein G_s, resulting in activation of the adenylyl cyclase and increased intracellular cAMP levels, direct or indirect measurement of cAMP levels is the most commonly used method for measuring signaling activities of these receptors. Indirect measurement of cAMP measures the reporter gene activity driven by increased intracellular cAMP levels. A commonly used reporter gene is luciferase. Before the assay, cells are serum starved for several hours (for example, 8 h), and then different concentrations of ligands are added to the cells and incubated for 16 additional hours to allow the gene transcription and translation to occur. Finally, the enzyme activity is measured, usually using a commercially available kit. If no ligand is added, the activity measured is the basal activity

of the receptor. This assay is very sensitive due to additional amplification, from cAMP to increased transcription and translation of luciferase.

Our lab has been using direct measurement of cAMP to monitor signaling. Cells stimulated with ligands are lysed with perchloric acid. For measurement of intracellular cAMP, 0.5 N perchloric acid with 180 μg/ml theophylline (an inhibitor of phosphodiesterase to block the breakdown of cAMP) is used (Tao and Segaloff, 2003). Lysate is neutralized with 0.72 *M* KOH/0.6 *M* $KHCO_3$. After a 10-min centrifugation at 4 °C, the supernatant is saved for radioimmunoassay.

Before assay, cAMP samples are acetylated to increase sensitivity and specificity and to reduce interference. This is done as follows: add 20 μl of 5 N KOH to a 12 × 75 mm glass tube with 500 μl standards or samples, add 5 μl of acetic anhydride, immediately vortex for 3–5 s, and let it sit at room temperature for 30 min. Then the samples are placed on ice and used for assay within an hour.

The cAMP standard used is from Sigma (cat # A9501). Succinyl cAMP tyrosine methyl ester (Sigma cat # M2257) is iodinated with chloramines T method and used as the tracer. Bulk cAMP–antibody can be purchased from various commercial sources. We obtained our antibody from Strategic Biosolutions (Newark, DE). The radioimmunoassay is performed as originally described in Steiner *et al.* (1969) except that polyethylene glycol 8000 is used for precipitation of the cAMP–antibody complex (Fan *et al.*, 2008b). This method involves the use of radioactive material. Appropriate local regulations regarding safety training and proper disposal should be strictly followed.

3. Naturally Occurring Constitutively Active MC4R Mutants

MC4R activation results in decreased food intake and increased energy expenditure. Loss-of-function mutations in the *MC4R* result in obesity (Tao, 2009). Loss of constitutive activity is suggested to be one mechanism that mutations in *MC4R* cause obesity (Srinivasan *et al.*, 2004). We showed that indeed some MC4R mutants have decreased basal activities whereas some mutants retain normal basal activities (Fan and Tao, 2009; Rong *et al.*, 2006; Roth *et al.*, 2009; Tao and Segaloff, 2005). Constitutively active mutations in the *MC4R* are expected to be associated with a constitutional lean phenotype, perhaps even anorexia nervosa. Indeed, the higher constitutive activity of I251L was proposed to be responsible for the negative association of this variant with body mass index (Xiang *et al.*, 2006). Paradoxically, six constitutively active mutations were identified from obese patients, including H76R, S127L, D146N,

H158R, P230L, and L250Q (reviewed in Tao, 2010). The reason for these mutations in causing obesity is not well established (Tao, 2008). The constitutive activities of S127L and P230L are modest, less than threefold (Fan and Tao, 2009). However, the constitutive activities of the other four mutants are substantial, 6- to 15-fold higher than the basal activity of the WT MC4R (Hinney *et al.*, 2006; Tao, 2010; Vaisse *et al.*, 2000). We transfected HEK293T cells with increasing concentrations of plasmids of H76R, D146N, and L250Q. As shown in Fig. 14.1, the basal cAMP levels increase with increasing concentrations of plasmids transfected, reaching a plateau at higher concentrations for L250Q.

