

# Pharmacological analyses of two naturally occurring porcine melanocortin-4 receptor mutations in domestic pigs<sup>☆</sup>

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## Abstract

The melanocortin-4 receptor (MC4R) is critical in regulating mammalian food intake and energy expenditure. Numerous mutations in the MC4R gene have been identified from obese humans. So far two naturally occurring porcine MC4R (pMC4R) mutations, D298N and R236H, have been identified from various strains of pigs and D298N is being utilized as a genetic marker to screen performance traits of pigs. In this study, we performed functional analyses of pMC4R D298N and R236H, including their ligand binding and signaling properties in transiently transfected HEK293T cells. Ligand binding assays showed that both D298N and R236H pMC4Rs had similar binding capacities and affinities for the natural agonist  $\alpha$ -MSH and the natural antagonist Agouti-related protein as wild-type pMC4R. In signaling assays, both mutants had normal EC<sub>50</sub> and maximal signaling to  $\alpha$ -MSH. In summary, pMC4R mutants D298N and R236H do not have any overt functional defects; therefore we suggest caution using these mutations as selection markers in breeding programs.

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**Keywords:** Melanocortin-4 receptor; Ligand binding; Signaling; Porcine; Naturally occurring mutations

## 1. Introduction

The melanocortin-4 receptor (MC4R) is a member of the superfamily of G protein-coupled receptors (GPCRs) consisting of the hallmark seven transmembrane domains (TMs) connected by alternating extracellular and intracellular loops, with an extracellular NH<sub>2</sub> terminus and intracellular COOH terminus. Upon hormone stimulation, MC4R couples to the stimulatory heterotrimeric G protein (Gs) and then

activates adenylyl cyclase to promote the intracellular accumulation of cAMP. The endogenous agonist of MC4R,  $\alpha$ -melanocyte stimulating hormone ( $\alpha$ -MSH), is derived from proopiomelanocortin (POMC) via tissue-specific posttranslational processing. Agouti-related protein (AgRP) is the endogenous antagonist of the MC4R.

During the past decade, ample evidences demonstrated the central role of the MC4R in regulating energy homeostasis of rodents (reviewed in [1]). MC4R or POMC knockout mice have increased food intake and obesity [2,3]. Mice over-expressing AgRP are hyperphagic, obese and hyperinsulinemic [4,5] whereas mice administered with  $\alpha$ -MSH or melanotan II, an analogue of  $\alpha$ -MSH, had decreased food intake [6,7]. Furthermore, mice co-administrated melanotan II and the MC4R antagonist SHU9119 showed a normal food intake, sug-

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gesting that SHU9119 inhibits the effect of melanotan II on food intake [6,7]. MC4R activation also increases energy expenditure [8,9].

Human genetic studies also showed that the MC4R plays a critical role in controlling food intake and body weight in humans. So far more than 110 distinct human MC4R (hMC4R) mutations have been identified from obese patients of various ethnic populations [10]. These mutants are caused by frameshift, in-frame deletion, non-sense and missense mutations. Although some mutants do not have obvious functional defects as determined by *in vitro* assays and might represent rare polymorphic variants, others are defective in cell surface expression, ligand binding or G protein coupling/activation (reviewed in [11]). Up to 6% of patients with severe early-onset obesity are caused by mutations in *MC4R* gene in some cohorts, thus representing the most common monogenic form of obesity [12].

