Structure Activity Studies of the Melanocortin-4 Receptor by *in Vitro* Mutagenesis: Identification of Agouti-Related Protein (AGRP), Melanocortin Agonist and Synthetic Peptide Antagonist Interaction Determinants†

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ABSTRACT: *In vitro* mutagenesis of the mouse melanocortin-4 receptor (mMC4R) has been performed, based upon homology molecular modeling and previous melanocortin receptor mutagenesis studies that identified putative ligand−receptor interactions. Twenty-three mMC4 receptor mutants were generated and pharmacologically characterized using several melanocortin-based ligands [α-MSH, NDP-MSH, MTII, DNal (1′)^7-MTII, Nal(2′)^7-MTII, SHU9119, and SHU9005]. Selected mutant receptors possessing significant differences in the melanocortin-based peptide agonist and/or antagonist pharmacology were further evaluated using the endogenous antagonist agouti-related protein fragment hAGRP(83−132) and hAGRP(109−118) molecules. These studies of the mouse MC4R provide further experimental data suggesting that the conserved melanocortin receptor residues Glu92 (TM2), Asp114 (TM3), and Asp118 (TM3) (mouse MC4R numbering) are important for melanocortin-based peptide molecular recognition. Additionally, the Glu92 and Asp118 mMC4R residues are important for molecular recognition and binding of AGRP(83−132). We have identified the Phe176 (TM4), Tyr179 (TM4), Phe254 (TM6), and Phe259 (TM6) receptor residues as putatively interacting with the melanocortin-based ligand Phe^7 by differences between α-MSH and NDP-MSH agonist potencies. The Glu92, Asp118, and Phe253 mMC4R receptor residues appear to be critical for hAGRP(83−132) molecular recognition and binding while Phe176 appears to be important for functional antagonism of AGRP(83−132) and AGRP(109−118) but not molecular recognition. The Phe253 mMC4R residue appears to be important for AGRP(83−132) molecular recognition and general mMC4 receptor stimulation. The Phe254 and Phe259 mMC4R amino acids may participate in the differentiation of agonist versus antagonist activity of the melanocortin-based peptide antagonists SHU9119 and SHU9005, but not AGRP(83−132) or AGRP(109−118). The Met192 side chain when mutated to a Phe results in a constitutively active mMC4R that does not effect agonist ligand binding or potency. Melanocortin-based peptides modified at the 7 position of MTII with DPhe, DNal(1′), Nal(2′), and DNal-(2′) have been pharmacologically characterized at these mutant mouse MC4Rs. These data suggest a revised hypothesis for the mechanism of SHU9119 antagonism at the MC4R which may be attributed to the presence of a "bulky" naphthyl moiety at the 7 position (original hypothesis), and additionally that both the stereochemistry and naphthyl ring position (2′ versus 1′) are important for positioning of the ligand Arg^8 residue with the corresponding mMC4R amino acids.

The melanocortin system is a unique G-protein coupled receptor (GPCR)[1] pathway that not only includes endogenous agonists and receptors, but also the only two identified naturally occurring antagonists of GPCRs (agouti/ASP and agouti-related protein/AGRP) (*1*, *2*). The melanocortin peptides (α-, *β*-, *γ*-melanocyte stimulating hormones and adrenocorticotropin hormone, ACTH) are the endogenous agonist ligands for the melanocortin receptors and are derived by posttranslational processing of the proopiomelanocortin (POMC) gene transcript. All the melanocortin agonist peptides contain the His-Phe-Arg-Trp consensus sequence that is also considered to be the message sequence of these ligands important for melanocortin receptor selectivity and stimulation. The melanocortin family contains five receptors (MC1R-MC5R) cloned to date (*3*−*9*) and stimulates the cAMP second messenger signal transduction pathway.

The melanocortin pathway includes five genetic factors that have been linked to energy homeostasis and obesity in mice and humans. The mouse agouti protein (ASP) (*10*, *11*)

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[1] Abbreviations: mMC4R, mouse melanocortin-4 receptor; AGRP, agouti-related protein; GPCR, G-protein coupled receptor; ASP, mouse form of the agouti protein; α-MSH, α-melanocyte stimulating hormone; POMC, proopiomelanocortin; cAMP, cyclic 3′5′-adenosine monophosphate; HEK-293 cells, human embryonic kidney cells.

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was first characterized as an antagonist of the skin MC1R and brain MC4R (*1*). The AGRP protein was demonstrated pharmacologically to competitively antagonize the MC3R and MC4R brain melanocortin receptors (*12*), and when ectopically expressed, resulted in an obese phenotype (*2*, *13*). The brain melanocortin receptor (MC3R and MC4R) knock out animals have been identified as physiologically participating in the regulation of energy homeostasis (*14*−*16*). Furthermore, a genetic modification of the gene transcripts for POMC (from which the agonist ligands are derived) (*17*) and MC4R (*18*−*20*) in obese humans has been identified.

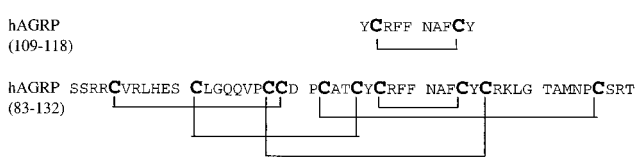
Studies of the agouti protein identified the Arg-Phe-Phe residues, conserved in both agouti and AGRP, as critical for its antagonistic properties and provided experimental evidence (Arg replacement by Ala resulted in a complete loss of antagonist activity) to support the hypothesis that these residues may mimic the melanocortin agonist Phe-Arg-Trp interactions with the melanocortin receptors (*21*). A report identifying the agouti and AGRP decapeptides Ac-c[CRFFGSAC]-NH_2 and Yc[CRFFNAFC]Y, respectively, as antagonists of the brain melanocortin receptors (*22*) demonstrated that this is at least one structural component of these endogenous antagonists required for inhibitory actions at the MC4R. Further work by our laboratory characterized the AGRP decapeptide Yc[CRFFNAFC]Y as an agonist at the mMC1R (*23*) which is normally involved in the regulation of pigmentation and coat color and is not antagonized or stimulated by AGRP (*2*, *12*). Additionally, we demonstrated that the AGRP decapeptide Yc[CRFFNAFC]Y was able to bind to the mouse MC1, MC3, MC4, and MC5 receptors, albeit with micromolar binding affinities. The three-dimensional ^{1}H NMR AGRP(87−132) structure characterized the AGRP Arg-Phe-Phe (111−113) residues as located in an external loop surface (*24*) which would potentially allow for interactions of these residues with the melanocortin receptor putative binding pocket.

Little is known about the exact structural features (primary, secondary, and tertiary) of the only two identified naturally occurring GPCR antagonists. This study was designed to help decipher the mechanism by which AGRP antagonizes (blocking the agonist binding pocket due to its size and bulk, allosterically modifying the agonist binding pocket, possessing the same ligand−receptor interactions as the agonist, or possessing some overlapping and distinct receptor interactions as the agonist) the brain MC4R. Recently, chimeric melanocortin receptors have been utilized to identify the exoloops of the MC4R as being important for AGRP and agouti interactions (*25*, *26*), but the specific ligand−receptor interactions remain to be identified.

The melanocortin-4 receptor, due to its direct involvement in feeding behavior (icv administered agonist, eat less; icv administered antagonist, eat more), is a current drug target for the design of selective agonist therapeutics to treat obesity and the design of selective antagonists for potential treatment of anorexia. This study was undertaken to characterize putative ligand−receptor interactions for selected melanocortin structural-based peptide agonists and antagonists and determine if there are similar ligand−receptor interactions for the endogenous AGRP(83−132) antagonist. These data can then potentially be applied to homology molecular modeling studies using the recently reported 2.8 Å resolution structure of the GPCR rhodopsin (*27*) and aid in the design of therapeutic agents for the treatment of obesity associated diseases. During the preparation of this manuscript a report of 24 point mutations of the human MC4R has been published (*28*). These mutagenesis data, in addition to the data presented herein, provide substantial informational insights into putative ligand−receptor interactions, receptor residues important for receptor stimulation, and differentiation of melanocortin agonist versus antagonist activities.

Table 1: Primary Structure of the Peptides Used in This Study

Abbreviation	Sequence*
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
NDP	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
DNal(1')^7 MTII	Ac-Nle-c[Asp-His-**DNal(1')**-Arg-Trp-Lys]-NH_2
Nal(2')^7 MTII	Ac-Nle-c[Asp-His-**Nal(2')**-Arg-Trp-Lys]-NH_2
SHU9119	Ac-Nle-c[Asp-His-**DNal(2')**-Arg-Trp-Lys]-NH_2
SHU9005	Ac-Ser-Tyr-Ser-**Nle**-Glu-His-**p(I)DPhe**-Arg-Trp-Gly-Lys-Pro-Val-NH_2



[a] The brackets [] in the MTII peptides denotes a cyclic lactam bridge formed between the Asp and Lys side chains via an amide bond.

