Pharmacological Characterization of 40 Human Melanocortin-4 Receptor Polymorphisms with the Endogenous Proopiomelanocortin-Derived Agonists and the Agouti-Related Protein (AGRP) Antagonist[†,‡]

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ABSTRACT: The melanocortin-4 receptor (MC4R) is a G-protein coupled receptor (GPCR) that is expressed in the central nervous system and has a role in regulating energy homeostasis and obesity. Up to a remarkable 6% of morbidly obese adults and children studied possess single nucleotide polymorphisms (SNPs) of the MC4R. Upon stimulation by agonist, the MC4R signals through the intracellular adenylate cyclase signal transduction pathway. Posttranslational modification of the pro-opiomelanocortin (POMC) gene transcript results in the generation of several endogenous melanocortin receptor agonists including α-, *β*-, *γ*-melanocyte stimulating hormones (MSH) and adrenocorticotropin (ACTH) ligands. The endogenous MC4R antagonist, agouti-related protein (AGRP), is expressed in the brain and is only one of two naturally occurring antagonists of GPCRs identified to date. Herein, we have generated 40 hMC4 polymorphic receptors and evaluated their cell surface expression by flow cytometry as well as pharmacologically characterized their functionality using the endogenous agonists α-MSH, *β*-MSH, γ_2-MSH, ACTH(1−24), the antagonist hAGRP(87−132), and the synthetic agonists NDP-MSH and MTII. This is the first study in which polymorphic hMC4Rs have been pharmacologically characterized simultaneously with multiple endogenous ligands. Interestingly, at the N97D, L106P, and C271Y hMC4Rs *β*-MSH was more potent than the other endogenous agonists α-MSH, γ_2-MSH, ACTH(1−24). The S58C and R165Q/W hMC4Rs possessed significantly reduced endogenous agonist potency (15- to 90-fold), but the synthetic ligands NDP-MSH and MTII possessed only 2−9-fold reduced potency as compared to the wild-type receptor, suggesting their potential as therapeutic ligands to treat individuals with these polymorphisms.

The melanocortin pathway has been implicated in the regulation of a number of diverse physiological pathways including pigmentation (*1*), sexual function (*2*), and energy homeostasis (*3*, *4*). The melanocortin system is comprised of five G-protein coupled receptors (GPCRs) identified to date (*5*−*11*) that stimulate the adenylate cyclase signal transduction pathway. The endogenous ligands are derived by posttranslational processing of the pro-opiomelanocortin (POMC) gene transcript by prohormone convertases PC1 and PC2 (*12*, *13*) to generate adrenocorticotropin (ACTH) and the α-, *β*-, *γ*-melanocyte stimulating hormones (MSHs) (Figure 1, Table 1). The melanocortin pathway also has the only two known endogenous antagonists of GPCRs, agouti (*14*, *15*) and agouti-related protein (AGRP) (*16*).

The role of the central melanocortin pathway in energy homeostasis has become an area of intense investigation. This interest is based upon results from genetic studies (*17*−*19*), knockout and transgenic mice (*4*, *16*, *20*−*24*), human polymorphisms (*25*−*39*), and central administration of agonists (decreased food intake), antagonists (increased food intake), and the sustained increased food intake (days) upon icv administration of AGRP (*3*, *24*, *40*−*44*). The MC4R mRNA is expressed in the hypothalamus of the brain (*45*, *46*), POMC is processed in the human brain to generate the endogenous melanocortin agonist peptides ACTH, α-, *β*-,

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[‡]This work is dedicated to Mrs. Rozella May Haskell (lived between September 10, 1922 and May 6, 2005) who passed away due to complications of Type 2 diabetes.

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[1] Abbreviations: POMC, pro-opiomelanocortin; CA, constitutively active; GPCR, G-protein coupled receptor; cAMP, cyclic adenosine monophosohate; AGRP, agouti-related protein; MSH, melanocyte stimulating hormone; ACTH, adrenocorticotropin hormone; TM, transmembrane domain; hMC4R, human melanocortin-4-receptor; NDP-MSH, 4-norleucine-7-D-phenylalanine α-MSH; MTII, Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2; hAGRP, human agouti-related protein; HEK-293, human embryonic kidney-293; CRE, cyclic adenosine monophosohate response element; DMEM, Dulbecco's modified Eagle's medium; ONPG, *o*-nitrophenyl-*β*-D-galactopyranoside; FACS, fluorescence activated cell sorting.

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Table 1: Amino Acid Sequences of the Endogenous and Synthetic Melanocortin Ligands Examined in This Study

Name	Amino Acid Sequence
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
β-MSH	Ala-Glu-Lys-Lys-Asp-Glu-Gly-Pro-Tyr-Arg-Met-Glu-His-Phe-Arg-Trp-Gly-Ser-Pro-Pro-Lys-Asp
γ_2-MSH	Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly
ACTH(1-24)	Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Tyr-Pro-Asn
NDP-MSH	Ac-Ser-Tyr-Ser-**Nle**-Glu-His-**DPhe**-Arg-Trp-Gly-Lys-Pro-Val-NH_2
MTII	Ac-**Nle**-c[Asp-His-**DPhe**-Arg-Trp-Lys]-NH_2
hAGRP(87-132)	CVRLHESCLGQQVPCCDPCATCYC-**Arg-Phe-Phe**-NAFCYCRKLGTAMNPCSRT

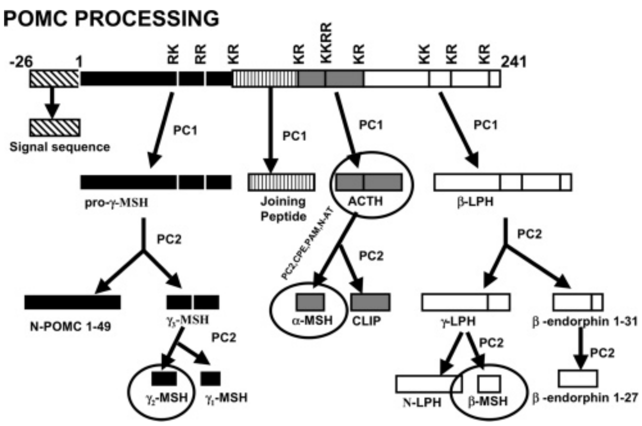

FIGURE 1: Schematic representation of the posttranslational processing the proopiomelanocortin (POMC) gene transcript by the prohormone convertases (PC) enzymes to generate the endogenous melanocortin agonists ACTH, α-MSH, β-MSH, and γ-MSHs (circled). The abbreviations are defined as LPH, lipotrophin; CLIP, corticotrophin-like intermediate lobe peptide; CPE, carboxypeptidase E; PAM, peptidyl γ-amidating mono-oxygenase; N-AT, nacetyltransferace.

and γ-MSH (*47*), and the endogenous antagonist AGRP is also expressed in the hypothalamus of the brain (*48*, *49*).

This study presented herein performs a side-by-side *in vitro* pharmacological comparison of 40 human MC4R polymorphisms (Figure 2) using multiple endogenous agonists, the synthetic agonists NDP-MSH and MTII, and the antagonist hAGRP(87−132). Since there are multiple putative endogenous melanocortin agonists (α-MSH, β-MSH, γ-MSH, and ACTH, Figure 1, Table 1), it might be envisioned that while one endogenous ligand might maintain equipotency or modified potency at a hMC4 polymorphic receptor, other endogenous agonists or the antagonist AGRP might possess normal or modified potencies. Thus, this study was performed to test the hypothesis that the mutant human MC4R polymorphisms might interact differentially with one or more of the endogenous melanocortin ligands.