3.1. Inverse agonism of AgRP at the MC4R

The MCRs are unique in having two endogenous antagonists, agouti and AgRP (agouti-related protein). Agouti is an antagonist for melanocortin-1 receptor, whereas AgRP is an antagonist for MC3R and MC4R (reviewed in Cone, 2006). Previous studies have shown that a fragment of AgRP, AgRP (83–132), is an inverse agonist for WT human MC4R (Nijenhuis *et al.*, 2001) and a constitutively active mouse MC4R mutant (Haskell-Luevano and Monck, 2001). Later studies showed that full-length AgRP, a smaller fragment of AgRP, AgRP (87–120), as well as two short peptides derived from AgRP, are all inverse agonists at the MC4R (Chai *et al.*, 2003).

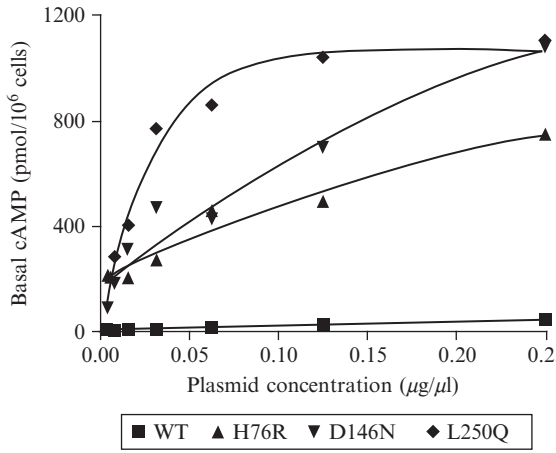

Figure 14.1 Basal activities of three naturally occurring *MC4R* mutations, H76R, D146N, and L250Q. Different concentrations of plasmids were transfected into HEK293T cells. Empty vector pcDNA3 was used to normalize the amount of plasmid DNA added to each well. Basal cAMP levels were measured 48 h after transfection in the presence of phosphodiesterase inhibitor for 75 min. Cyclic AMP levels were measured with radioimmunoassay (Fan *et al.*, 2008a).

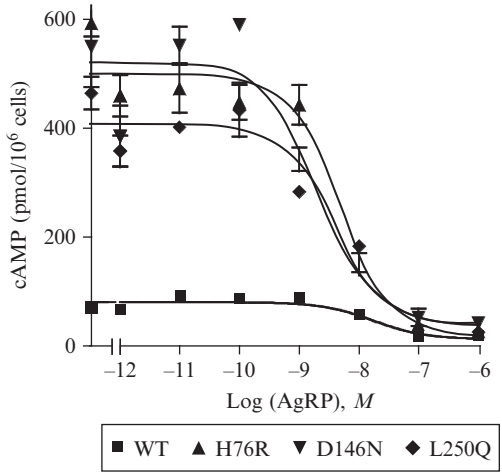

Figure 14.2 Inverse agonism of AgRP at the MC4R. Cells expressing WT or constitutively active MC4R mutants, H76R, D146N, and L250Q, were treated with different concentrations of AgRP. Cyclic AMP levels were measured with radioimmunoassay (Fan *et al.*, 2008a).

Table 14.1 Inverse agonism of AgRP and Ipsen 5i at the MC4R

	AgRP		Ipsen 5i	
MC4R	EC_{50}	Basal activity remaining at 10^{-6} *M* AgRP	EC_{50}	Basal activity remaining at 10^{-6} *M* Ipsen 5i
WT	23.46 ± 4.56	21 ± 3	17.46 ± 3.23	33 ± 5
H76R	5.56 ± 0.51	4 ± 0	9.28 ± 1.00	44 ± 4
D146N	3.50 ± 1.00	6 ± 1	5.43 ± 1.19	36 ± 3
L250Q	2.22 ± 0.93	6 ± 1	16.32 ± 6.32	35 ± 1

Data shown are from three or more experiments, with the mean ± standard error of the mean listed in the table.