In 2000, Rothschild and co-workers identified a missense mutation in the porcine MC4R (pMC4R) gene that change a highly conserved Asp in TM7 in melanocortin receptors (MCRs) to Asn [13]. This Asp, designated 7.49 according to the numbering scheme of Ballesteros and Weinstein [14], lies in the highly conserved N/DPxxY motif. The majority of Family A (rhodopsin-like) GPCRs has an Asn at this position. MCRs, together with gonadotropin-releasing hormone receptor and prostanoid receptors, are some of the few receptors that have an Asp at position 7.49. Within the MCR subfamily, however, this Asp is fully conserved in all the MCRs cloned to date, pointing to its potential functional importance. In our structure-function studies on the hMC4R, we generated D298N hMC4R and found that the mutant receptor has relatively normal functions. These results are dramatically different with the results reported for the pMC4R mutant, where a total loss of signaling was reported [15]. Subsequent genotype-phenotype association studies on this locus resulted in divergent conclusions, with some studies supporting the original association [16–19] while other studies failed to find an association [20–22]. Based on extensive studies on other GPCRs, we hypothesized that D298N pMC4R has normal function. We therefore generated the mutant pMC4R and analyzed its function. A novel pMC4R mutant, R236H, was recently identified [19]. This mutant was identified in a Piétrain founder pig, a relatively slow-growing pig. It was not found in the Mangalitsa pigs in the same breeding program. R236 is located in the third intracellular loop that could be important for G protein coupling. However, the functional properties of this mutant were not investigated in the original study. Detailed functional studies are necessary to establish a

causal relationship between the variant identified from genetic screening and any potential contribution of the variant to growth traits [11]. We therefore studied its functional properties.

## 2. Materials and methods

### 2.1. Peptides and supplies

[Nle<sup>4</sup>, D-Phe<sup>7</sup>]- $\alpha$ -MSH (NDP-MSH), a superpotent analogue of  $\alpha$ -MSH, was obtained from Phoenix Pharmaceuticals (Belmont, CA).  $\alpha$ -MSH was purchased from Bachem (King of Prussia, PA). Human AgRP(86-132) was obtained from Peptides International (Louisville, KY). <sup>125</sup>I-iodinated NDP-MSH was purchased from the Peptide Radioiodination Service Center at The University of Mississippi (University, MS) with a specific activity of 2176 Ci/mmol. Tissue culture plastic wares were purchased from Corning (Corning, NY). Dulbecco's modified Eagles medium (DMEM) and other cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

### 2.2. Molecular cloning of pMC4R

The pMC4R coding region was amplified directly from porcine genomic DNA (Novagen, San Diego, CA) using a primer pair (sense primer 5'-AAGAATTCATGAACCTCAACCCATCAC-3' and anti-sense primer 5'-CCTCTAGATTAATATCTGCTAGACAAATC-3') designed based on the published nucleotide sequence of pMC4R (GenBank access no. AB021664) incorporating EcoRI and XbaI restriction sites in sense and anti-sense primers, respectively (underlined). PCR amplification was performed in a 50  $\mu$ l mixture containing 100 ng of the porcine genomic DNA, 0.25 mM dNTPs, 0.4  $\mu$ M of each primer, 1  $\times$  pfu DNA polymerase buffer, 1.5 mM of MgCl<sub>2</sub>, and 2.5 U pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) with the following cycling parameters: 2 min at 95 °C for one cycle and 1 min at 95 °C, 45 s at 56 °C, and 90 s at 72 °C for 35 cycles followed by a final cycle of extension at 72 °C for 10 min. The PCR products of expected size as visualized by agarose gel electrophoresis were purified with Qiagen PCR purification kit (Qiagen, Valencia, CA) and double digested with EcoRI and XbaI (New England Biolabs, Beverly, MA). The PCR fragment was further purified, ligated into the expression vector pcDNA3.1, and transformed into JM109 competent cells. Cells were grown overnight on LB agar plates containing ampicillin and 8 clones were selected for growing in LB medium. Plasmid

DNA was extracted with Qiaprep spin miniprep kit (Qiagen) to screen clones with the insert of expected size after digestion with EcoRI and XbaI. The nucleotide sequence of the cloned pMC4R was determined by sequencing three independent plasmids performed at the DNA Sequencing Facility of University of Chicago Cancer Research Center. Plasmid DNA containing the pMC4R of correct sequence was prepared with Qiagen plasmid maxi kit (Qiagen) for site-directed mutagenesis or transfection as described below.