MATERIALS AND METHODS

Materials. Radiolabeled [^{125}I]NDP-MSH, MTII, SHU9119, and AGRP(83−132) were supplied by NEN Life Sciences. NDP-MSH is iodinated at the Tyr^2 position, MTII and SHU9119 are iodinated at the His position, and AGRP(83−132) is iodinated at the Tyr amino acids (Table 1). MTII was purchased from Peninsula Lab, and SHU9119 was purchased from Phoenix Pharmaceuticals, Inc. Iodination of MTII: ^{125}I label was prepared by iodination with carrier free Na^{125}I using the chloramine T as the oxidizing agent. The product was purified using a C-8 reversed-phase column (Zorbax SB 300A C-8, 4.6 mm × 75 mm) with a linear gradient from 25 to 40% acetonitrile in 0.1% TFA over 40 min. The [^{125}I]MTII eluted around 20 min of the gradient. The product was isolated, then diluted, and stored in a solution containing 50% water, 0.1% TFA, 0.4% BSA, 23% 1-propanol, and 27% acetonitrile. Iodination of SHU9119: [^{125}I]SHU9119 was prepared using a similar procedure as for [^{125}I]MTII. The noniodinated peptide NDP-MSH was purchased from Bachem, MTII and SHU9119 were provided by Dr. Victor Hruby's laboratory (University of Arizona) or purchased from Bachem. The SHU9005, DNal(1′)-MTII and Nal(2′)-MTII peptides were provided by Dr. Victor Hruby's laboratory at the University of Arizona (Tucson, AZ) and the pharmacology at the mouse MC1, MC3, MC4, and MC5 receptors has been previously reported (*29*). Primers were synthesized and desalted by Gibco-BRL.

Receptor Mutagenesis. Mouse MC4R cDNA (1.6 Kb fragment) was subcloned into pBluescript (Stratagene) and was used for mutagenesis. Mutants were prepared by the polymerase chain reaction (PCR) using pfu polymerase (Stratagene) and a complementary set of primers containing the nucleotide mutation(s) resulting in the desired amino acid residue change. After completion of the PCR reaction (95° 30 s, 12 cycles of 95° 30 s, 55° 1 min, 68° 9 min) the product was purified (Qiaquick PCR reaction, Qiagen) and eluted in water. Subsequently, the sample was cut with Dpn1 (Biolabs)

to lineralize 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 *Hin*dIII/*Xba*I restriction sites of the $pCDNA_3$ expression vector (Invitrogen). Complete mutant mMC4R sequences were confirmed free of PCR nucleotide base errors by DNA sequencing (Vollum Institute and University of Florida sequencing core facilities).

Cell Culture and Transfection. Briefly, HEK-293 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum 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. Stable receptor populations were generated using G418 selection (1 mg/mL) for subsequent bioassay analysis.

Receptor Binding Studies. HEK-293 cells stably expressing the mutant and wild-type receptors were maintained as described above. One day preceding the experiment, $(0.1-0.3) \times 10^6$ cells/well were plated into Primera 24 well plates (Falcon). The peptides NDP-MSH, MTII, SHU9119, and AGRP(83−132) were used to competitively displace the ^{125}I-radiolabeled peptides (100 000 cpm/well) NDP-MSH, MTII, SHU9119, and AGRP(83−132), respectively. [^{125}I]AGRP-(83−132) binding studies were selectively performed on mMC4R mutant receptors that possessed interesting pharmacological properties for the melanocortin-based peptide agonists or antagonists. Dose−response curves (10^{-6} to 10^{-12} M) and IC_{50} values were generated and analyzed by nonlinear least-squares analysis (*30*) and the PRISM program (version 3.0, GraphPad Inc.). Each experiment was performed using duplicate data points and repeated in at least two independent experiments. The standard deviation errors are derived from the IC_{50} values.

cAMP β-Galactosidase Bioassay. HEK-293 cells stably expressing wild-type and mutant receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described (*31*, *32*). Briefly, 5000−15000 posttransfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. Forty-eight hours posttransfection, the cells were stimulated with 100 μL of peptide ($10^{-6}-10^{-12}$ 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, 200 mg of 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 determined by adding 200 μL 1:5 dilution Bio-Rad G250 protein dye:water to the 10 μL 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. Assays were performed using duplicate or triplicate data points and repeated in at least two independent experiments. Data analysis and EC_{50} values were determined using nonlinear regression analysis with the PRISM program (version 3.0, GraphPad Inc.). The antagonistic properties of AGRP(83−132) (Phoenix Pharmaceuticals), SHU9005 (Victor Hruby, University of Arizona), SHU9119 (Bachem), and AGRP(109−118) were determined by the ability of these ligands to competitively displace the NDP-MSH or MTII agonist (Bachem) in a dose-dependent manner. The pA_2 values were generated using the Schild analysis method (*33*). The standard deviation errors are derived from the agonist EC_{50} or antagonist pA_2 values.

Transient Transfection Bioassay. 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 DNA's were transfected at different concentrations using the calcium phosphate method. 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 forskolin values were measured (*34*).

Concentration of DNA Used in the Transient Transfection Bioassay

	concentration plotted					
(DNA transfected)	0	5 ng	25 ng	50 ng	75 ng	100 ng
mutant/WT	0	5 ng	25 ng	50 ng	75 ng	100 ng
pCDNA3 plasmid	100 ng	95 ng	75 ng	50 ng	25 ng	0
CRE-β-gal	4 μg	4 μg	4 μg	4 μg	4 μg	4 μg

Yc[CRFFNAFC]Y Peptide Synthesis. Peptide synthesis was performed using standard Fmoc methodology in a manual reaction vessel (*35*). The amino acids Fmoc-Tyr-(tBu), Fmoc-Cys(Trt), Fmoc-Arg(Pbf), Fmoc-Phe, Fmoc-Asn(Trt), and Fmoc-Ala were purchased from Peptides International (Louisville, KY). The peptide was assembled on Fmoc-Tyr(tBu) Wang resin (0.49 mequiv/g substitution), purchased from Peptides International (Louisville, KY). All reagents were ACS grade or better. Briefly, the Fmoc protecting groups were removed using 20% piperidine (Sigma Aldrich) in DMF, amino acid coupling (3-fold excess) was accomplished using BOP (3-fold excess), HOBt (3-fold excess), and DIEA (3.1-fold excess). Completion of amino acid coupling and Fmoc deprotection steps were monitored using the ninhydrin test (*36*). Final peptide cleavage from the resin and amino acid side chain protecting group removal was performed using (15:3:1:1) TFA:1,2-ethanedithiol:*p*-cresol:water. Peptide cyclization was performed in solution according to previously published methods (*37*). HPLC purification was performed using a Shimadzu chromatography system with a photodiode array detector. Final peptide purification was achieved using a semipreparative RP-HPLC C_{18} bonded silica column (Vydac 218TP1010, 1.0×25 cm). The purified peptide was >99% pure as determined by analytical RP-HPLC, 2D ^{1}H NMR, and had the correct molecular mass (University of Florida protein core facility).

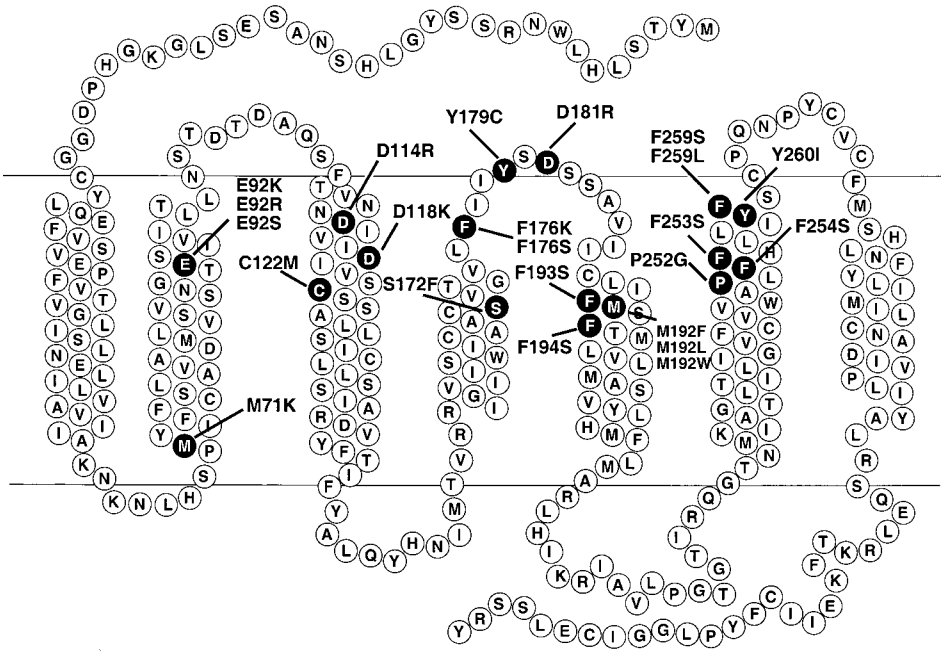


FIGURE 1: Two-dimensional model of the mouse melanocortin-4 receptor. The putative transmembrane spanning domains and loop regions are based upon the 2.8 Å structure of rhodopsin (*27*) with slight extension of the TM5 domain and the addition of two amino acids as part of the second extracellular loop.

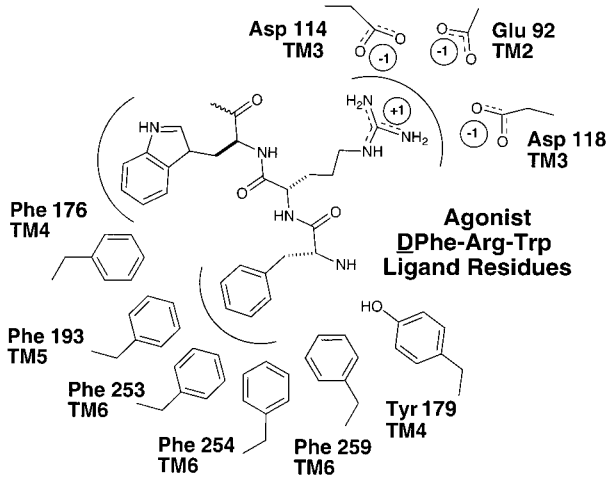


FIGURE 2: Putative mMC4R amino acids (periphery) that interact with the melanocortin ligand DPhe^7-Arg^8-Trp^9 residues (center). This model was generated using previous methodology described for the hMC1R (*38*).