MATERIALS AND METHODS

Peptides used in this study were purchased from commercial sources, α-MSH, NDP-MSH, MTII, ACTH(1−24), β-MSH, γ_2-MSH (Bachem), and hAGRP(87−132) (Peptides International).

hMC4R In Vitro Receptor Mutagenesis. The human wild-type N-terminally Flag tagged MC4R cDNA was generously provided by Dr. Robert Mackenzie (*50*) and was subcloned into the pBluescript plasmid (Stratagene) for subsequent mutagenesis. Mutant hMC4Rs were generated using a polymerase chain reaction (PCR) based mutagenesis strategy, as previously described by our laboratory (*51*). A complementary set of PCR primers were designed containing the reported nucleotide base pair changes resulting in the modified amino acid. After completion of the PCR reaction (95 °C 30 s, 12 cycles of 95 °C 30 s, 55 °C 1 min, 68 °C 9 min) the product was purified (Qiaquick PCR reaction, Qiagen) and eluted in water. Subsequently, the sample was cut with *Dpn*1 (Biolabs) to linearize the wild-type template DNA leaving only nicked circularized mutant DNA. This was transformed into competent DH5α *E. coli*. Single colonies were selected, and the presence of the desired mutant was checked by DNA sequencing. The DNA containing the mutant was then excised and subcloned into the *Hind*III/*Xba*I restriction sites of the $pCDNA_3$ expression vector (Invitrogen). Complete mutant hMC4R sequences were confirmed free of PCR nucleotide base errors by DNA sequencing (University of Florida sequencing core facilities).

Generation of Stable Cell Lines. HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS) and seeded 1 day prior to transfection at $(1-2) \times 10^6$ cell/100-mm dish. Mutant and wild-type DNA in $pCDNA_3$ expression vector (20 μg) were transfected using the calcium phosphate method (*52*). Stable receptor populations were generated using G418 selection (0.7−1 mg/mL) for subsequent bioassay analysis.

cAMP-Based Functional Bioassay. HEK-293 cells stably expressing the mutant and wild-type melanocortin receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described (*51*, *53*). Briefly, 5000 to 15 000 post transfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. Forty-eight hours post-transfection, the cells were stimulated with 100 μL of peptide (10^{-5}−10^{-12} M) or forskolin (10^{-4} M) control in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated and 50 μL of lysis buffer (250 mM Tris-HCl pH = 8.0 and 0.1% Triton X-100) was added. The plates were stored at −80 °C overnight. The plates containing the cell lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96-well plate for relative protein determination. To the cell lysate plates, 40 μL of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 2 mg/mL ONPG) was added to each well, and the plates were incubated at 37 °C. The sample absorbance, OD_{405}, was measured using a 96-well plate reader (Molecular Devices). The relative protein was

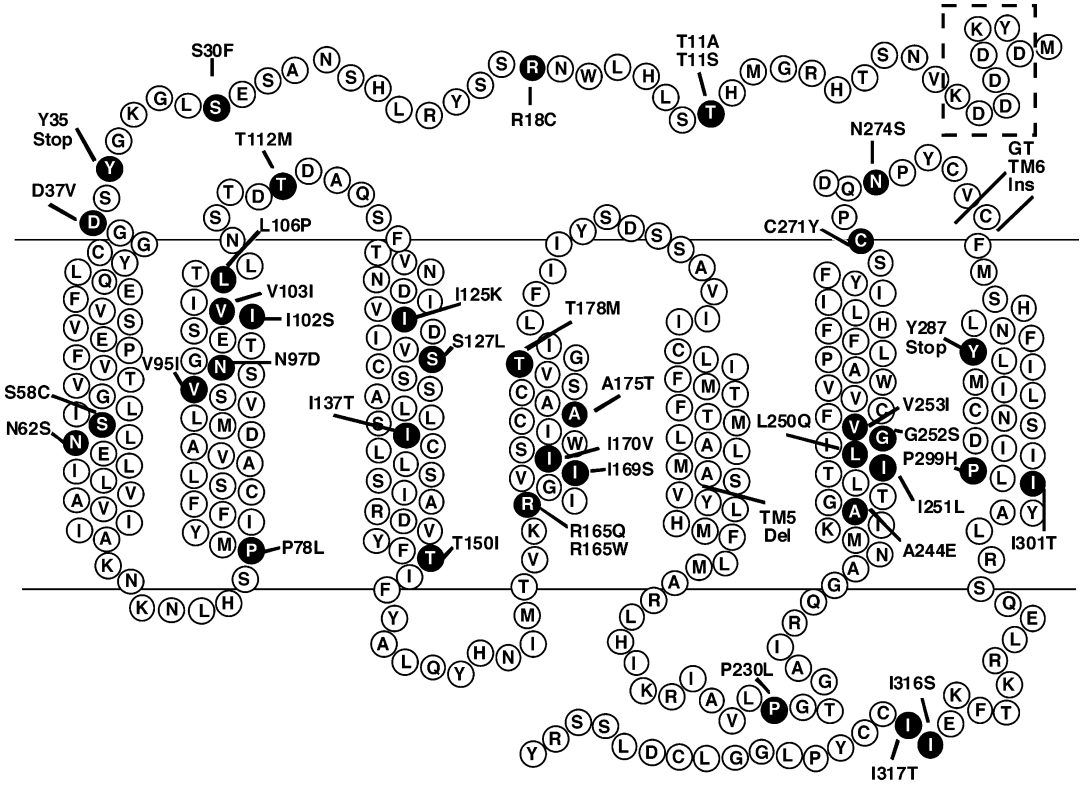

FIGURE 2: Summary of the 40 hMC4R polymorphisms examined in this study. The dashed box indicates the N-terminal FLAG sequence used for immunohistochemical cell expression studies. The normal amino acid is indicated in a black circle with white text, and the mutation is indicated in the text adjacent to the receptor residue.

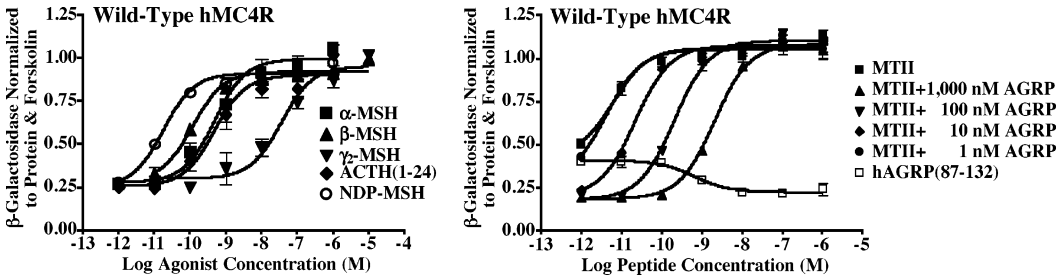

FIGURE 3: Pharmacological profile of the ligands examined in this study at the wild-type hMC4R.

determined by adding 200 μL of 1:5 dilution Bio Rad G250 protein dye/water to the 10 μL of cell lysate sample taken previously, and the OD_{595} was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and nonreceptor dependent forskolin stimulation. The antagonistic properties of hAGRP-(87−132) were evaluated by the ability of hAGRP(87−132) to competitively displace the MTII agonist (Bachem) in a dose-dependent manner, at up to 10 μM concentrations (*51*). The pA_2 values were generated using the Schild analysis method (*54*).

Data Analysis. EC_{50} and pA_2 values represent the mean of duplicate experiments performed in triplicate or more independent experiments. EC_{50} and pA_2 estimates, and their associated standard errors of the mean, were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (v4.0, GraphPad Inc.).