### 2.3. Site-directed mutagenesis

Nucleotide sequencing of the cloned pMC4R showed that it is D298N pMC4R. Wild-type (WT) pMC4R was generated by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene) according to the protocol described previously [23]. R236H pMC4R was made using the WT pMC4R as the template by the same method. Wild-type hMC4R attached with a myc epitope at its N terminus (after the initiating Met) has been described previously [23]. The hMC4R mutant D298N was generated using this construct as template by the same mutagenesis method.

### 2.4. Cells and transfection

Human embryonic kidney (HEK) 293T cells (American Type Culture Collection, Manassas, VA) were grown at 5% CO<sub>2</sub> in DMEM supplemented with 10% newborn calf serum, 10 mM HEPES, and 100 units/ml of penicillin and 100 µg/ml streptomycin. For transient expression of the MC4Rs, cells were plated on gelatin-coated 35-mm 6-well plates and transfected using the calcium precipitation method [24]. Four-microgram plasmid in 2 ml media was used per 35-mm well. The transfection cocktail included 86 µl water, 10 µl 2.5 M CaCl<sub>2</sub>, 4 µl of plasmid DNA, and 100 µl of 2 × BSS (consisting of 280 mM NaCl, 1.5 mM Na<sub>2</sub>HPO<sub>4</sub>, 50 mM *N,N*-bis[2-hydroxy]-2-aminoethane sulfonic acid, pH 6.95). After 15 min incubation in the hood at room temperature, 1.8 ml of growth media was combined with the cocktail and put into a well in 6-well clusters. Forty-eight hours after transfection, cells were used for measuring ligand binding and hormone stimulation of cAMP generation.

### 2.5. Radioligand binding assay

Forty-eight hours after transfection, cells were washed twice with warm Waymouth's MB752/1 media (Sigma–Aldrich, St. Louis, MO) modified to contain

1 mg/ml BSA (referred to as Waymouth/BSA). One milliliter of fresh Waymouth/BSA was added to each well, and then 100,000 cpm of <sup>125</sup>I-NDP-MSH (50 µl) was added to each well, with or without different concentrations of unlabeled α-MSH or NDP-MSH. The final concentration of unlabeled ligands ranged from 10<sup>-10</sup> to 10<sup>-5</sup> M (for α-MSH) or 10<sup>-11</sup> to 10<sup>-6</sup> M (for NDP-MSH). For the AgRP binding assay, cells were plated in 100 mm dishes and transfected when the cells reached 50–70% confluency. After an overnight incubation in transfection cocktail, cells were trypsinized and replated into 24-well plates. The total volume for binding assay in the 24-well plate was 200 µl. (The reason for performing the AgRP binding assays in 24-well plate is to save the amount of AgRP used.) The final concentration of unlabeled AgRP ranged from 10<sup>-11</sup> to 10<sup>-6</sup> M. After incubation at 37 °C for 1 h, cells were placed on ice, washed twice with cold Hanks' balanced salt solution (Sigma–Aldrich) modified to contain 1 mg/ml BSA (referred as HBSS/BSA). Then 100 µl of 0.5 N NaOH was added to each well. Lysed cells were collected using cotton swabs, and ligand binding was counted in a gamma counter. Binding capacity and IC<sub>50</sub> were calculated using Prism software version 4 (GraphPad Software, San Diego, CA).

### 2.6. Ligand stimulation of intracellular cAMP generation

Forty-eight hours after transfection, cells were washed twice with warm Waymouth/BSA. Then 1 ml of fresh Waymouth/BSA containing 0.5 mM isobutylmethylxanthine (Sigma–Aldrich) was added to each well. After incubation at 37 °C for 15 min, either buffer alone or different concentrations of α-MSH or NDP-MSH were added. The final concentrations ranged from 10<sup>-12</sup> to 10<sup>-6</sup> M (for NDP-MSH) or 10<sup>-11</sup> to 10<sup>-5</sup> M (for α-MSH). After incubation at 37 °C for 1 h, cells were then placed on ice, media aspirated, and intracellular cAMP extracted by the addition of 0.5 N perchloric acid containing 180 µg/ml theophylline, and measured using radioimmunoassay. All determinations were performed in triplicate. Iodinated cAMP was prepared using chloramines T method. Polyclonal antibody against cAMP was obtained from Strategic Biosolutions (Newark, DE). The radioimmunoassay was performed as described before [25] except that polyethylene glycol 8000 was used to precipitate the antibody-bound fraction of cAMP instead of a second antibody in the original publication. Maximal responses (*R*<sub>max</sub>) and EC<sub>50</sub> values were calculated using Prism software version 4 (GraphPad Software).