In our experiments with WT and three constitutively active mutant (CAM) MC4Rs, we showed that AgRP is a potent inverse agonist (Fig. 14.2 and Table 14.1). In WT MC4R, AgRP is a full inverse agonist, decreasing basal cAMP level to the detection limit of our radioimmunoassay. In the CAM MC4Rs, AgRP also decreased the basal cAMP levels to just above the detection limit of our assays (Fig. 14.2 and Table 14.1). Therefore, AgRP can also be considered as a full inverse agonist in the CAM MC4Rs.

3.2. Small molecule MC4R inverse agonists

We recently reported that ML00253764, 2-[2-[2-(5-bromo-2-methoxy-phenyl)-ethyl]-3-fluorophenyl]-4,5-dihydro-1*H*-imidazole, is a MC4R inverse agonist, decreasing the basal activities of two constitutively active MC4R mutants, H76R and D146N (Tao, 2010). However, it seems that ML00253764 is only a partial inverse agonist. At 10 μ*M* concentration, the WT MC4R does not have any residual constitutive activity. However, the two mutants still retain high constitutive activities. The low affinity of ML00253764 for the MC4R might be responsible for the partial inverse agonism. Originally described by Vos *et al.* (2004) at Millennium Pharmaceuticals, the Ki of ML00253764 for human MC4R is 0.16 μ*M*.

We recently tested the inverse agonist activity of another small molecule antagonist, Ipsen 5i. Described by Roubert and colleagues at Ipsen, the Ki of this compound for the MC4R is 2 n*M* (Poitout *et al.*, 2007). Enzo Life Sciences International, Inc. (Plymouth Meeting, PA) synthesized the compound for us. We transfected HEK293T cells with WT or mutant MC4Rs. Forty-eight hours after transfection, cells were incubated with different concentrations of Ipsen 5i for 1 h. As shown in Fig. 14.3, intracellular cAMP levels decrease with increasing concentrations of Ipsen 5i for both WT and mutant MC4Rs, H76R, D146N, and L250Q. The maximal inhibition ranged from 56% to 63% (Table 14.1). Therefore, Ipsen 5i is also a partial inverse agonist for the MC4R.

4. Inverse Agonism of AgRP at the MC3R

Although it is well known that AgRP is an inverse agonist for the MC4R (see above), it was not known whether this endogenous antagonist is also an inverse agonist for the MC3R. The WT MC3R has little or no constitutive activity (Tao, 2007) and no constitutively active MC3R mutant had been reported. Therefore, there was no tool to study this question. We recently showed that F347A has increased basal activity, with a basal activity about 6.9-fold that of the WT MC3R (Wang and Tao, 2010). Using this newly found tool, we showed herein that AgRP is also an inverse agonist for the MC3R, with EC_{50} of 0.17 n*M* (Fig. 14.4). When the cells were treated with 1 μ*M* AgRP, the basal cAMP of F347A was reduced to 33% that of the untreated cells.

5. Computational Modeling of the Constitutively Active MC4R Mutants

The recent X-ray studies revealed the structure of opsin in complex with the C-terminal peptide of transducin stabilizing the photoactivated structure of rhodopsin (Scheerer *et al.*, 2008). This structure may be