Transient Transfection Bioassays were performed as previously reported (*51*). Briefly, HEK-293 cells are maintained in DMEM with 10% fetal calf serum and seeded 1 day prior to transfection at 2×10^6 cells/100-mm dish. Mutant and wild-type plasmid DNAs were transfected simultaneously at different concentrations using the calcium phosphate method (*52*). Cells were incubated overnight at 35 °C and 3% CO_2, and the colorimetric reporter gene bioassays were performed as described above, with the exception that dose−response curves of compounds are omitted; only basal and nonreceptor dependent forskolin values were measured (*51*, *55*). The average of twelve data points at each concentration was determined in four independent experiments.

Concentration of DNA used in the transient transfection bioassay is listed below.

conc plotted → (DNA transfected) (ng)	0	5	25	50	75	100
mutant/WT (ng)	0	5	25	50	75	100
$pCDNA_3$ plasmid (ng)	100	95	75	50	25	0
CRE-β-gal (μg)	4	4	4	4	4	4

Competitive Displacement Binding Assays. NDP-MSH Iodination. ^{125}I-NDP-MSH was prepared using a modified chloramine-T method as previously described by Yang et

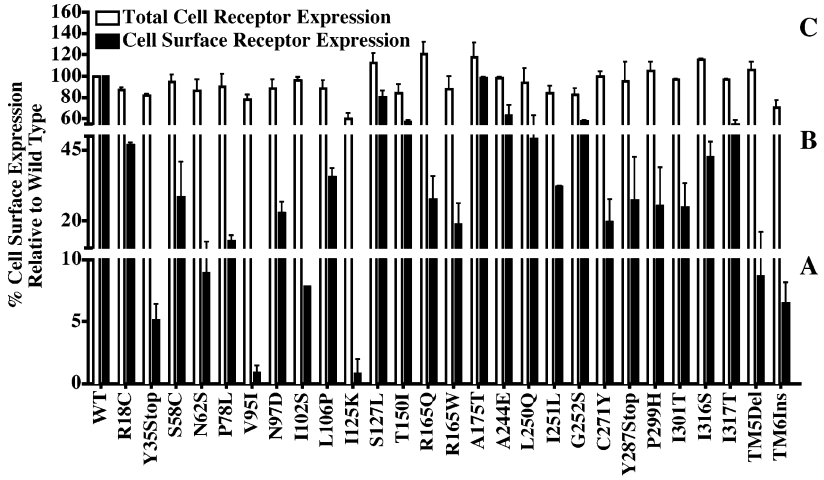

FIGURE 4: Fluorescence activated cell sorting analysis (FACS) of the hMC4R polymorphisms in stably expressed in HEK-293. The total cell receptor expression levels were determined using permeabilized cells measuring both cell surface and intracellular protein expression. The cell surface expression levels were determined using nonpermeabilized cells. Cell expression levels are presented relative to the wild-type hMC4R control. A indicates 0−10% expression, B indicates 11−50% expression, and C indicates 55−160% expression levels.

al. (*56*). Using 50 mM sodium phosphate buffer pH 7.4 as the reaction buffer, we added ^{125}I-Na (0.5 mCi, Amersham Life Sciences, Inc., Arlington Heights, IL) to 20 μg of NDP-MSH (Bachem, Torrance, CA) in 5 μL of buffer. To initiate the reaction, 10 μL of a 2.4 mg/mL solution of chloramine T (Sigma Chemical Co., St. Louis, MO) was added for 15 s with gentle agitation. This reaction was terminated by the addition of 50 μL of a 4.8 mg/mL solution of sodium metabisulfite (Sigma Chemical Co.) for 20 s with gentle agitation. The reaction mixture was then diluted with 200 μL of 10% bovine serum albumin, and the resultant mixture layered on a Bio-Gel P2 (Bio-Rad Labs, Hercules, CA) column (1.0 × 30 cm Econocolumn, Bio-Rad Labs) for separation by size exclusion chromatography using 50 mM sodium phosphate buffer, pH 7.4 as column eluant. Fifteen drop fractions (approximately 500 μL) were collected into glass tubes containing 500 μL of 1% BSA. Each fraction was then counted on the Apex Automatic Gamma counter (ICN Micromedic Systems Model 28023, Huntsville, AL with RIA AID software, Robert Maciel Associates, Inc., Arlington, MA) to determine peak ^{125}I incorporation fractions.

Receptor Competitive Displacement Binding Studies. HEK-293 cells stably expressing the mutant and wild-type MC4 receptors were maintained as described above. Before the experiment, 0.3 to 0.5 × 10^6 cells/well were plated into Primera 12-well plates (Falcon) and grown to confluency. For the assays, the media was removed, and the cells were washed twice with a freshly prepared assay buffer (DMEM and 0.1% BSA). A 500 μL sample of the NDP-MSH peptide concentration being tested and 150 000 cpms of ^{125}I-radiolabeled NDP-MSH were added to the well and incubated at 37 °C for 1 h. The medium was subsequently removed, and each well was washed twice with assay buffer. The cells were lysed by the addition 0.5 mL of 0.1 M NaOH and 0.5 mL of 1%Triton X-100. The mixture was left to lyse the cells for 10 min, and the contents of each well were transferred to a labeled 16 × 150-mm glass tube and quantified on a Apex Automatic Gamma counter. Dose−response curves (10^{-6} to 10^{-12} M) of NDP-MSH and IC_{50} values were generated and analyzed by a one-site competition nonlinear least-squares analysis (*57*) and the PRISM program (v4.0, GraphPad Inc.). The percent total specific binding was determined based upon the nonspecific values obtained using 10^{-6} M NDP-MSH. Each experiment was performed using duplicate data points and repeated in at least two independent experiments. The standard deviations were derived from the IC_{50} values from at least two independent experiments and using the PRISM program (v4.0, GraphPad Inc.)

Immunohistochemical Analysis of Wild-Type FLAG-Tagged hMC4R. Flow cytometric analysis (FACS) of intracellular FLAG-tagged wild-type hMC4R was performed as described previously for the detection of the intracellular protein cyclooxygenase/prostaglandin synthatase 2 (COX2/PGS2) (*58*). Briefly, cells were dissociated from monolayer culture dishes using cold Cell Dissociation buffer (Cellgro, Mediatech), centrifuged at 600*g* for 5 min, room temperature, and the pelleted cells were resuspended in sterile-filtered FACS buffer (1% BSA, 0.1% Na azide, in 1× PBS pH 7.2; Sigma Chemical, St. Louis, MO). The cells were distributed to multiple FACS tubes (Falcon, Fisher Scientific) at one million cells per tube. The cells were treated with 10 mg/mL unconjugated mouse IgG (Upstate Biotech or Sigma) to block nonspecific antibody binding. To determine cell surface receptor protein expression, the cells were then incubated for 45 min at room temperature with anti-FLAG-PE (Prozyme, San Leandro, CA). To determine the total cellular receptor protein expression, the cells were fixed with 2% methanol free formaldehyde in 1× PBS (Ted Pella or EM Scientific, Fisher Scientific) for 10 min, permeabilized for 20 min with saponin buffer [0.5% saponin (Sigma) in FACS buffer, pH 7.2], and subsequently washed with saponin buffer. After centrifugation (600*g*, 5 min), cell aliquots were conjugated with anti-FLAG-APC antibodies (Prozyme) for 1 h at room temperature to label the total (intracellular and surface) FLAG-tagged molecules. After the anti-FLAG antibody incubation, the labeled cells were washed 1 mL of saponin buffer three times prior to resuspension in FACS buffer for analysis. The PE- and APC-conjugated nonspecific antibodies (BD Biosciences-Pharmingen, CalTag, Burlingame, CA) served as isotype controls for the anti-FLAG antibody conjugates used in these analyses and were used to set the background for fluorescence staining detection on BD Biosciences FACS Calibur flow cytometers. Data were