### 2.7. Statistic analysis

Statistical calculations were performed using Prism 4 [26]. For comparisons on maximal binding and signaling, one sample *t*-test was used. For comparisons on  $IC_{50}$  and  $EC_{50}$ , an unpaired *t*-test was used.

## 3. Results

### 3.1. Ligand binding and signaling properties of the MC4R mutants using NDP-MSH as the ligand

HEK293T cells have been used extensively as an *in vitro* system for evaluation of functional properties of MC4Rs from various species. Herein, WT and mutant pMC4Rs were transiently transfected into HEK293T cells and their ligand binding and signaling properties were analyzed. Wild-type and D298N hMC4Rs were included in these experiments for comparison. The superpotent analogue of  $\alpha$ -MSH widely used in MCR studies, NDP-MSH, was used in these studies. As shown in Fig. 1A and Table 1, WT, D298N and R236H pMC4Rs bound NDP-MSH with an  $IC_{50}$  of 2.46, 6.70

and 14.00 nM, respectively. Maximal binding ( $B_{max}$ ) of the two mutants were similar to that of WT pMC4R (Table 1). Similar results were obtained for hMC4Rs: WT and D298N hMC4Rs bound NDP-MSH with  $IC_{50}$  of 7.70 and 14.07 nM, respectively, and the  $B_{max}$  of hMC4R D298N was at the same level as that of WT hMC4R (Fig. 1A and Table 1).

The signaling properties of the WT and mutant MC4Rs were analyzed upon stimulation with NDP-MSH in HEK293T cells transiently transfected with these receptor constructs. The results showed that NDP-MSH induced dose-dependent increases of intracellular cAMP in all groups (Fig. 1B). As shown in Table 1, NDP-MSH stimulated cAMP accumulation with  $EC_{50}$  of 2.80, 27.62 and 15.43 nM for pMC4R WT, D298N and R236H, respectively. The maximal responses of pMC4R D298N and R236H were reduced to 76% and 65% of that of WT pMC4R, respectively. NDP-MSH stimulated cAMP generation with  $EC_{50}$ s of 1.63 and 12.96 nM for hMC4R WT and D298N, respectively. The maximal responses were similar for hMC4R WT and D298N (Table 1). We also measured the basal (constitutive) activities of the expressed receptors. The results showed