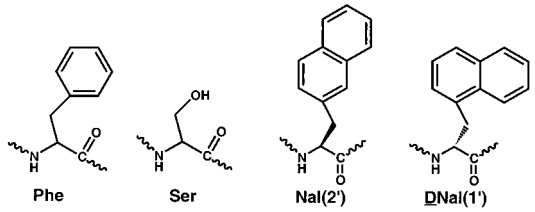


FIGURE 3: Amino acid side chains comparing the Phe to Ser substitutions of the receptor and the differences between the Nal(2′) and DNal(1′) residues used to substitute the DPhe^7 in the cyclic agonist MTII peptide template.

RESULTS

Figure 1 summarizes the mouse MC4R mutations generated in this study. Figure 2 illustrates the putative agonist ligand−receptor interactions based upon homology molecular modeling (C. Haskell-Luevano, unpublished material) of the mouse MC4R using methods previously described for the human MC1R (*38*). This model was built prior to the 2.8 Å resolution structure of rhodopsin (*27*), although subsequent rebuilding of the MC4R model based upon the 2.8 Å resolution structure of rhodopsin resulted in the observation of similar putative ligand−receptor interactions. Receptor mutations were systematically selected based upon the putative ligand−receptor interactions in Figure 2, and previous receptor mutations of the skin MC1R which were identified as important for ligand−receptor interactions and/or receptor activation (*32*, *39*, *40*). The Phe to Ser mutations were selected due to dramatic differences in amino acid side chain aromaticity, hydrophobicity, and electronegativity. A study examining amino acid side chain packing in proteins (*41*) reported that Phe−Ser side chain to side chain interactions were less favorable than other potential amino acid side chain packing interactions. Therefore, by substituting the Phe receptor side chain functionality with a Ser residue (Figure 3) which might repulse the ligand aromatic Phe or Trp residue, one might speculate that large differences in ligand affinity or efficacy might result. Similarly, the Pro252 receptor residue was replaced with the nonchiral highly structurally flexible Gly residue to release any constraint the Pro residue would have on TM 6. The electrostatic mMC4R residues were substituted to mutations that were previously identified as resulting in constitutive activation of the mouse MC1R (*32*, *40*).

Ligand Binding. Table 1 lists the primary sequences of the peptides pharmacologically characterized at these mutant mMC4 receptors. The term "melanocortin-based antagonists" used throughout this text refers to the peptide agonists α-MSH, NDP-MSH, MTII, DNal(1′)^7-MTII, and Nal(2′)^7-MTII in addition to the SHU9119 and SHU9005 antagonists (Table 1) which have been derived from the lead agonist peptides MTII and NDP-MSH, respectively (*42*, and unpublished data). Multiple ^{125}I-radiolabed peptides were used for competitive displacement studies at the mutant MC4 recep-

Table 2: Competitive Binding Affinity IC_{50} Results at Mutant mMC4Rs

		Binding IC_{50} (nM)							
mutant	TM	[^{125}I]NDP-MSH	fold difference	[^{125}I]MTII	fold difference	[^{125}I]SHU9119	fold difference	[^{125}I]AGRP (83−132)	fold difference
mMC4R		0.88 ± 0.28	1.0	0.50 ± 0.13	1.0	0.38 ± 0.10	1.0	0.66 ± 0.46	1.0
M71K	2	1.12 ± 0.51	1	0.57 ± 0.18	1	0.27 ± 0.10	1		
E92K	2	32.1 ± 2.3	36	37.1 ± 40.5	74	1.09 ± 0.24	3	>1000	
E92R	2	>1000		>1000		15.3 ± 3.81	40		
E92S	2	>1000		>1000		50.1 ± 9.7	132		
D114R	3	>1000		19.6 ± 14.2	39	37.5 ± 48.3	99	0.44 ± 0.22	−1.5
D118K	3	>1000		>1000		70.3 ± 19.2	185	>1000	
C122M	3	0.66 ± 0.17	1	0.48 ± 0.17	1	0.58 ± 0.08	2		
S172F	4	1.02 ± 0.42	1	2.77 ± 2.6	6	1.54 ± 1.65	4		
F176S	4	>1000		>1000		59.1 ± 5.9	155		
F177K	4	5.70 ± 0.14	6	2.75 ± 0.21	6	1.46 ± 0.37	4	3.69 ± 2.77	6
Y179C	4	0.89 ± 0.67	1	1.29 ± 0.08	3	0.48 ± 0.26	1	3.35 ± 1.23	5
D181R	EL2	0.96 ± 0.04	1	0.84 ± 0.52	1	0.26 ± 0.01	1		
M192F	5	1.67 ± 0.33	2	1.17 ± 0.01	3	0.92 ± 0.14	2	0.91 ± 0.09	1
M192L	5	2.64 ± 2.6	3	2.35 ± 2.1	5	2.14 ± 2.8	6		
M192W	5	1.22 ± 0.39	1	0.46 ± 0.08	1	0.36 ± 0.08	1		
F193S	5	2.87 ± 0.28	3	1.44 ± 0.13	3	0.74 ± 0.34	2	4.37 ± 0.30	7
F194S	5	1.15 ± 0.45	1	0.95 ± 0.08	2	0.70 ± 0.35	2		
P252G	6	0.97 ± 0.38	1	0.75 ± 0.29	2	0.17 ± 0.04	−2		
F253S	6	2.74 ± 1.33	3	8.07 ± 2.64	16	0.40 ± 0.06	1	>1000	
F254S	6	9.31 ± 7.2	11	3.48 ± 2.84	7	4.01 ± 4.5	11	2.52 ± 1.79	4
F259S	6	3.26 ± 0.71	4	2.39 ± 0.54	5	1.67 ± 0.86	4	1.06 ± 0.23	2
F259L	6	0.89 ± 0.15	1	0.84 ± 0.15	2	0.35 ± 0.22	1		
Y260I	6	0.77 ± 0.24	1	0.96 ± 0.21	2	0.53 ± 0.38	1		

[a] Noniodinated NDP-MSH, MTII, SHU9119, and AGRP(83−132), respectively, were used to competitively displace the corresponding ^{125}I-radiolabeled peptide. The standard deviation errors are derived from the IC_{50} values obtained from at least two independent experiments. A blank space for the [^{125}I]AGRP(83−132) label indicates that this mutant receptor was not characterized with this radiolabeled peptide.

tors. [^{125}I]NDP-MSH is a 13 amino acid linear agonist peptide, [^{125}I]MTII is a seven amino acid cyclic agonist peptide, [^{125}I]SHU9119 is a seven amino acid cyclic antagonist, and [^{125}I]AGRP(83−132) is a 50 amino acid antagonist peptide putatively containing five disulfide bridges. Each of these radiolabeled peptides possesses different potential binding characteristics and tertiary structures. These radioligands were selected in our experimental design to increase our chances for identifying differences between agonist and antagonist interactions, in addition to potentially characterizing melanocortin-based compound−receptor interactions as similar or different for the endogenous AGRP−receptor interactions. As shown in Table 2 for the E92R, E92S, D114R, D118K, and F176S mMC4 receptors our rationale of using multiple radiolabels was beneficial as the agonist NDP-MSH and MTII radiolabels were unable to bind these mutant receptors, whereas the antagonist SHU9119 was able to bind. The fact that the radiolabeled antagonist SHU9119 was able to bind demonstrated that these receptors are expressed on the cell surface. The observation that the antagonist peptide was able to bind these receptors whereas the agonist peptides were not, is in agreement with the GPCR ternary complex model for activation (*43*, *44*) in which there is a larger proposed receptor population that the antagonist can bind versus a lower population in which the agonist can bind.

Table 2 summarizes the pharmacological IC_{50} results of the radiolabeled (^{125}I) ligand competitive displacement experiments at these mutant mMC4Rs. For each radiolabeled peptide [NDP-MSH, MTII, SHU9119, and AGRP(83−132)] the respective noniodinated, or cold, peptide was used to competitively displace the labeled compound resulting in the binding affinity IC_{50} values summarized in Table 2. Figure 4 illustrates the binding and functional pharmacology at the wild-type mMC4R. Small differences (1−6-fold) in radioligand affinity were observed for several of the mutant receptors (M71K, C122M, S172F, F176K, Y179C, D181R, M192F/L/W, F193S, F194S, P252G, F259S/L, and Y260I) compared to the wild-type receptor. The E92K mutant receptor possessed 36−74-fold decreased ligand affinity for the agonist peptides NDP-MSH and MTII, while the antagonist SHU9119 possessed nearly equal affinity as the wild-type receptor. However, AGRP(83−132) was unable to bind to this mutant mMC4R. The E92R, E92S, D118K, and F176S mutant mMC4Rs were unable to bind to the agonist labeled peptides NDP-MSH and MTII, but 40-, 132-, 185-, and 155-fold decreased binding affinities, respectively, were observed for the antagonist SHU9119 ligand as compared with the wild-type receptor. At the D118K mMC4R, the seven amino acid antagonist SHU9119 was able to bind to this receptor; however, the AGRP(83−132) 50 amino acid antagonist lost all binding affinity. Interestingly, at the F253S mMC4R, the melanocortin-based ligands retain nearly equal binding affinities as at the wild-type receptor, with the exception of MTII which possesses a 16-fold decreased affinity, but AGRP(83−132) has lost all ability to bind to this receptor. The F254S mMC4R possesses 11-fold decreased binding affinities for NDP-MSH and SHU9119, 7-fold decreased affinity for MTII, 4-fold decreased affinity for AGRP(83−132), compared with the wild-type receptor.