Table 2: Summary of the Endogenous Melanocortin Agonist Ligand Pharmacology at the hMC4R Polymorphisms[a]

mutation	TM	α-MSH EC_{50} (nM)	β-MSH EC_{50} (nM)	γ_2-MSH EC_{50} (nM)	ACTH(1−24) EC_{50} (nM)
WT[#]		0.65 ± 0.19	0.42 ± 0.13	73 ± 24	0.65 ± 0.15
T11S	N-term	0.23 ± 0.051	0.24 ± 0.08	37 ± 7.7	0.57 ± 0.26
T11A	N-term	0.29 ± 0.075	0.16 ± 0.08	34 ± 8.5	0.38 ± 0.18
R18C	N-term	0.69 ± 0.32	0.32 ± 0.12	72 ± 34	0.35 ± 0.30
S30F	N-term	0.42 ± 0.16	0.29 ± 0.14	79 ± 32	0.58 ± 0.21
Y35Stop	N-term	no stim	no stim	no stim	no stim
D37V	N-term	1.55 ± 0.85	0.15 ± 0.06	82 ± 14	1.84 ± 0.44
S58C	1	18.5 ± 6.05**	15.3 ± 6.60*	2360 ± 960*	24.4 ± 6.63**
N62S	1	153 ± 99* (partial agonist)	147 ± 64* (partial agonist)	2300 ± 1800* (partial agonist)	156 ± 61* (partial agonist)
P78L	2	no stim	no stim	no stim	no stim
V95I	2	no stim	no stim	no stim	no stim
N97D	2	>10	660 ± 47**	>10	>10
I102S	2	260 ± 58**	150 ± 52*	3620 ± 1022*	480 ± 89**
V103I	2	0.32 ± 0.11	0.057 ± 0.016	67 ± 23	0.60 ± 0.10
L106P	2	50% at 1μM	356 ± 53**	2660 ± 370*	40% at 1μM
T112M	EL1	0.90 ± 0.42	0.12 ± 0.08	50 ± 19	1.12 ± 0.42*
I125K	3	no stim	no stim	no stim	no stim
S127L	3	5.49 ± 0.80**	2.22 ± 0.88*	930 ± 170**	11.4 ± 5.37*
I137T	3	2.00 ± 0.43**	0.43 ± 0.036	260 ± 38	3.88 ± 1.27*
T150I	3	8.14 ± 3.2*	6.53 ± 2.81*	1300 ± 760*	14.0 ± 4.87*
R165Q	4	18.7±10.8*	6.31 ± 2.94*	1070 ± 330*	39 ± 6.2**
R165W	4	21.5 ± 5.54*	7.91 ± 2.34*	1140 ± 350*	58 ± 19.3*
I169S	4	0.77 ± 0.27	0.43 ± 0.09	300 ± 92	0.59 ± 0.10
I170V	4	0.82 ± 0.28	0.58 ± 0.27	60 ± 21	0.73 ± 0.37
A175T	4	0.54 ± 0.23	0.63 ± 0.32	66 ± 30	0.82 ± 0.57
T178M	4	0.52 ± 0.053	0.36 ± 0.11	115 ± 30	1.00 ± 0.19
P230L	IL3	0.40 ± 0.16	0.62 ± 0.31	157 ± 84	0.46 ± 0.11
A244E	6	1.13 ± 0.22*	0.93 ± 0.27	228 ± 1.04	3.49 ± 1.04*
L250Q	6	4.32 ± 1.10**	3.55 ± 1.67	380 ± 200	3.63 ± 0.82**
I251L	6	2.06 ± 0.86	0.42 ± 0.18	81 ± 25	5.18 ± 2.32
G252S	6	6.11 ± 1.75*	0.53 ± 0.12	409 ± 50**	10.9 ± 3.89*
V253I	6	0.60 ± 0.24	0.45 ± 0.18	116 ± 18	4.02 ± 2.12
C271Y	6/EL3	48% at 1μM	154 ± 12**	58% at 10μM	42% at 1μM
N274S	EL3	0.45 ± 0.069	0.36 ± 0.12	105 ± 28	1.04 ± 0.36
Y287Stop	7	65 ± 16* (partial agonist)	17 ± 3.38** (partial agonist)	2490 ± 160** (partial agonist)	150 ± 12** (partial agonist)
P299H	7	no stim	no stim	no stim	no stim
I301T	7	8.42 ± 1.64**	2.44 ± 0.44*	370 ± 100*	16 ± 2.63*
I316S	C-term	4.30 ± 2.08	0.50 ± 0.042	160 ± 18**	4.26 ± 0.94**
I317T	C-term	0.60 ± 0.17	0.59 ± 0.19	96 ± 50	2.35 ± 1.45
TM5Del		no stim	no stim	no stim	no stim
TM6Ins		no stim	no stim	no stim	no stim

[a] Pound sign (#) indicates the average from greater than nine independent experiments at the wild type (WT) hMC4R. The values indicated represent the mean of at least three independent experiments with the standard error of the mean indicated. >10 indicates that an EC_{50} value was not reportable at up to 10 μM ligand concentrations. No stim indicates that the endogenous agonists were unable to stimulate the receptor polymorphisms at up to 1 μM concentrations. Partial agonist indicates that some stimulatory agonist pharmacology resulted, but the maximal stimulation levels were less then the nonreceptor dependent forskolin control level. A percentage value indicates that some stimulatory agonist pharmacology resulted at up to 10 μM concentrations, but the maximal stimulation levels were less then the nonreceptor dependent forskolin control level. Statistical analysis was performed using a student T-test compared to the wild-type receptor values with $*p < 0.05$, $**p < 0.01$.

collected as both stained cell percentages (either surface or total detected) and as mean fluorescence per cell from a minimum of 10 000 collected events for each sample run. Receptor cell surface expression and total cellular expression (using permeabilized cells) were determined as summarized in Figure 4.

RESULTS

The 40 human MC4 polymorphic receptors that were examined in this study are summarized in Figure 2. Figure 3 illustrates the functional receptor pharmacology of the ligands examined in this study at the wild-type hMC4R. Table 2 summarizes the functional endogenous α-MSH, β-MSH, γ_2-MSH, and ACTH(1−24) agonist potency of the 40 polymorphic hMC4Rs. Table 3 summarizes the synthetic melanocortin agonist NDP-MSH agonist potency and binding affinity in a competitive displacement assay using radiolabeled ^{125}I-NDP-MSH. Table 4 summarizes the MTII agonist EC_{50} values and antagonist pA_2 values of the hAGRP(87−132) hMC4R antagonist.

hMC4R Polymorphic Receptors That Possess Normal Pharmacology as Compared to the Wild-Type hMC4R. Of the 40 polymorphic hMC4 receptors characterized in this study, only nine resulted in pharmacological profiles similar to the wild-type hMC4R. These nine hMC4R polymorphic receptors are T11S, S30F, D37V, I169S, A175T, T178M, P230L, V253I, and N274S. The experimental parameters used to classify these polymorphic receptors as similar to wild-type hMC4R include agonist functional potency (Tables 2 and 3), hAGRP(87−132) antagonist potency (Table 4), percentage of the receptor expression at the cell surface (Figure 4), and the ability of radiolabeled ^{125}I-NDP-MSH to bind to the stably expressing HEK-293 cell line (Figure 5).