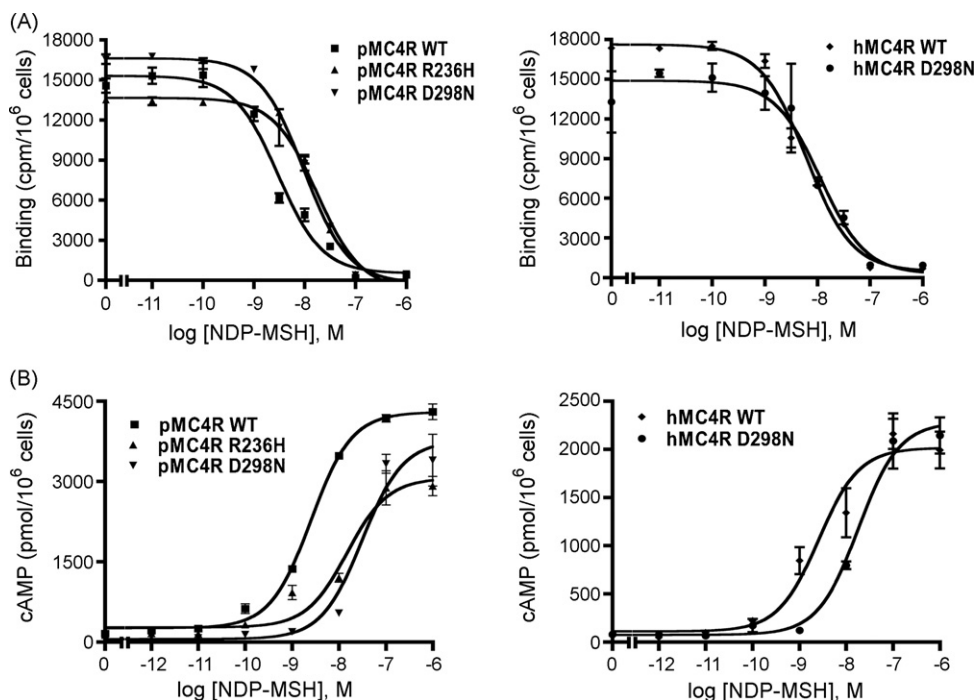


Fig. 1. Ligand binding and signaling properties of the WT and mutant MC4Rs using NDP-MSH as the ligand. HEK293T cells were transiently transfected with the indicated pMC4R and hMC4R constructs and binding and signaling assays were performed as described in Section 2. In (A) different concentrations of unlabeled NDP-MSH were used to displace the binding of <sup>125</sup>I-NDP-MSH to pMC4Rs or hMC4Rs on intact cells. Results shown are expressed as the mean  $\pm$  S.E.M. from duplicate determinations within one experiment. In (B) HEK293T cells transiently transfected with the indicated pMC4R or hMC4R constructs were stimulated with different concentrations of NDP-MSH. Intracellular cAMP levels were measured using radioimmunoassay. Results are expressed as the mean  $\pm$  S.E.M. of triplicate determinations within one experiment. All experiments were performed three times.

Table 1  
Ligand binding and agonist-stimulated cAMP generation of WT and mutant pMC4Rs and hMC4Rs

	n	NDP-MSH binding			NDP-MSH-stimulated cAMP			AgRP binding			α-MSH binding			α-MSH-stimulated cAMP		
		IC <sub>50</sub> (nM)	B <sub>max</sub> (% wt)	R <sub>max</sub> (% wt)	EC <sub>50</sub> (nM)	R <sub>max</sub> (% wt)	B <sub>max</sub> (% wt)	IC <sub>50</sub> (nM)	IC <sub>50</sub> (nM)	IC <sub>50</sub> (nM)	B <sub>max</sub> (% wt)	R <sub>max</sub> (% wt)	EC <sub>50</sub> (nM)	R <sub>max</sub> (% wt)		
pMC4R WT	3	2.46 ± 0.27	100	100	2.80 ± 0.41	100	100	3.87 ± 1.00	10.10 ± 1.67	10.10 ± 1.67	100	11.02 ± 0.85	100			
pMC4R D298N	3	6.70 ± 1.53	114 ± 7	76 ± 10	27.62 ± 3.16 <sup>a</sup>	76 ± 10	76 ± 10	3.42 ± 1.22	13.02 ± 2.58	13.02 ± 2.58	103 ± 7	12.03 ± 0.45	100 ± 2			
pMC4R R236H	3	14.00 ± 1.90 <sup>a</sup>	89 ± 10	65 ± 3 <sup>a</sup>	15.43 ± 1.02 <sup>b</sup>	65 ± 3 <sup>a</sup>	65 ± 3 <sup>a</sup>	2.08 ± 0.45	13.08 ± 3.60	13.08 ± 3.60	103 ± 3	11.43 ± 0.75	104 ± 4			
hMC4R WT	3	7.70 ± 1.56	100	100	1.63 ± 0.53	100	100	5.43 ± 1.52								
hMC4R D298N	3	14.07 ± 1.16 <sup>c</sup>	88 ± 8	116 ± 4	12.96 ± 2.77 <sup>c</sup>	116 ± 4	116 ± 4	4.02 ± 1.41								