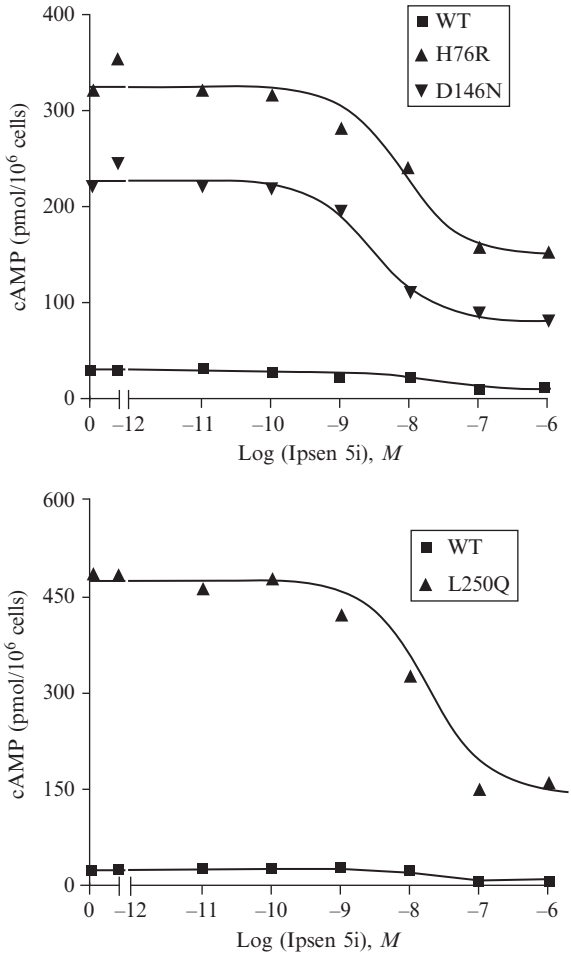

Figure 14.3 **Partial inverse agonism of Ipsen 5i at the MC4R. Cells expressing WT or constitutively active MC4R mutants, H76R, D146N, and l250Q, were treated with different concentrations of Ipsen 5i. Cyclic AMP levels were measured with radioimmunoassay (Fan *et al.*, 2008a).**

regarded as a prototype for the general activated structure of the rhodopsin-like GPCRs. We assumed that the X-ray structure of β_2-adrenoreceptor (Cherezov *et al.*, 2007; the PDB entry 2RH1) may be a template for the ground state of MC4R and the X-ray structure of opsin (the PDB entry 3DQB) may be a template for the activated state of MC4R.

Details of the modeling protocol are described elsewhere (Nikiforovich and Baranski, 2010). Briefly, modeling of the transmembrane (TM) regions of MC4R and the mutants involved several main steps: sequence alignment

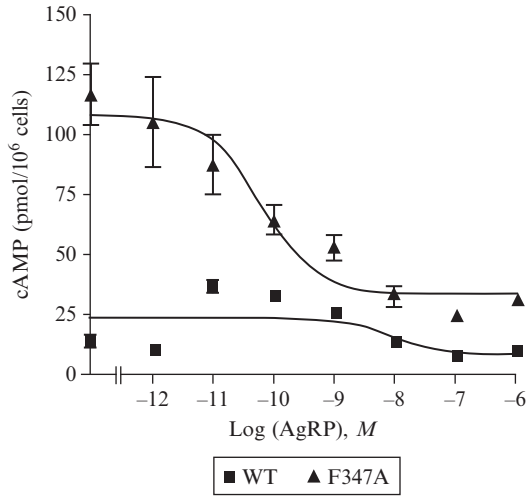

Figure 14.4 Inverse agonism of AgRP at the MC3R. Cells expressing WT or constitutively active MC3R mutant, F347A, were treated with different concentrations of AgRP. AgRP decreases the basal activities of F347A to that of the WT MC3R, which is devoid of basal activity. Cyclic AMP levels were measured with radioimmunoassay (Fan *et al.*, 2008a).

to the selected template to define boundaries of TM helices; conformational calculations for individual TM helices; structural alignment to the selected templates; and final energy minimization with optimization of the side chain packing.

Our modeling results revealed specific changes in system of contacts between the cytoplasmic parts of TM6 and TM5 upon transition from the presumed ground state to the presumed activated state of MC4R. Namely, the cytoplasmic part of TM6 moved away from TM7 and toward TM5. One more structural difference between the ground and activated states of MC4R was the orientation of the flexible side chain of R147 located in TM3. While in the ground state this orientation is determined almost exclusively by the salt bridge between the side chains of D146 and R147, in the activated state, the side chain of D146 is involved in the strong salt bridge with the side chain of R165 in TM4. As a result, interaction D146–R147 is significantly weakened, and the R147 side chain may change orientation, possibly contacting L250 in TM6 (compare Fig. 14.5A and B). This specific orientation of the side chain of R147 may be an important structural feature characteristic for activation of MC4R.