Table 3: Summary of the Synthetic Agonist NDP-MSH Functional (EC_{50}) and Competitive Displacement Binding Affinity Studies (IC_{50}) of the Polymorphic hMC4Rs Examined in This Study[a]

mutation	TM	NDP-MSH EC_{50} (nM)	NDP-MSH binding IC_{50} (nM)
WT[#]		0.030 ± 0.0096	14.0 ± 2.24
T11S	N-term	0.032 ± 0.008	16.6 ± 2.2
T11A	N-term	0.046 ± 0.017	8.24 ± 3.06
R18C	N-term	0.048 ± 0.018	3.16 ± 0.83
S30F	N-term	0.069 ± 0.020	8.14 ± 0.84
Y35Stop	N-term	no stim	>10
D37V	N-term	0.007 ± 0.001*	21.6 ± 11.7
S58C	1	0.13 ± 0.022*	15.7 ± 11.6
N62S	1	4.74 ± 2.26*	>1
P78L	2	no stim	>1
V95I	2	no stim	>1
N97D	2	1.31 ± 0.09*	96.3 ± 32*
I102S	2	0.66 ± 0.21*	32.5 ± 6.93
V103I	2	0.061 ± 0.021	9.30 ± 4.95
L106P	2	0.42 ± 0.076*	9.30 ± 4.95
T112M	EL1	0.011 ± 0.005*	22.5 ± 9.55
I125K	3	no stim	>1
S127L	3	0.028 ± 0.003	15.9 ± 1.06
I137T	3	0.12 ± 0.096	9.86 ± 2.32
T150I	3	0.31 ± 0.06*	5.66 ± 2.32
R165Q	4	0.13 ± 0.021*	12.6 ± 6.21
R165W	4	0.23 ± 0.046*	20.7 ± 11.8
I169S	4	0.021 ± 0.004	8.55 ± 0.93*
I170V	4	0.072 ± 0.040	6.39 ± 0.89
A175T	4	0.033 ± 0.014	6.67 ± 2.33*
T178M	4	0.025 ± 0.007	9.39 ± 3.12
P230L	IL3	0.033 ± 0.009	4.95 ± 1.39
A244E	6	0.019 ± 0.004*	7.95 ± 1.85
L250Q	6	0.25 ± 0.07*	5.91 ± 0.47*
I251L	6	0.025 ± 0.008	11.8 ± 5.01
G252S	6	0.081 ± 0.060	18.9 ± 12.8
V253I	6	0.026 ± 0.006	9.46 ± 3.32
C271Y	6/EL3	1.74 ± 0.23*	34.1 ± 29.6
N274S	EL3	0.019 ± 0.004*	6.43 ± 1.21*
Y287Stop	7	0.80 ± 0.22*	>1
P299H	7	no stim	>1
I301T	7	0.063 ± 0.003	11.3 ± 1.95
I316S	C-term	0.024 ± 0.0003	9.84 ± 3.91
I317T	C-term	0.030 ± 0.006	4.83 ± 0.52*
TM5Del		no stim	>1
TM6Ins		no stim	>1

[a] ^{125}I-NDP-MSH was used to competitively displace nonlabeled NDP-MSH in a dose−response fashion. Pound sign (#) indicates the average from greater than 10 independent experiments. No stim indicates that the endogenous agonists were unable to stimulate the receptor polymorphisms at up to 1 μM concentrations. >1 indicates that an IC_{50} could not be determined at up to 1 μM concentrations, or no competitive displacement binding of NDPMSH was observed. Statistical analysis was performed using a student T-test compared to the wild-type receptor values with $*p < 0.05$.

Table 4: Summary of the Polymorphic hMC4R Pharmacology of the Endogenous C-Terminal Antagonist Agouti-related Protein Ligand [hAGRP(87−132)] and the Synthetic Agonist MTII

mutation	TM	MTII agonist EC_{50} (nM)	antagonist pA_2 hAGRP (87−132)	hAGRP(87−132) inverse agonist activity observed
WT[#]		0.017 ± 0.007	8.28 ± 0.11	yes
T11S	N-term	0.020 ± 0.004	8.02 ± 0.25	yes
T11A	N-term	0.008 ± 0.002	8.74 ± 0.29	yes
R18C	N-term	0.006 ± 0.004	8.73 ± 0.20	not observed
S30F	N-term	0.009 ± 0.003	8.34 ± 0.10	yes
Y35Stop	N-term	No stim	ND	ND
D37V	N-term	0.005 ± 0.001*	8.53 ± 0.28	yes
S58C	1	0.045 ± 0.008*	8.02 ± 0.13	not observed
N62S	1	No stim	ND	ND
P78L	2	No stim	ND	ND
V95I	2	No stim	ND	ND
N97D	2	2.30 ± 0.18**	7.44 ± 0.11*	not observed
I102S	2	0.43 ± 0.07**	8.17 ± 0.10	not observed
V103I	2	0.035 ± 0.016	7.93 ± 0.18*	yes
L106P	2	0.92 ± 0.13**	8.27 ± 0.12	not observed
T112M	EL1	0.011 ± 0.003	8.33 ± 0.15	yes
I125K	3	No stim	ND	ND
S127L	3	0.027 ± 0.006	7.06 ± 0.16**	yes
I137T	3	0.012 ± 0.002	8.33 ± 0.12	not observed
T150I	3	0.15 ± 0.02**	8.34 ± 0.06	not observed
R165Q	4	0.030 ± 0.003	8.57 ± 0.059	not observed
R165W	4	0.15 ± 0.03**	8.52 ± 0.17	not observed
I169S	4	0.014 ± 0.002	8.33 ± 0.04	yes
I170V	4	0.009 ± 0.001	8.43 ± 0.23	yes
A175T	4	0.009 ± 0.005	8.54 ± 0.30	yes
T178M	4	0.019 ± 0.008	8.24 ± 0.15	yes
P230L	IL3	0.006 ± 0.005	8.86 ± 0.40	yes
A244E	6	0.027 ± 0.017	8.21 ± 0.20	yes
L250Q	6	0.058 ± 0.04**	7.42 ± 0.09	yes
I251L	6	0.047 ± 0.033*	8.59 ± 0.29	yes
G252S	6	0.040 ± 0.011*	8.53 ± 0.02	yes
V253I	6	0.014 ± 0.003	8.41 ± 0.17	yes
C271Y	6/EL3	0.60 ± 0.11**	8.45 ± 0.03	not observed
N274S	EL3	0.019 ± 0.009	8.35 ± 0.25	yes
Y287Stop	7	0.61 ± 0.16** (partial agonist)	9.10 ± 0.08	not observed
P299H	7	no stim	ND	ND
I301T	7	0.028 ± 0.004	8.60 ± 0.07	not observed
I316S	C-term	0.008 ± 0.001	8.71 ± 0.06	yes
I317T	C-term	0.016 ± 0.003	8.33 ± 0.12	not observed
TM5Del		no stim	ND	ND
TM6Ins		no stim	ND	ND

[a] Pound sign (#) indicates the average from greater than nine independent experiments. The MTII agonist EC_{50} values indicated represent the mean of at least three independent experiments with the standard error of the mean indicated. No stim indicates that MT11 was unable to stimulate the receptor polymorphisms at up to 1 μM concentrations. The antagonistic pA_2 values were determined using the Schild analysis and the agonist MTII ($K_i = -\log pA_2$). The indicated errors for the functional data (pA_2) represent the standard error of the mean determined from at least three independent experiments. ND indicates that the pA_2 value could not be determined since MTII was unable to potently stimulated the polymorphic receptor. Statistical analysis was performed using a student T-test compared to the wild-type receptor values with $*p < 0.05$, $**p < 0.01$.

hMC4R Polymorphic Receptors that Are Expressed at the Cell Surface and Functionally Respond Differently to the Endogenous POMC Derived agonists. The hMC4R polymorphisms that are expressed at the cell surface and possess decreased potency of the endogenous melanocortin agonists examined in this study [α-MSH, β-MSH, γ_2-MSH, and ACTH(1−24), Table 2] as compared to the wild-type receptor can be subdivided into two classes. The first subclass that possesses statistically significantly reduced potency for all these endogenous melanocortin agonists and the second subclass that possesses statistically significantly reduced potency only at one or more of the endogenous agonists. The first subclass consists of 11 polymorphic receptors including S58C, N97D, I102S, L106P, S127L, T150I, R165Q, R165W, C271Y, Y287Stop, and I301T. The second subclass consists of the six T112M, I137T, A244E, L250Q, G252S, and I316S hMC4 polymorphic receptors.