Agonist Potency and Efficacy. Table 3 summarizes the agonist stimulatory response at the wild-type and mutant mMC4 receptors. Figure 4 illustrates the agonist ligand potencies at the wild-type mMC4R. Interestingly, the DNal-(1′)-MTII analogue is only a partial agonist whereas Nal(2′)-MTII is a full agonist and the DNal(2′) derivative, SHU9119 is a competitive antagonist (*29*). The ligands used in this study (Table 1) were selected to try and differentiate

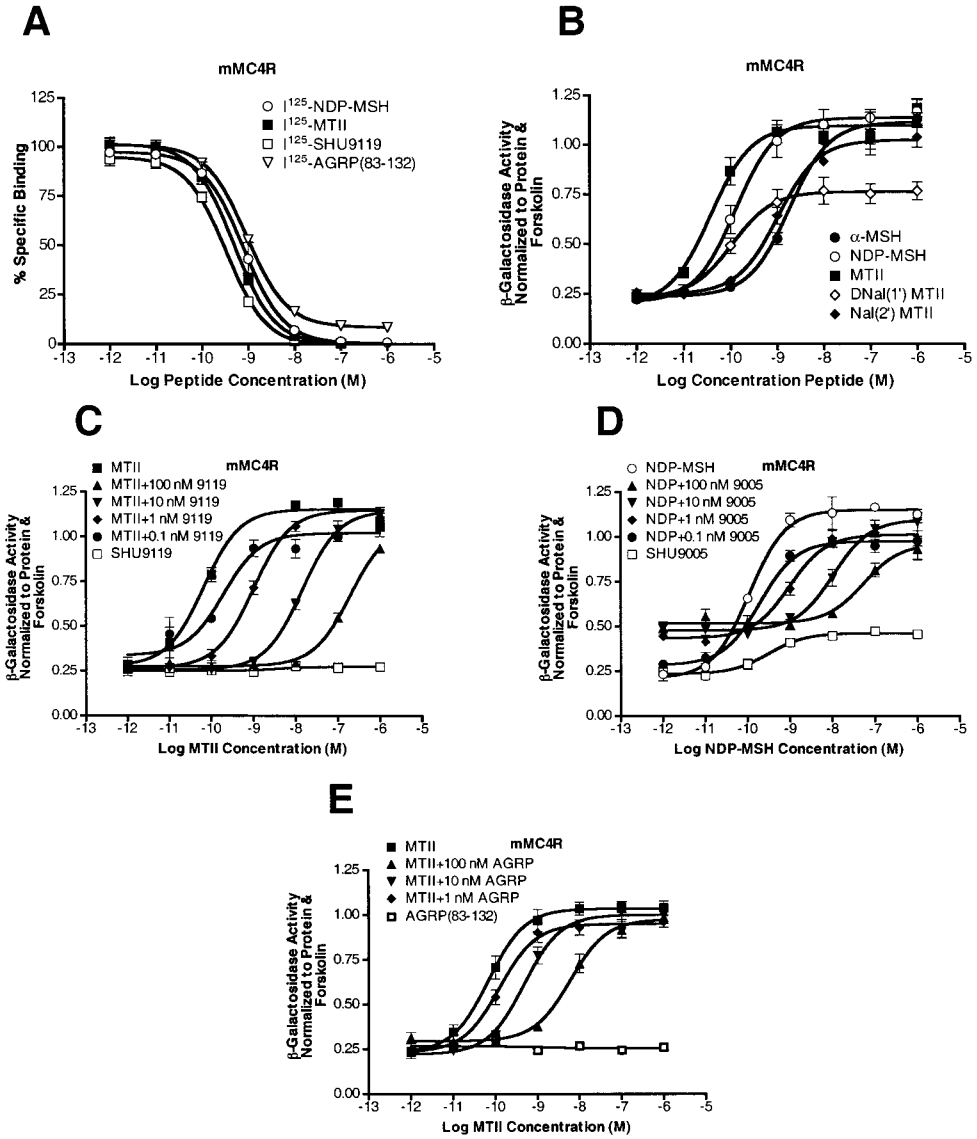


FIGURE 4: (A) Binding affinities of the linear 13 amino acid [^{125}I]NDP-MSH agonist, the cyclic seven amino acid [^{125}I]MTII agonist, the cyclic seven amino acid [^{125}I]SHU9119 antagonist, and the 50 amino acid multicyclic [^{125}I]hAGRP(83−132) antagonist at the wild-type mMC4R. (B) Ligand agonist potencies at the wild-type mMC4R where all the compounds are full agonists with the exception of DNal(1′) MTII which is only a partial agonist. (C) Competitive antagonist curve of SHU9119 in the presence and absence of the MTII agonist at the wild-type mMC4R, illustrating that there is no agonist activity for SHU9119, denoted in open squares. (D) Competitive antagonist curve of SHU9005 in the presence and absence of the NDP-MSH agonist at the wild-type mMC4R, illustrating that there is partial agonist activity for SHU9005, denoted in open squares. (E) Competitive antagonist curve of hAGRP(83−132) in the presence and absence of the MTII agonist at the wild-type mMC4R, illustrating that there is no agonist activity for hAGRP(83−132), denoted in open squares.

ligand−receptor interactions between the linear agonist peptides (α-MSH, NDP-MSH) and the linear antagonist SHU9005, and differential putative aromatic residue interactions at the 7 position (Figure 3) in the cyclic agonists [MTII, DNal(1′)^7-MTII and Nal(2′)^7-MTII] versus the cyclic antagonist [SHU9119 which contains DNal(2′)^7]. The most dramatic decreases in agonist ligand potencies, compared to the wild-type receptor, occurred at the E92, D114, D118, and F176 mutant mMC4 receptors. Unexpectedly, melanocortin-based peptides possessed only partial agonist activity at the P252G and F253S mutant mMC4 receptors, as compared to the wild-type receptor, while they possessed relatively normal NDP-MSH, MTII, and SHU9119 binding affinities (Figure 5).

Antagonist Pharmacology. Table 4 summarizes the pharmacology for the antagonists SHU9119, SHU9005, and AGRP(83−132) at the mMC4 receptor mutations. Figure 4 illustrates the competitive antagonist curves of SHU9119 and AGRP(83−132) at the wild-type mMC4R, both of which do not possess any partial agonist activity, and are nearly identical to previous reports (*2*, *12*, *29*, *42*, *45*). The SHU9005 antagonist however, possesses both antagonistic and partial agonist activity at the mMC4R, and is reminiscent of the pharmacological profile observed for SHU9119 at the MC3R (*29*, *42*). Differences in relative potencies (5−13-fold) between the cyclic SHU9119 and linear SHU9005 antagonists are observed at the C122M, S172F, F176K, Y179C, D181R, and Y260I mutant mMC4 receptors. No agonist or antagonist activity for AGRP(83−132) is observed at the E92K, D114R, D118K, and F176K mMC4Rs. At both the D114R and F176K mMC4 receptors, radiolabeled AGRP(83−132) was observed to bind with nM ligand affinity (Table 2, Figure 6). Interestingly, the M192F, F254S,

Table 3: Functional Activity of the Melanocortin Agonists (Table 1) at the Wild-Type and Mutant mMC4 Receptors