If these results are valid, conformational transitions from the ground state of MC4R to the activated state may be facilitated either by weakening the salt bridge D146–R147 (destabilization of the ground state) or by enhancing interaction between the side chain of R147 and that in position 250

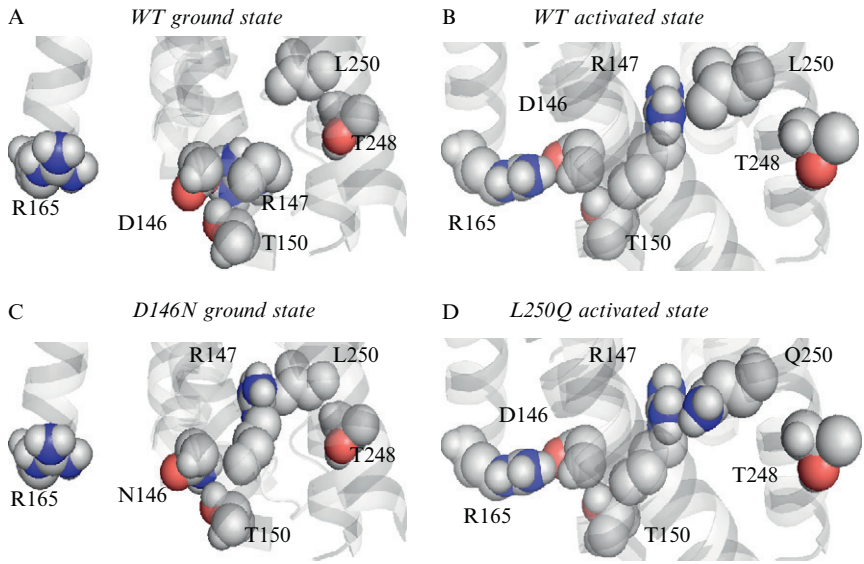

Figure 14.5 Sketches of the cytoplasmic portion of the TM region of MC4R. Presented are MC4R ground state (A), MC4R activated state (B), mutant D146N ground state (C), and mutant L250Q activated state (D). Side chains of D/N146, R147, T150, R165, T248, and L/Q250 are shown as space-filled models. TM helices are shown as semitransparent cartoons.

(stabilization of the activated state). Indeed, further modeling showed that in mutant D146N, where the salt bridge D146–R147 is replaced by a weaker hydrogen bonding, orientation of the R147 side chain characteristic for the activated state may be already adopted in the ground state (Fig. 14.5C). On the other hand, according to our modeling, the salt bridge D146–R147 remained unchanged in the ground state of mutant L250Q, but the side chain of R147 is involved in hydrogen bonding with the side chain of Q250 in the activated state (Fig. 14.5D). The same hydrogen bonding was suggested for the activated state of mutant L250Q in the earlier modeling study (Proneth *et al.*, 2006). Our further modeling showed that the discussed specific orientation of R147 was stabilized in L250N by interactions with N250, but was destabilized by steric clashes with the side chain of F250 in L250F. Since L250N and L250Q are known as strong CAMs, and L250F is not (Proneth *et al.*, 2006), that structural feature found by modeling may be considered significant for displaying constitutive activity in the L250x mutant series.

In summary, our molecular modeling employed an assumption that the ground state of MC4R may be based on the X-ray structure of β_2-adrenoreceptor and the activated state may be based on the X-ray structure of opsin cocrystallized with the transducin peptide. Modeling successfully

rationalized the mutagenic data on several CAMs of MC4R by suggesting that transition to the activated state may be facilitated by mutations that either destabilize the ground state (D146N) or stabilize the activated state (L250Q), two ways to generate CAMs (Tao *et al.*, 2000). These results, in turn, confirm our general assumption.

ACKNOWLEDGMENTS

These studies were supported by National Institutes of Health (R15DK077213) and intramural support from Auburn University, including Animal Health and Disease Research Program and Interdisciplinary Research Program. J.N.W. was partially supported by Auburn University Undergraduate Research Fellowship.

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