Functional and Competitive Displacement Binding Characterization of the Synthetic Melanocortin Agonist NDP-MSH. NDP-MSH (Table 1) is a highly potent and stable synthetic melanocortin receptor agonist (*59*). Because of its potency, chemical stability, and ease of iodination, NDP-MSH has become the ^{125}I-radiolabeled ligand of choice for characterization of the melanocortin receptors. Table 3

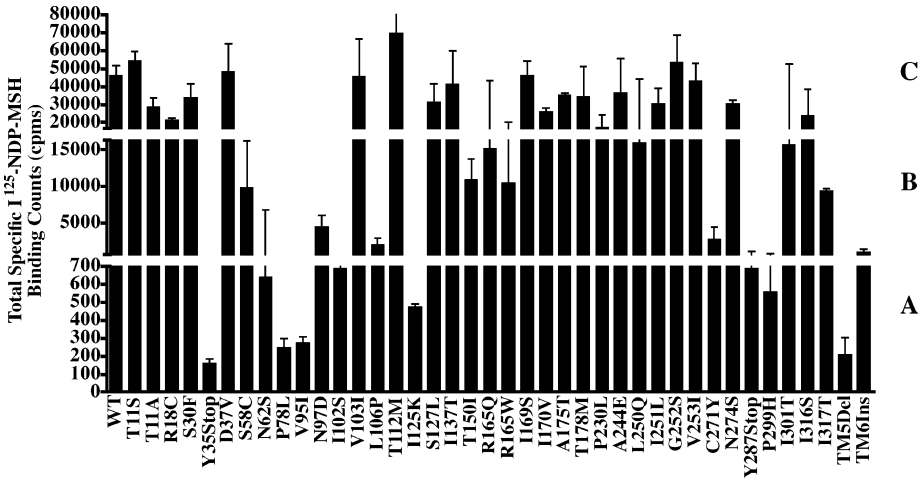

FIGURE 5: Summarizes the total specific binding counts (cpms) of ^{125}I-NDP-MSH binding to the polymorphic hMC4R stable HEK-293 cell lines generated in this study. This figure correlates with the FACS experiment Figure 4, demonstrating that receptor protein expressed at the cell surface is properly folded to allow for ligand binding. A indicates specific binding up to 700 cpms, B indicates specific binding between 700 and 16 250 cpms, and C indicates the range of greater than 17 000 cpms total specific binding that we considered to be within a "normal" range of cell surface receptor expression levels.

summarizes the functional agonist EC_{50} values and competitive displacement binding IC_{50} pharmacological results of the NDP-MSH ligand. Unlabeled "cold" NDP-MSH was used to competitively displace the ^{125}I-NDP-MSH radioligand. Of the 40 polymorphic hMC4 receptors expressed at the cell surface and examined in this study, 18 (T11A, T11S, R18C, S30F, V103I, S127L, I137T, I169S, I170V, A175T, T178M, P230L, I251L, G252S, V253I, I301T, I316S, and I317T) resulted in equipotent NDP-MSH agonist stimulation as compared to the wild-type hMC4R, 11 (S58C, N62S, N97D, I102S, L106P, T150I, R165Q, R165W, L250Q, C271Y, and Y287Stop) resulted in statistically significant decreased NDP-MSH agonist potency, and four (D37V, T112M, A244E, and N274S) interestingly possessed increased NDP-MSH agonist potency.

Comparison of the ability of NDP-MSH to competitively displace radiolabeled ^{125}I-NDP-MSH resulted in polymorphic hMC4Rs that were equipotent or possessed modest statistically significant increased binding affinity (I169S, A175T, L250Q, N274S, and I317T) for NDP-MSH (Table 3). Unexpectedly, only the N97D hMC4R possessed greater than 6-fold decreased NDP-MSH binding affinity as compared to the wild-type hMC4R. Figure 5 summarizes the total specific binding values for the 40 hMC4R polymorphic and wild-type stably expressing HEK-293 cell lines generated in this study and used for pharmacological comparisons. Of these cell lines, only the Y35Stop, N62S, P78L, V95I, I102S, I125K, Y287Stop, P299H, TM5Del, and TM6Ins hMC4R expressing cells did not bind to radiolabeled ^{125}I-NDP-MSH above an average of 750 counts per minute (cpms), suggesting that these cell lines do not express very much of the conformationally required receptor protein at the cell surface or that the amino acid change in the receptor has changed the ability of ^{125}I-NDP-MSH to interact with the putative binding pocket at the concentrations examined in this study (Table 3 and Figure 5).

Pharmacological Characterization of the AGRP Endogenous Antagonist. Since the full-length 132 amino acid hAGRP antagonist ligand is not readily synthetically accessible by chemical means (*60*), the studies presented herein utilized the commercially available hAGRP(87−132) fragment that has been demonstrated to be equipotent to the full-length 132 amino acid hAGRP ligand (*16*). Table 4 summarizes the antagonist pA_2 values of the MC4R antagonist hAGRP(87−132) fragment. Figure 3 illustrates the hAGRP-(87−132) antagonist pharmacology at the wild-type hMC4R. Consistent with previous reports, hAGRP(87-132) possessed inverse agonist activity (*61*−*63*) and a potent K_i value of 5 nM (*16*, *56*), where $K_i = -\log pA_2$ (*54*). For the Y35Stop, N62S, P78L, V95I, I125K, P299H, TM5Del, and TM6Ins hMC4Rs, the potency of the hAGRP(87−132) antagonist could not be determined as the MTII agonist used in the assay was unable to potently stimulate the receptor. This lack of MTII agonist induced stimulation can be associated with both poor cell surface expression (Figure 4) and ^{125}I-NDP-MSH binding affinity (Figure 5). Interestingly, the N97D (K_i = 36.3 nM), V103I (K_i = 11.7 nM), and S127L (K_i = 87.1 nM) possessed significantly reduced hAGRP(87−132) antagonist potency, while the Y287Stop (K_i = 0.8 nM) possessed an increased hAGRP(87−132) antagonist potency.

Constitutively Active hMC4R Polymorphism Candidates. Consistent with previous studies, we have observed an elevated basal activity of the L250Q hMC4R mutation (Figure 6), indicative of a constitutively active receptor modification (*33*, *64*). Unexpectedly, we also observed an increase in basal activity for the I251L hMC4R mutation (Figure 6). One explanation to increased basal activity of a cell line stably expressing a receptor might be attributed to an increase in the number of receptors expressed on the cell surface, and hence an increase in the number of basal adenylate cyclase enzymes stimulated. Figure 4 summarizes the relative expression of the L250Q (50%) and I251L (35%) hMC4Rs at the cell surface in stably expressing cell lines generated in this study. Figure 5 compares the total counts per minute (cpms) of the I251L and wild-type hMC4R stably expressing cell lines. Taken together, these latter data indicate that the I251L hMC4R stably expressing cell line does not possess a significantly increased number of receptors expressed at the cell surface, discounting the hypothesis that the I251L increased basal activity is attributed to increased receptor cell surface expression. However, a key experiment in determining if a receptor mutation is indeed constitutively