The data are expressed as the mean ± S.E.M. of three independent experiments. The maximal binding (B<sub>max</sub>) of intact HEK293T cells expressing WT pMC4R or hMC4R were 15601 ± 1568 and 15046 ± 1214 cpm bound/10<sup>6</sup> cells for NDP-MSH, respectively. The maximal responses (R<sub>max</sub>) were 4520 ± 359 and 1513 ± 271 pmol cAMP/10<sup>6</sup> cells for WT pMC4R and hMC4R, respectively. IC<sub>50</sub> is the concentration of NDP-MSH, AgRP or α-MSH that is needed to cause 50% inhibition in the binding assay. EC<sub>50</sub> is the concentration of NDP-MSH or α-MSH that results in 50% stimulation of the maximal response.

<sup>a</sup> Significantly different from corresponding WT receptor, *p* < 0.01.

<sup>b</sup> Significantly different from corresponding WT receptor, *p* < 0.001.

<sup>c</sup> Significantly different from corresponding WT receptor, *p* < 0.05.

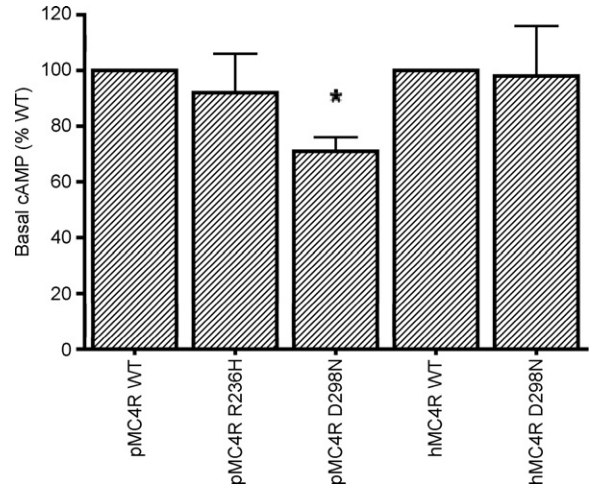


Fig. 2. Basal activities of WT and mutant pMC4Rs and hMC4Rs. The basal activities of WT and mutant MC4Rs were measured by assaying intracellular cAMP levels in the absence of NDP-MSH. The results for the mutants were normalized to the corresponding WT MC4R (designated as 100%) and shown herein as mean percentage ± S.E.M. from three independent experiments. The basal cAMP levels for WT pMC4R and hMC4R were 194 ± 48 pmol/10<sup>6</sup> cells and 155 ± 23 pmol/10<sup>6</sup> cells, respectively.

that pMC4R D298N, but not R236H, had decreased basal signaling compared with WT pMC4R whereas hMC4R D298N has normal constitutive activity (Fig. 2).

### 3.2. Ligand binding and signaling properties of the MC4R mutants using α-MSH as the ligand

Although NDP-MSH is widely used in MCR studies, it is a superpotent long-lasting analog of the natural agonist derived from POMC, α-MSH. Different pharmacological properties of the mutant receptors might be observed using NDP-MSH vs. α-MSH. Therefore, we also measured ligand binding and signaling properties of the mutant pMC4Rs using α-MSH as the ligand. These results showed that there is no difference between the WT pMC4R and either of the pMC4R mutants, R236H and D298N, in any of the parameters measured (Fig. 3 and Table 1).

### 3.3. Ligand binding properties of the MC4R mutants using the antagonist AgRP

Since the balance between the agonist α-MSH and the antagonist AgRP determines the function of the MC4R, it is important to determine whether the mutant receptors have increased affinity for AgRP. Increased affinity for AgRP, even in the face of normal affinity for and response to α-MSH, would result in decreased function