		potency EC_{50} (nM)									
mutant	TM	α-MSH	fold difference	NDP-MSH	fold difference	MTII	fold difference	*D*Nal(1′)-MTII	fold difference	Nal(2′)-MTII	fold difference
mMC4R		1.86 ± 0.28	1.0	0.13 ± 0.007	1.0	0.036 ± 0.029	1.0	0.12 ± 0.05	1.0	0.89 ± 0.55	1.0
M71K	2	0.78 ± 0.26	−2.4	0.09 ± 0.05	−1.4	0.022 ± 0.025	−1.6	0.04 ± 0.04	−3	0.25 ± 0.30	−4
E92K	2	>1000		9.25 ± 5.4	71	4.50 ± 4.7	125	11.9 ± 7.4	99	30.4 ± 27	34
E92R	2	>1000		>1000		>1000		>1000		>1000	
E92S	2	>1000		>1000		>1000		>1000		>1000	
D114R	3	>1000		47.8 ± 28.3	368	144 ± 10	4000	810 ± 306	6750	205 ± 107	230
D118K	3	>1000		46.2 ± 18.9	355	166 ± 64	4611	>1000		>1000	
C122M	3	1.76 ± 0.01	1	0.034 ± 0.018	−4	0.024 ± 0.007	1	0.064 ± 0.008	2	0.19 ± 0.08	−5
S172F	4	3.43 ± 0.13	2	0.071 ± 0.011	−2	0.046 ± 0.021	1	0.21 ± 0.035	2	1.18 ± 0.62	1
F176S	4	>1000		>1000		>1000		>1000		>1000	
F176K	4	413 ± 57	222	2.15 ± 0.78	17	2.75 ± 0.92	76	10.6 ± 3.9	88	107 ± 46	120
Y179C	4	37.0 ± 32.5	20	0.20 ± 0.028	1	0.11 ± 0.03	3	1.01 ± 0.13	8	3.05 ± 0.91	3
D181R	EL2	6.55 ± 1.34	4	0.60 ± 0.42	5	0.16 ± 0.007	4	0.40 ± 0.28	3	2.55 ± 0.21	3
M192F	5	0.72 ± 0.30	−3	0.29 ± 0.21	2	0.07 ± 0.014	2	0.08 ± 0.06	−2	0.58 ± 0.58	−2
M192L	5	0.53 ± 0.27	−4	0.049 ± 0.025	−3	0.044 ± 0.013	1	0.078 ± 0.015	−2	0.89 ± 0.52	1
M192W	5	1.10 ± 0.78	1	0.20 ± 0.11	1	0.067 ± 0.047	2	0.095 ± 0.092	1	0.19 ± 0.24	−5
F193S	5	8.65 ± 8.83	5	0.38 ± 0.33	3	0.16 ± 0.11	4	0.70 ± 0.03	6	4.48 ± 1.52	5
F194S	5	6.30 ± 3.68	3	0.16 ± 0.14	1	0.05 ± 0.014	1	0.16 ± 0.04	1	1.02 ± 0.32	1
P252G	6	Partial agonist		Partial agonist		Partial agonist		Partial agonist		Partial agonist	
F253S	6	Partial agonist		Partial agonist		Partial agonist		Partial agonist		Partial agonist	
F254S	6	17.7 ± 3.32	10	0.30 ± 0.08	2	0.47 ± 0.31	13	0.64 ± 0.04	5	6.98 ± 2.83	8
F259S	6	32.6 ± 18.0	18	0.42 ± 0.06	3	0.22 ± 0.06	6	0.22 ± 0.10	2	4.26 ± 4.21	5
F259L	6	3.05 ± 1.59	2	0.20 ± 0.01	2	0.18 ± 0.11	5	0.55 ± 0.16	5	2.21 ± 1.0	2
Y260I	6	2.60 ± 1.98	1	0.089 ± 0.044	1	0.095 ± 0.050	3	0.52 ± 0.25	4	2.21 ± 1.10	2

[a] The indicated errors represent the standard deviation of the mean determined from at least two independent experiments. Partial agonist denotes that some stimulatory response was observed but not enough to determine an EC_{50} value.

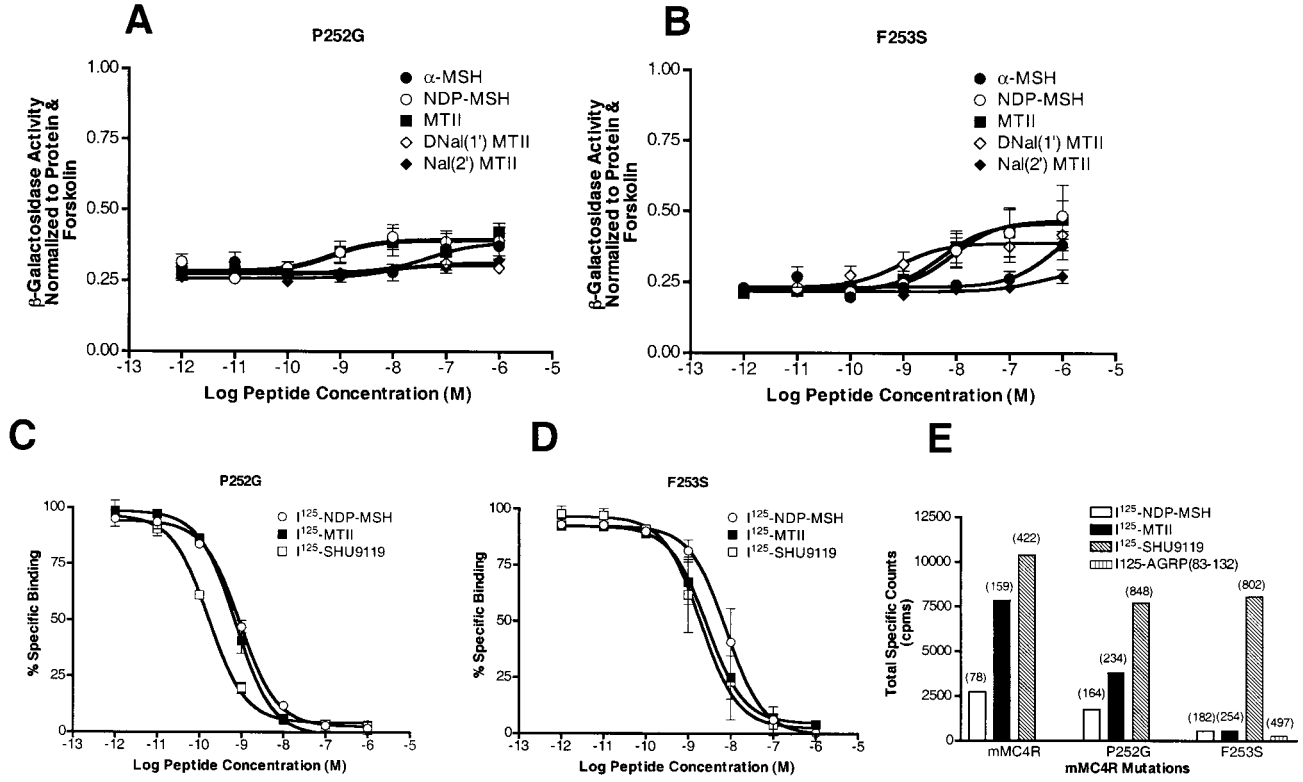


FIGURE 5: (A and B) Functional activity of the mMC4R agonists illustrating their slight agonist activity at the P252G and P253S mutant mMC4Rs. (C and D) Illustrate that both the P252G and F253S are able to bind to [^{125}I]NDP-MSH, MTII, and SHU9119 peptides with nearly wild-type affinities. (E) Summarizes the total specific binding cpms observed for [^{125}I]NDP-MSH, MTII, SHU9119, and hAGRP-(83−132) at the wild-type mMC4R, the P252G mMC4R and the F253S mMC4R. Nonspecific counts were determined using a 10^{-6} M concentration of the respective noniodinated peptide and are indicated in the parentheses. Additionally, at the F253S mMC4R, the SHU9119 antagonist was able to bind with greater than 7500 cpms whereas the AGRP(83−132) antagonist only had approximately 275 total specific counts.

and F259S mutant mMC4Rs resulted in agonist activity of the SHU9119 antagonist and increased agonist activity of the SHU9005 partial agonist/antagonist (Figure 7). Additionally, the M192F mMC4R possesses a slight increase (ca. 25%) above the basal activity of the wild-type and other mutant mMC4R's (Figure 8).

Table 4: Functional Activity of the Antagonists at the Wild-Type and Mutant mMC4 Receptors

		antagonist pA_2 value					
mutant	TM	SHU9119	fold difference	SHU9005	fold difference	AGRP (83−132)	fold difference
mMC4R		10.4 ± 0.5	1.0	9.7 ± 0.1	1.0	9.39 ± 0.1	1.0
M71K	2	10.0 ± 0.06	2.5	10.2 ± 0.6	1		
E92K	2	8.9 ± 0.4	31	8.0 ± 0.4	50	no activity	
E92R	2	ND[a]		ND[a]			
E92S	2	ND[a]		ND[a]			
D114R	3	ND[a]		ND[a]		no activity	
D118K	3	ND[a]		ND[a]		no activity	
C122M	3	9.5 ± 0.4	8	10.1 ± 0.2	1		
S172F	4	9.3 ± 0.1	13	9.8 ± 0.1	1		
F176S	4	ND[a]		ND[a]			
F176K	4	8.8 ± 0.4	40	8.9 ± 0.1	6	no activity	
Y179C	4	9.6 ± 0.1	6	9.9 ± 0.3	1	8.40 ± 0.6	10
D181R	EL2	8.4 ± 0.6	10	9.9 ± 0.3	1		
M192F	5	9.9 ± 0.4	3	10.2 ± 0.4	1	9.57 ± 0.02	1
				agonist			
				0.39 ± 0.44 nM	agonist		
M192L	5	10.2 ± 0.4	1.5	10.3 ± 0.9	1		
M192W	5	10.6 ± 0.7	1	10.3 ± 0.3	1		
F193S	5	9.8 ± 0.4	4	9.9 ± 0.1	1	8.70 ± 0.22	5
F194S	5	10.0 ± 0.1	2.5	9.7 ± 0.1	1.0		
P252G	6	ND[a]		ND[a]			
F253S	6	ND[a]		ND[a]			
F254S	6	10.2 ± 0.7	1.5				
		agonist EC_{50} =		agonist EC_{50} =	agonist	8.80 ± 0.06	4
		0.83 ± 0.17 nM	agonist	0.21 ± 0.11 nM			
F259S	6	agonist EC_{50} =	agonist	agonist EC_{50} =	agonist	9.09 ± 0.71	2
		0.56 ± 0.36 nM		0.22 ± 0.10 nM			
F259L	6	9.9 ± 0.8	3	9.2 ± 0.2	3		
Y260I	6	9.7 ± 0.6	5	9.8 ± 0.6	1		

[a] The indicated errors represent the standard deviation of the mean determined from at least two independent experiments. The fold difference of the value at the mutant verses wild-type receptors were determined by converting the pA_2 value to the Ki value where $K_i = -\log pA_2$. ND under an antagonist indicates that a pA_2 value was not technically able to be generated as these particular mutant receptors lacked the agonist potency required for the Schild pA_2 analysis (these antagonist experiments were performed, however). No activity indicates that the compound was examined at the mutant receptor, but neither agonist or antagonist activity resulted.