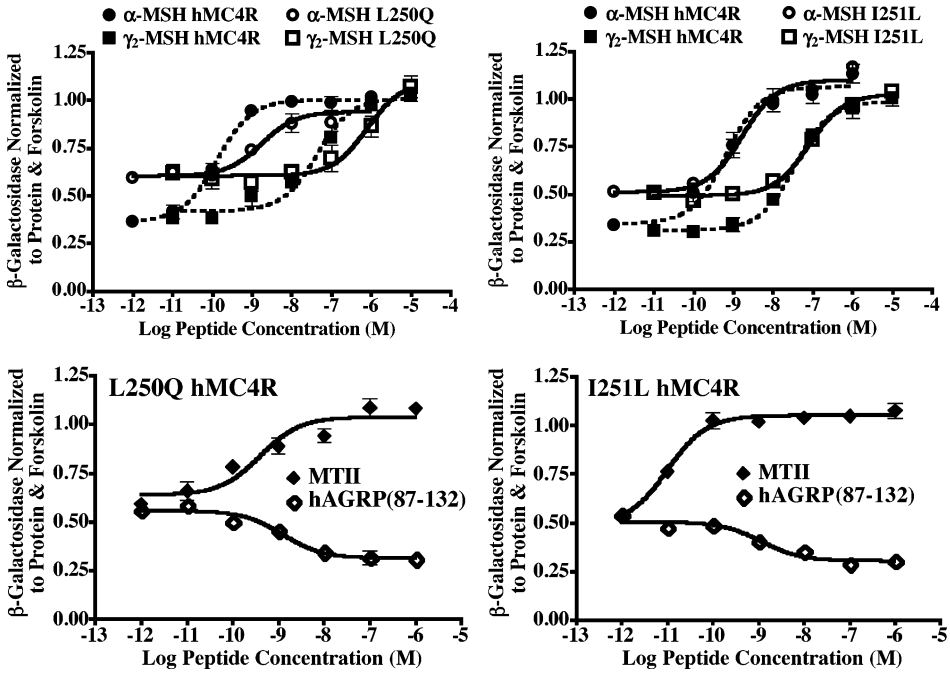

FIGURE 6: Pharmacological comparison of the L250Q and I251L hMC4Rs with the endogenous melanocortin agonists and the hAGRP-(87−132) antagonist. Both these receptors possess an increased basal level of activity as compared to the wild-type hMC4R indicated by dotted lines. Both the L250Q and I251L hMC4Rs possess inverse agonist activity for hAGRP(87−132).

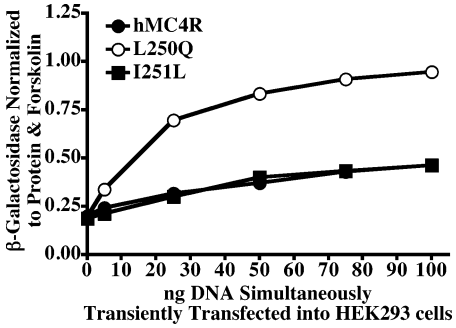

FIGURE 7: Transient transfection experiment comparing basal levels of the wild-type hMC4R, L250Q hMC4R, and I251L hMC4R upon increasing concentration of receptor plasmid. These data demonstrate that the L250Q hMC4R possesses constitutive activity whereas the wild-type and I251L hMC4 receptors do not.

active is to perform a transient transfection experiment in which different amounts of wild-type and mutant receptor DNA are simultaneously examined (*51*, *55*, *65*). Figure 7 summarizes the results of a transient transfection experiment comparing the wild-type, L250Q, and I251L mutant receptor DNA. These latter data clearly indicate that while the L250Q hMC4R mutation is indeed constitutively active, the I251L hMC4R mutation is not constitutively active and possessed the same profile as the wild-type hMC4R in this experiment. Thus, it might be possible to explain the increased basal activity of the I251L hMC4R by alteration of downstream intracellular events associated with GPCR cAMP signal transduction. These events might include an increase of the interactions of this I251L hMC4 mutant receptor with more G-proteins, an upregulation and expression of the adenylate cyclase protein, a downregulation of phosphodiesterase activity, or perhaps an upregulation of the catalytic protein kinase A. These speculations remain to be experimentally verified however.

Cell Surface Expression of Selected hMC4R Polymorphic Receptors in Stably Expressing HEK-293 Cells. Previous studies of the hMC4R polymorphic receptors have observed differences in the ability of the mutant receptors to be expressed at the cell surface in transiently transfected (*38*, *64*, *66*) and stably expressing (*33*) HEK-293 cell lines. Previously reported polymorphic hMC4Rs that were observed to possess reduced cell surface expression levels, those generated in this study based upon total receptor binding of ^{125}I-NDP-MSH that fell below 17 000 cpms (Figure 5), and hMC4 receptors possessing differences in ligand pharmacology between them and the wild-type hMC4R were selected for cell surface expression level experiments. The wild-type hMC4R possessed total specific binding counts of 46000 ± 5500 (n = 8). Figure 4 summarizes the percentage of total cellular receptor protein expression and receptor cell surface expression of selected mutant hMC4 receptors stably expressed in HEK-293, relative to the wild-type hMC4R, using the N-terminal FLAG-hMC4Rs and fluorescence activated cell sorting (FACS) quantification. These data demonstrate that for all the polymorphic hMC4Rs generated in this study, the protein was stably expressed in the cell lines derived. Using a combination of intracellular labeling on saponin-permeabilized cells and surface labeling on nonpermeabilized cells, it was observed that the Y35Stop, P78L, V95I, I125K, P299H, TM5Del, and TM6Ins hMC4R polymorphisms are producing detectable receptor protein within the cell but are unable to transport these hMC4R molecules to the surface or generate stable expression and function (Figure 4). These data suggest that failure of cell surface hMC4R protein expression may be a result of the inability to transport the polymorphic protein to the cell surface, or alternatively an unstable receptor conformation may result that might not be retained at the cell surface.

DISCUSSION

Obesity is becoming a significant health risk in the United States of America as well as internationally. The MC4R has

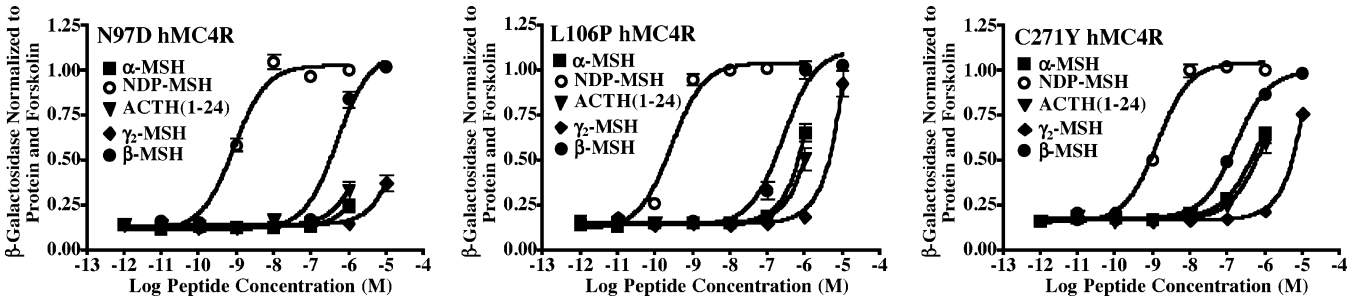

FIGURE 8: Characterization of the N97D, L106P, and C271Y hMC4 receptors demonstrating that the endogenous agonist β-MSH is able to maximally stimulate these receptors, whereas the endogenous agonists α-MSH, γ_2-MSH, and ACTH(1−24) are unable to stimulate a maximal response at up to micromolar concentrations.