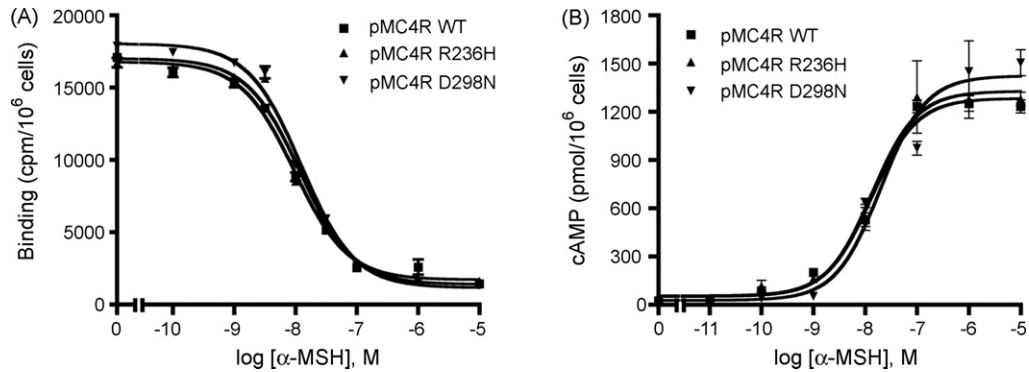


Fig. 3. Ligand binding and signaling properties of the WT and mutant pMC4Rs using  $\alpha$ -MSH as the ligand. HEK293T cells were transiently transfected with the indicated pMC4R constructs and binding and signaling assays were performed as described in Section 2. In (A) different concentrations of unlabeled  $\alpha$ -MSH were used to displace the binding of  $^{125}\text{I}$ -NDP-MSH to pMC4Rs on intact cells. Results shown are expressed as the mean  $\pm$  S.E.M. from duplicate determinations within one experiment. In (B) HEK293T cells transiently transfected with the indicated pMC4R constructs were stimulated with different concentrations of  $\alpha$ -MSH. Intracellular cAMP levels were measured using RIA. Results are expressed as the mean  $\pm$  S.E.M. of triplicate determinations within one experiment. All experiments were performed three times.

of the MC4R. Therefore, we also determined the binding properties of the mutant receptors to AgRP. As shown in Fig. 4, both WT and mutant pMC4Rs bound AgRP with similar high affinity (Table 1). Similarly, WT and D298N hMC4Rs bound to AgRP with the same affinities (Fig. 4 and Table 1).

#### 4. Discussion

More than 110 distinct mutations have been identified from obese humans in the *MC4R* gene. Functional studies are essential for establishing a causal relationship between the mutations identified and the obesity phenotype [11,27]. This is highlighted by several recent studies where functional studies provided strong support for the genetic studies [12,28,29].

Studies on the involvement of pig MC4R in feed intake and energy balance have resulted in conflicting results. Barb et al. showed that intracerebroventricular

administration of NDP-MSH decreased feed intake, but treatments with the synthetic antagonist SHU9119 or AgRP, the natural antagonist of the MC4R, failed to stimulate feed intake [30], which differs from the results obtained in rodents [6,31,32] and sheep [33]. *In vitro*, these ligands did act as antagonists [30]. We showed herein that WT and D298N pMC4Rs have similar binding affinities for AgRP (Fig. 4 and Table 1). Therefore, these data, while conforming the *in vitro* data of Barb et al., cannot provide an explanation for the lack of effect of AgRP on feed intake in pigs as observed by Barb et al. [30].

Rothschild and co-workers identified a missense mutation in pMC4R, D298N, which mutated the conserved Asp in N/DPxxY motif in TM7. They showed that this mutation was associated with fatness, growth, and feed intake traits [13]. Subsequent functional studies on this mutant revealed that the mutation did not affect ligand binding but totally abolished ligand-stimulated