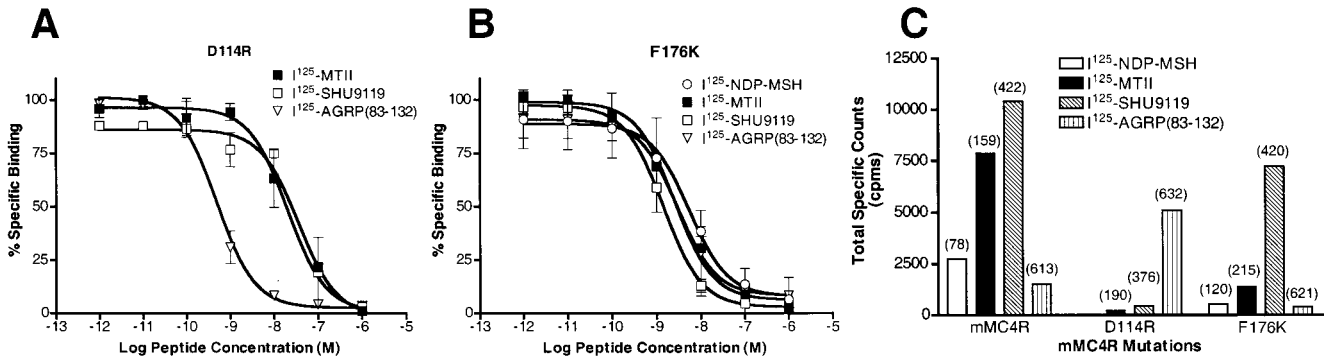


FIGURE 6: (A and B) Illustrate the binding affinities of the radiolabeled peptides at the D114R and F176K mutant mMC4Rs. (C) Summarizes the total specific binding cpms observed for [^{125}I]NDP-MSH, MTII, SHU9119, and hAGRP(83−132) at the wild-type mMC4R, the D114R mMC4R, and the F176K mMC4R. At the D114R mMC4R, AGRP(83−132) binds with a total specific count of 5000 cpms, and at the F176K mMC4R, SHU9119 binds with a total specific count of 7200 cpms. Nonspecific counts were determined using a 10^{-6} M concentration of the respective noniodinated peptide and are indicated in the parentheses.

AGRP(109−118) at the F176K, F254S, and F259S mMC4 Receptors. As noted above, AGRP(83−132) is a 50 amino acid multi-cyclic antagonist peptide and SHU9119 is a monocyclic seven amino acid melanocortin-based peptide antagonist. Significant differences between these two peptides and how they interact respectively with the receptor can be conceived. We therefore decided to test the AGRP(109−118) decapeptide antagonist at the F176K, F254S, and F259S mMC4 receptors to determine if these results would correlate with the larger AGRP peptide antagonist or the smaller SHU9119 melanocortin-based peptide antagonist. At the wild-type mMC4R, AGRP(109−118) possessed a binding $IC_{50} = 0.28 \pm 0.062$ μM and antagonist $pA_2 = 6.8 \pm 0.4$ (*23*), while at the F176K mMC4R, AGRP(109−118) bound to the receptor ($IC_{50} = 3.66 \pm 1.58$ μM) with 13-fold decreased affinity compared with wild-type mMC4R, but was unable to generate an antagonist response (Figure 9), similar to that observed for AGRP(83−132), data not shown.

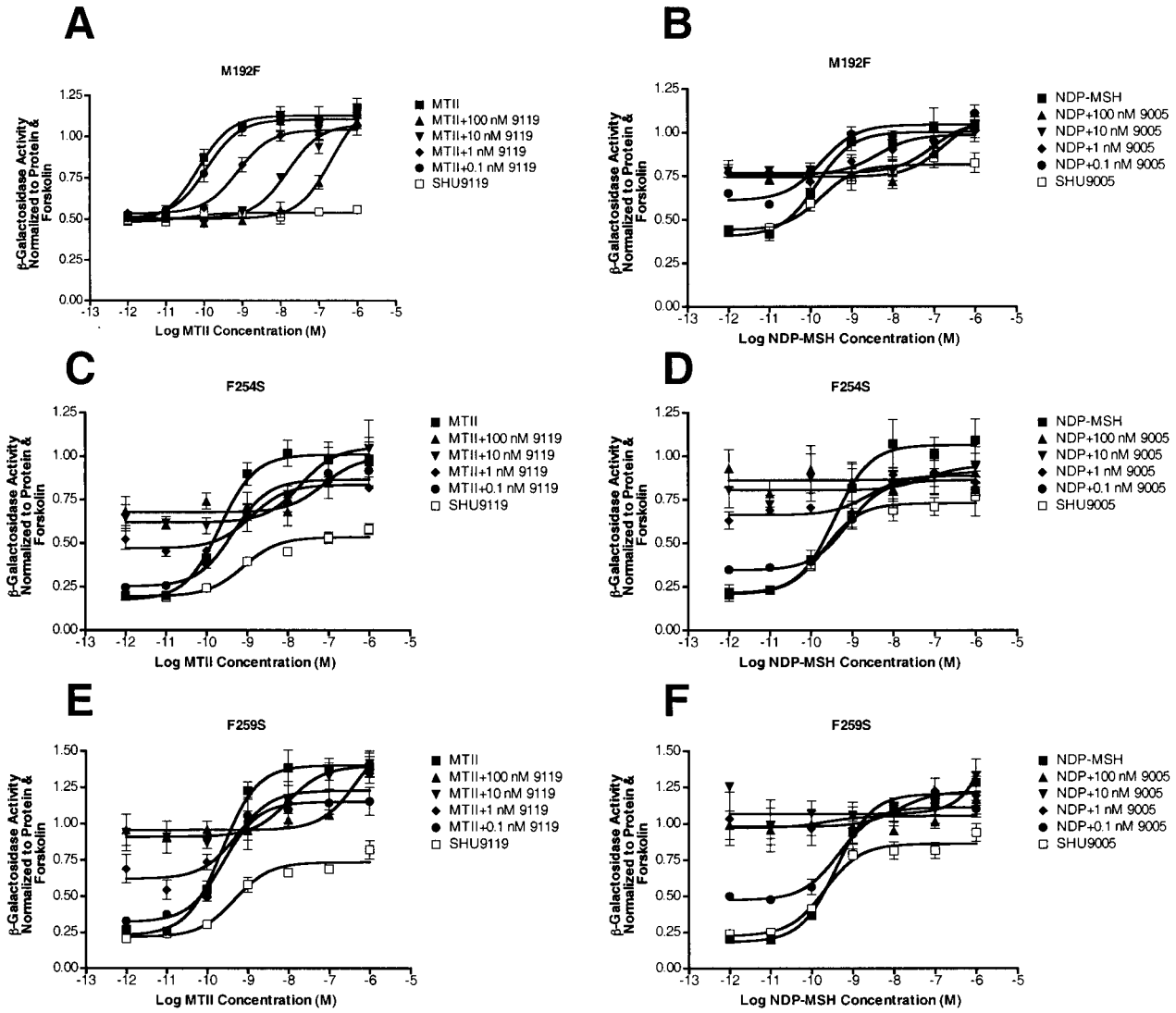


FIGURE 7: (A) Competitive antagonist curves of SHU9119 at the M192F mMC4R, illustrating that there is no agonist activity for the mMC4R SHU9119 antagonist, denoted in open squares. (B) Competitive antagonist curves of SHU9005 at the M192F mMC4R, illustrating that there is increased agonist activity for the mMC4R SHU9005 peptide compared with the wild-type mMC4R (Figure 4), denoted in open squares. (C and E) Competitive antagonist curves of SHU9119 in the presence and absence of the MTII agonist at the F254S and F259S mMC4Rs, illustrating that there is agonist activity for the mMC4R SHU9119 antagonist, denoted in open squares. (D and F) Competitive antagonist curves of SHU9005 in the presence and absence of the NDP-MSH agonist at the F254S and F259S mMC4Rs, illustrating that there is increased agonist activity for the SHU9005 peptide compared with the wild-type mMC4R (Figure 4), denoted in open squares.

Interestingly, AGRP(109−118) did not possess any agonist activity at the F254S and F259S mutant receptors (Figure 10).

DISCUSSION

The melanocortin system has been identified as a major participating factor in feeding behavior, obesity, and energy homeostasis (*1*, *2*, *14*, *15*, *46*). This study was undertaken to identify putative ligand−receptor interactions that may be used to aid in the rational design of MC4R specific therapeutic agents for the treatment of obesity and related diseases. Additionally as little is known about the exact mechanism by which the only two known naturally occurring antagonists of GPCRs (agouti and AGRP) function, this study was undertaken to help decipher the mechanism by which AGRP antagonizes the MC4R. During the preparation of this manuscript, a report of 24 mutations of the human MC4R was published (*28*). MC4R residues of both the human and mouse were performed at the E92 (TM 2), D114 (TM 3), D118 (TM3), C122 (TM3), M192 (TM5), F253 (TM 6), F259 (TM6), and Y260 (TM6) (mMC4R numbering for comparisons herein).

General Ligand−Receptor Interactions. Figure 2 summarizes the putative ligand−receptor interactions predicted to be involved in the melanocortin ligand and mouse MC4 receptor interactions. This model and hypothesis were generated based upon three-dimensional homology molecular modeling and previous studies of similar work at the skin melanocortin-1 receptor (MC1R) (*32*, *38*−*40*). The rationale for marking these particular receptor side chain amino acid substitutions has been described above, and these mutant receptors have resulted in some interesting pharmacological differences between the ligands examined compared to their respective pharmacology at the wild-type mMC4R. Previous studies have suggested that the melanocortin-based ligand Arg^8 putatively interacts with one or several of the conserved melanocortin receptor residues E92 (TM2), D114 (TM3), and D118 (TM3) (mouse MC4R numbering) (*28*, *32*, *38*−*40*).