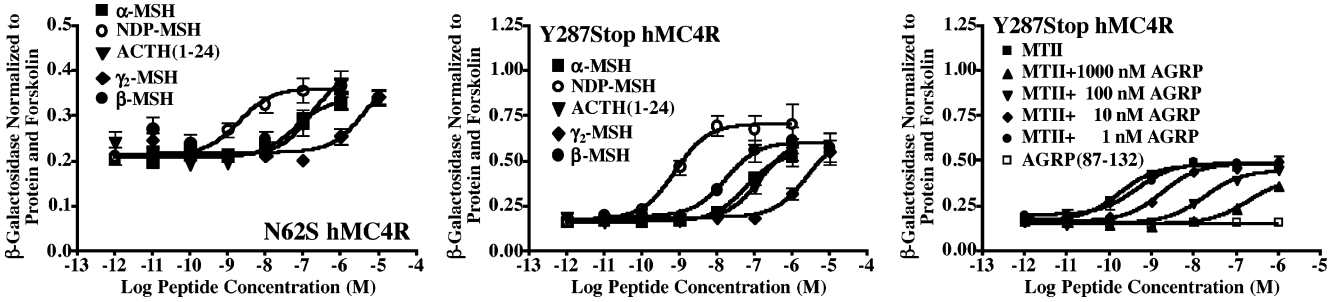

FIGURE 9: Illustration of the partial agonist activity of the endogenous melanocortin agonists at the N62S hMC4R and Y287Stop hMC4R and hAGRP(87−132) antagonist pharmacology at the Y287Stop hMC4R relative to nonreceptor dependent maximal stimulation by forskolin control which is defined as 1.0 on the *y*-axis.

been demonstrated in both mice and humans to be involved in the regulation of feeding behavior and energy homeostasis (*3*, *4*, *29*, *32*, *33*). Thus, the discovery of MC4R polymorphisms in morbidly obese humans may be an important genetic linkage between human obesity and a genetic risk factor and obesity. In attempts to understand the molecular mechanism by which these polymorphic hMC4Rs may be dysfunctional, *in vitro* pharmacological characterization experiments provide an initial forum to identify which polymorphic receptors may result in an unnatural response to endogenous ligands or be susceptible to improper cell surface trafficking. The physiological relevance of *in vitro* heterologous cell assay systems needs to be verified however using *in vivo* models to more definitively establish a link between humans with genetically modified MC4Rs and a specific molecular dysfunctional mechanism.

A number of research laboratories have performed *in vitro* pharmacological characterization experiments on a large number of the hMC4R polymorphisms identified in humans. However, the systematic comparisons of these polymorphic MC4 receptors with the multiple endogenous melanocortin ligands has not been previously reported and is the focus of the studies presented herein. Herein, we have generated 40 polymorphic hMC4 receptors and pharmacologically characterized them for changes in cell surface expression, endogenous agonist ligand potency, antagonist hAGRP(87−132) efficacy, and the ability of the synthetic NDP-MSH ligand to stimulate and bind these receptors. These studies have been undertaken for a systematic side-by-side comparison of these receptors using a combination of pharmacological assays in attempts to associate putative molecular mechanism defects with the human obesity phenotype. The seven Y35Stop, P78L, V95I, I125K, P299H, TM5Del, and TM6Ins hMC4R polymorphic proteins were unable to bind ^{125}I-NDP-MSH, did not possess greater than 10% cell surface expression levels relative to the wild-type hMC4R but were stably expressed in HEK-293 cells (Figure 4). Another set of nine polymorphic hMC4R receptors (T11S, S30F, D37V, I169S, A175T, T178M, P230L, V253I, and N274S) were characterized in this study to possess ligand agonist and antagonist efficacy equipotent to the wild-type receptor and possess relatively normal levels of cell surface expression. Consistent with these findings, the T178M (*67*) and V253I (*32*, *38*, *68*) hMC4Rs have been identified in nonobese patient controls. For the remaining seven hMC4R pharmacologically characterized to possess profiles similar to the wild-type hMC4R, potential molecular mechanisms associating them with the obese phenotype remain to be hypothesized and experimentally tested.

A group of 11 polymorphic receptors including S58C, N97D, I102S, L106P, S127L, T150I, R165Q, R165W, C271Y, Y287Stop, and I301T possess statistically significant decreased potency for the endogenous melanocortin agonists examined. Yet, only the N97D and S127L polymorphic hMC4Rs also possess significantly reduced hAGRP(87−132) antagonist potency. Furthermore, of these two hMC4Rs, only the N97D also possesses less than 50% receptor cell surface expression as compared to the wild-type hMC4R, and decreased NDP-MSH potency and affinity. Thus, the N97D appears to be the only hMC4R examined in this study that was significantly different than the wild-type receptor in all the assays performed herein. Interestingly, the N97D, L106P, and C271Y hMC4Rs are not maximally stimulated by any of the endogenous agonist ligands at the micromolar concentrations examined in this study, with the exception of the β-MSH ligand, which can maximally stimulate these receptors (Figure 8), albeit with reduced potency as compared to the wild-type hMC4R.

Six polymorphic hMC4 receptors (T112M, I137T, A244E, L250Q, G252S, and I316S) possess statistically significant

reduced potency only for one or more of the endogenous agonists, supporting our hypothesis that the hMC4R polymorphisms may respond normally to some putative endogenous melanocortin ligands and differently to others. Interestingly, the I137T, A244E, and L250Q possess decreased agonist potency for both α-MSH and ACTH(1−24) from which α-MSH is derived (Figure 1). The S127L hMC4R possessed both decreased endogenous agonist and hAGRP-(87−132) potency as compared to the wild-type hMC4R. The hMC4R V103I polymorphism has been reported in both obese and control human patients (*29*, *32*, *33*, *35*, *36*, *67*−*71*). Since this hMC4R V103I polymorphism possesses a normal endogenous agonist ligand affinity profile and normal receptor expression levels at the cell surface as determined from the total specific binding of ^{125}I-NDP-MSH (Figure 5), it has been difficult to link this mutation with a potential molecular defect. Herein, however, we have observed that this V103I hMC4R possesses a modest 2-fold statistically significant decrease in antagonist hAGRP(87−132) potency (Table 4), which is not consistent with the obese phenotype observed in some patients.

The N62S and Y287Stop hMC4R polymorphisms examined in this study resulted in receptor proteins that possessed less than 50% cell surface expression (Figure 4) and yet were still able to be partially stimulated by the endogenous agonists relative to the nonreceptor dependent forskolin control values and to the wild-type hMC4R (Figure 9). These N62S and Y287Stop hMC4R polymorphic receptors possess decreased agonist potency for all the agonist ligands examined in this study. The Y287Stop hMC4R interestingly possesses a 6-fold increased hAGRP(87−132) potency of 0.8 nM relative to the wild-type hMC4R.

SUMMARY

For the first time, we have performed an extensive side-by-side pharmacological characterization study of 40 human MC4R polymorphisms examining multiple endogenous melanocortin agonists [α-MSH, β-MSH, γ_2-MSH, and ACTH-(1−24)] and antagonist [hAGRP(87−132)] potency and receptor cellular expression levels. This study has identified nine hMC4R polymorphisms that are expressed at the cell surface and respond similarly to the wild-type receptor in the experiments performed, three hMC4R polymorphisms that respond normally to the endogenous ligands yet have reduced cell surface expression, 19 hMC4R polymorphisms that resulted in decreased endogenous agonist, synthetic NDP-MSH agonist, or hAGRP(87−132) antagonist potency as compared to the wild-type hMC4R, two hMC4R polymorphisms that resulted in reduced cell surface expression and ligand partial agonist pharmacological profiles, and seven hMC4R polymorphisms that resulted in less than 50% cell surface expression, relative to the wild-type hMC4R. From these results, six polymorphic hMC4Rs were identified that possessed significantly reduced potency only at one or more of the endogenous agonists, supporting our hypothesis that the hMC4R polymorphisms may respond normally to some putative endogenous melanocortin ligands and differently to others.

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