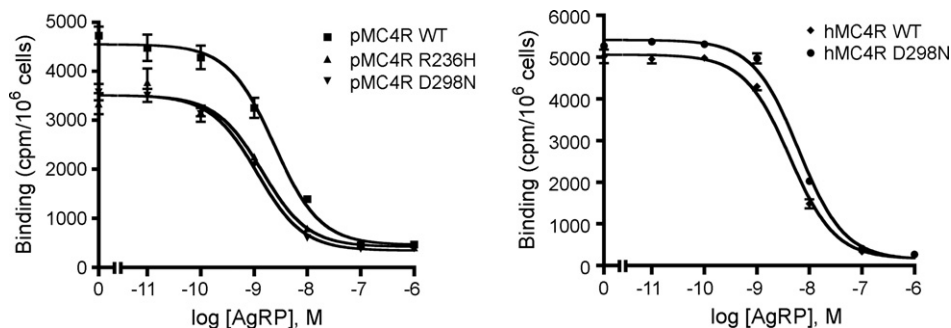


Fig. 4. Ligand binding properties of the WT and mutant MC4Rs on AgRP. HEK293T cells were transiently transfected with the indicated pMC4R and hMC4R constructs and binding assays were performed using AgRP as the competitor. Different concentrations of AgRP were used to displace the binding of  $^{125}\text{I}$ -NDP-MSH to pMC4Rs or hMC4Rs on intact cells. Results shown are expressed as the mean  $\pm$  S.E.M. from duplicate determinations within one experiment. All experiments were performed three times.

signaling [15] therefore belonging to the Class IV category according to our classification scheme [11,23]: mutants defective in G protein coupling/activation. From a structure-function point of view, this mutation is interesting in that a fully conserved Asp in MCRs is mutated to Asn, the most commonly found amino acid in Family A GPCRs. Since Asn is found in most of the Family A GPCRs, it can be reasoned that Asn should confer normal functions for the receptor. Our preliminary results with hMC4R showed that indeed D298N hMC4R is fully functional in terms of both ligand binding and signaling. Therefore, we were interested in identifying the molecular determinants dictating the dramatic differences between our own data on hMC4R and the data on the pMC4R. We cloned the pMC4R from genomic DNA purchased from a commercial vendor and determined that the cDNA we cloned was in fact the mutant D298N. We then generated the WT cDNA using site-directed mutagenesis. As reported herein, our results differ from those of Kim et al. [15]. The D298N pMC4R, similar to the D298N hMC4R, can bind radiolabeled NDP-MSH with normal affinity and generate cAMP with similar maximal signaling, although the EC<sub>50</sub>s are increased (Fig. 1 and Table 1). The reason for this discrepancy is not clear. We noticed that the maximal stimulation in the study by Kim et al., about 16 pmol per million cells, is extremely low in this transient transfection system using HEK293 cells. In our radioimmunoassay for cAMP, this is about the lowest level we can detect. Recently, Patten et al. also showed that D298N hMC4R retained cAMP formation in response to NDP-MSH stimulation [34].

Another mutation in pMC4R, R236H, was recently identified in another screening effort [19]. We showed here that the mutant had normal ligand binding and signaling properties compared to WT pMC4R when the natural ligand  $\alpha$ -MSH was used for these assays (Fig. 3 and Table 1), suggesting that it might not cause significant changes in feed intake and growth in pigs. The corresponding mutation was also identified in hMC4R from a patient and found to have normal functions, including cell surface expression as well as basal and stimulated activities [35]. This arginine is not conserved in the cloned MCRs. In other MCRs, histidine, tryptophan, or glutamine was observed. It should be pointed out that in functional studies of hMC4R mutations identified from obese patients, several groups have reported a number of mutations that result in no overt functional defects (reviewed in [11]). We classify these mutants as Class V: mutants with no known defects [11,23]. Similarly, in other GPCRs that result in diseases, mutations identified from patients that have normal functions were observed not infrequently (reviewed in [10]), suggesting

that these mutations were not the cause of the disease. One drawback of these experiments is that the MC4R is expressed in neurons. Our experiments in HEK293T cells may not reveal any neuron-specific aspects of the MC4R function.

Another interesting aspect that is worth mentioning is that from all the screening efforts in different strains of pigs, only two mutations in pMC4R were identified. In humans, numerous mutations were identified from both obese patients and the general population. The reason for this lower occurrence of pMC4R mutation is unknown at this time.

In summary, we reported here that the two mutations in pMC4R, R236H and D298N, do not result in overt defects in receptor functions, suggesting that they might not cause significant increases in feed intake and growth. We therefore suggest caution using these mutations as selection markers in breeding programs.

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