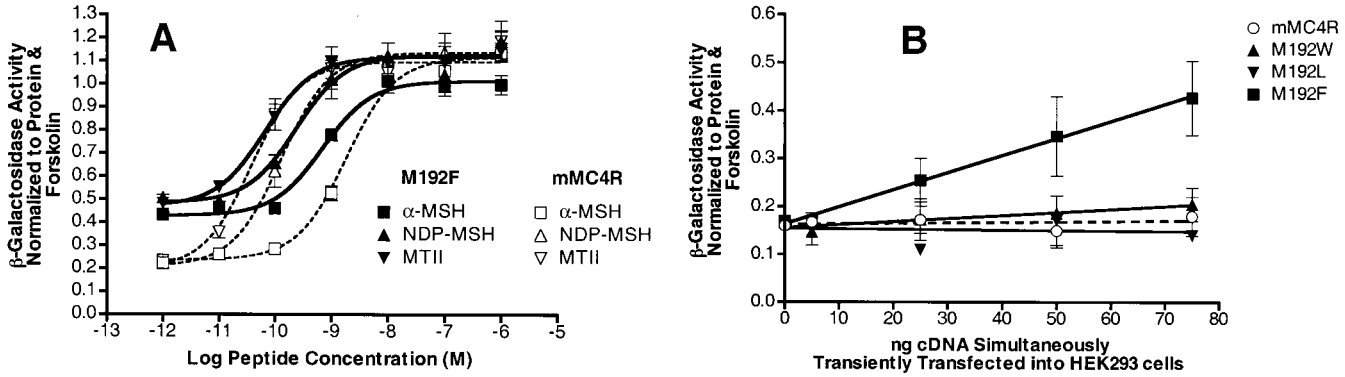


FIGURE 8: (A) Comparison of the wild-type mMC4R (open symbols and dotted lines) and the M192F mMC4R (solid symbols and lines) pharmacological profiles of the agonists α-MSH, NDP-MSH, and MTII. These agonists have equipotent potencies at both the wild-type and M192F mMC4Rs (within inherent experimental errors). The M192F mMC4R possesses approximately 25% increased basal β-galactosidase activity (based upon cAMP), compared with the wild-type mMC4R. (B) Summarizes the experiment where increasing amounts of mutant or wild-type cDNA are transiently transfected into the HEK293 cells (basal activity is normalized to the forskolin induced activity and protein content as controls). The wild-type mMC4R is denoted in an open circle with a dotted line. The wild-type, M192W and M192L mMC4Rs do not result in increased basal activity with increased DNA concentration and lack constitutive activity. However, the M192F mMC4R (solid square) results in increased basal activity in a dose−response relationship with increasing DNA concentration and therefore is indicative of possessing constitutive activity in the absence of agonist ligand.

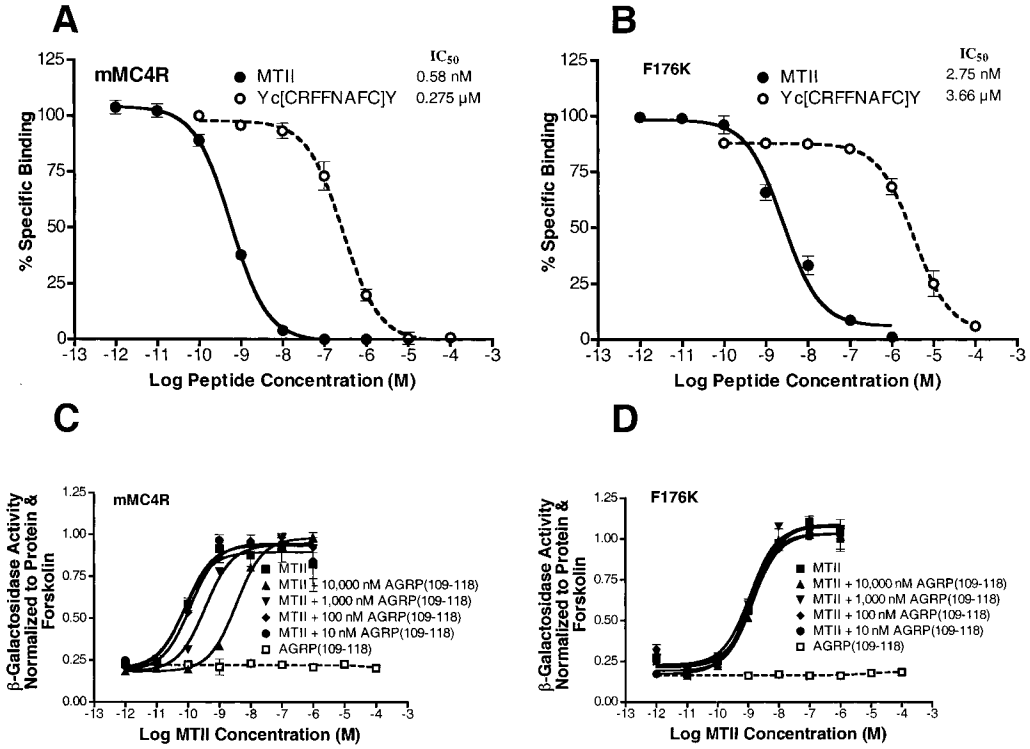


FIGURE 9: Pharmacology of hAGRP(109−118) at the wild-type and F176K mMC4Rs. (A and B) Illustrate the binding affinity of hAGRP-(109−118) at the wild-type and F176K mMC4Rs, respectively. (C and D) Illustrate the competitive antagonist curves of hAGRP(109−118) using MTII as the agonist. At the wild-type mMC4R, AGRP(109−118) is a competitive antagonist while at the F176K mMC4R, no antagonist activity is observed at up to 10 μM concentrations.

The studies presented herein are in agreement with the previous hypothesis, and further emphasize the importance of the Glu92 (TM2), Asp114 (TM3), and Asp118 (TM3) receptor residues for both agonist binding and functional activity. For the E92R and E92S mMC4Rs, the agonist radiolabeled peptides were unable to bind these receptors; however, the antagonist labeled [^{125}I]SHU9119 peptide was able to bind these mutant receptors demonstrating that they are expressed on the cell surface (Table 2). Radiolabeled peptide binding of NDP-MSH and MTII was not detected at the D114R and D118K mutant mMC4 receptors; however, functional stimulatory activity was observed for these ligands.

In the functional assay of the D118K mMC4R (Table 3) α-MSH did not result in any stimulatory activity (up to 10 μM), whereas NDP-MSH and MTII were able to stimulate the receptor but resulted in 355- and 4611-fold decreased potencies, compared with the wild-type receptor. It is worth noting that some total specific binding was observed for radiolabeled MTII (158 cpms), but not enough to unequivocally determine an IC_{50} value. One explanation why stimulatory EC_{50} values were determined for NDP-MSH and MTII, but not IC_{50} values could be that receptor competitive displacement studies are directly dependent upon receptor number, whereas functional studies are less dependent upon

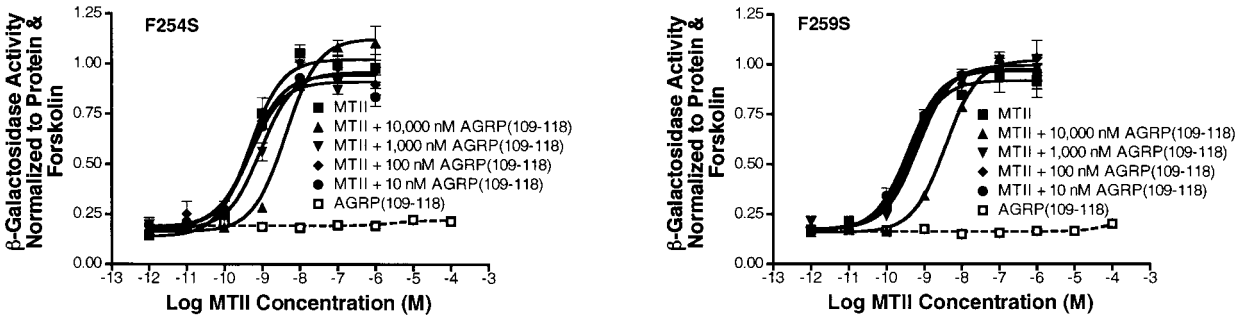


FIGURE 10: Pharmacology of the mMC4R AGRP(109−118) antagonist at the F254S and F259S mMC4Rs, where AGRP(109−118) does not possess any agonist activity (open square, dotted line), compared with the SHU9119 antagonist at these mutant receptors, Figure 7.

receptor number and are generally more sensitive assays (*47*). This is illustrated by the comparison of IC_{50} versus EC_{50} values for NDP-MSH and MTII (Tables 2 and 3) where differences of 6- and 14-fold, respectively are observed. In both the human (*28*) and mouse MC4Rs, the Asp 114 and Asp 118 (TM3) appears to be important for melanocortin-based ligand binding and agonist activity.

The differences between relative changes in potency of the linear agonists α-MSH and NDP-MSH (which primarily differ by the stereochemistry of the Phe in the 7 position, Table 1), are at the F176 (13-fold), Y179 (20-fold), F254 (5-fold), and F259 (6-fold) mutant mMC4 receptors implicate these receptor amino acids as participating in interactions with the Phe^7 of the α-MSH. The Phe254 mMC4R amino acid (conserved in all melanocortin receptor subtypes cloned to date) was mutated based on information proposed by three-dimensional homology molecular modeling of the hMC1R (*38*, *48*) and MC4R (C. Haskell-Luevano, unpublished material). It was proposed that the hMC1R receptor residue homologous to the Phe254 mMC4R residue may differentially interact with the linear NDP-MSH Phe^7 ligand residue and that this ligand−receptor interaction was absent in the MTII ligand binding. This original hypothesis has been demonstrated experimentally to be incorrect, and in actuality, it appears that this Phe receptor residue is involved in MTII ligand−receptor interactions and not NDP-MSH ligand−receptor interactions as previously proposed for the MC1R (*40*). At the mMC4R, the Phe254 to Ser mutation resulted in 7−11-fold decreased binding of radiolabeled NDP-MSH, MTII, and SHU9119, and only a 4-fold decrease in radiolabeled AGRP(83−132) binding (Table 2). Functionally, there was a 5-fold difference in agonist activity between α-MSH and NDP-MSH, suggesting that there may be putative interactions of this Phe254 receptor residue with the Phe^7 of α-MSH. These putative contacts between Phe^7 of α-MSH and the mMC4R Phe and Tyr residues presumably include primarily hydrophobic and Van der Waal interactions. The F254S and F259S mutant mMC4 receptors were able to bind to the radiolabeled peptides and resulted in nM stimulatory activities of the agonists. The most significant observation at these two mutant receptors is the agonist activity of the competitive antagonist SHU9119 and the increased agonist activity of the partial agonist SHU9005 (Table 4, Figure 7). These data would suggest that these Phe receptor amino acids are potentially involved in receptor activation. However, the F259L mMC4R did not possesses any agonist activity changes for the SHU9119 and SHU9005 antagonists. This suggests that the Phe259 to Ser change may be altering the receptor interaction(s) with the ligand (per experimental design) and forcing perhaps the aromatic ligand residues to find alternative interactions with other mMC4R residues. However, the observations that there are less than 20-fold differences in ligand binding and agonist EC_{50} values would support the hypothesis were these Phe mMC4R residues in TM 6 are important for the differentiation of agonist versus antagonistic activity and perhaps mMC4 receptor stimulation. At the F253S mMC4R, [^{125}I]MTII resulted in 16-fold decreased binding affinity whereas [^{125}I]-NDP-MSH and [^{125}I]SHU9119 possessed equipotency compared to the wild-type receptor (Table 2), and [^{125}I]AGRP-(83−132) was not able to bind to this receptor. The 16-fold difference in binding between the cyclic MTII and SHU9119 peptides suggest putative interactions of the MTII DPhe^7 amino acid (as the only difference between these two ligands is at the 7 position) with the mMC4 F253 receptor residue. Functionally, F253S mMC4R was only able to generate a partial agonist response of the agonist peptides examined in this study (Table 3, Figure 5). These data suggest that the Phe 253, 254, and 259 residue side chains in the mMC4R TM 6 may be involved in critical ligand−receptor interactions necessary for receptor stimulation.

The largest differences between the linear NDP-MSH agonist and the cyclic MTII agonist (both contain a DPhe^7) are at the D114 (10-fold), D118 (13-fold), F176 (4-fold), and F254 (6.5-fold) mutant mMC4Rs. The largest differences between the linear SHU9005 antagonist and cyclic SHU9119 antagonist are at the C122 (8-fold), S172 (13-fold), F176 (7-fold), Y179 (6-fold), D181 (10-fold), and Y260 (5-fold) mutant mMC4R's. Since notable differences between both the linear and cyclic agonists and antagonists occur at the F176, we can speculate that (1) variation in tertiary structure of the linear versus cyclic DPhe^7 containing agonist peptides may be discriminated by the D114 (TM3), D118 (TM3), and F176 (TM4) mMC4 receptor residues, and (2) variation between the melanocortin linear and cyclic antagonists may be discriminated by the C122 (TM3), S172 (TM4), Y179 (TM4), D181 (EL2), and Y260 (TM6) mMC4 receptor residues.

Differentiation of Antagonist versus Agonist Activity at the mMC4R Mutations. Interestingly, the difference between the melanocortin-based antagonists, SHU9119 and SHU9005 compared to the agonists MTII and NDP-MSH, respectively, is the addition of "bulky" substituents at the 7 position (Table 1, Figure 3). The hypothesis that a "bulky" modification at the 7 position of the melanocortin agonist MTII results in the SHU9119 antagonist activity has been proposed as the mechanistic difference between these compound's interactions with the MC4R (*42*). Interestingly, the F254S and

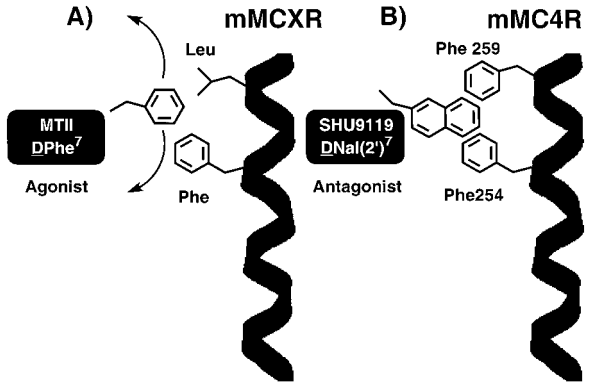


FIGURE 11: Illustration of the melanocortin receptor TM 6 region where (A) the murine melanocortin receptors, other than the MC4R possess a Leu residue in the homologous 259 position (mMC4R numbering) and where the agonist MTII DPhe^7 side chain can interact with these TM 6 residues to freely rotate (shown with the arrows. (B) Illustrates how the mMC4R TM 6 F259 and F254 aromatic receptor residues may be forming an "aromatic $\pi-\pi$ network" by interacting with the bulky aromatic functional moiety [DNal(2′)^7] of SHU9119 to hinder the rotation of TM 6 necessary for the mMC4R agonist activity.

F259S mMC4 receptors both resulted in increased agonist activity (Figure 7) of the SHU9119 and SHU9005 mMC4R antagonists (Figure 4). Analysis of the melanocortin receptor subtype primary amino acid sequence identified aromatic residues of the mMC4R in TM 6 that are not aromatic amino acids in the other mouse melanocortin receptors. Perhaps these mMC4R F259 and Y260 residues may be sterically and aromatically interacting with the bulky substituent at the antagonist ligand 7 position and not allowing the receptor structural modification(s) necessary to generate full agonist responses (*49*). To test this hypothesis, the F259 was mutated to a Leu and a second Y260I mutant receptor was generated. These later mutations were proposed to be similar to the other melanocortin receptor subtypes where SHU9119 and SHU9005 were either agonists or partial agonists. At the F259L mutation, SHU9119 demonstrated only a very slight agonist activity, and no discernible differences in agonist characteristics of SHU9005, as compared with wild-type mMC4R. At the Y260I mutation, SHU9119 lacked agonist activity and what appears to be a slight decrease in partial agonism of SHU9005 (although this may be within experimental error and not significant). Thus, these later mutations do not support our original hypothesis. However, the F254S mMC4R also possesses increased sensitivity to the agonist activity of both SHU9005 and SHU9119 (Figure 7), suggesting a revised hypothesis in which the mMC4R F254 and F259 residues may be sterically and aromatically interacting with the bulky substituents at the antagonist ligand 7 position and hindering the receptor to undergo the conformational and structural modification(s) necessary to generate full agonist responses. This concept is illustrated in Figure 11.

One alternative to explaining the F254S and F259S data may be related to our original choice of side chains used to substitute the Phe residue. One could speculate that by substituting the hydrophobic aromatic receptor residue with a hydrophilic residue, the ligand−receptor interactions may be modified. To this extent and in accordance with our experimental rationale, perhaps the bulky antagonist substituents at the ligand 7 position are repulsed by the Ser hydroxyl moiety and/or the presence of water(s), and then interact with the mMC4R in a different binding mode. This later speculation supports the lack of significant SHU9119 and SHU9005 agonism observed at the F259L and Y260I mutant MC4 receptors. While both SHU9119 and SHU9005 antagonize the MC3R and MC4R and the mMC4R Phe254 and Phe259 residues appear to be important for these ligands antagonistic properties (directly or indirectly), the fact remains that a homologous Phe259 residue is absent in the MC3R. This observation, supported by numerous agonist structure−function studies which clearly demonstrate different MC3R/MC4R receptor pharmacology (*40*, *42*, *50*−*52*), strongly implicates the possibility of different mechanisms of activation between the MC3R and MC4R. Additionally, SHU9119 and SHU9005 resulted in increased agonist activity at the TM 6 receptor F254S mutation. This mutation is putatively located one helical turn down the TM region from the F259 residue and is conserved in all the murine melanocortin receptor subtypes identified to date (excluding the MC2R).

Differences between Aromatic Residues at the 7 Position of MTII. The primary hypothesis regarding the mechanism of antagonism of both SHU9119 and SHU9005 at the MC4 receptor is attributed to the addition of a "bulky" moiety at the 7 position of the MTII and NDP-MSH agonists, respectively (*42*). To further test this hypothesis and identify putative mMC4R amino acids which may be interacting with these ligand residues at position 7, MTII-based agonist peptides containing DNal(1′) and Nal(2′) at the 7 position (*29*) were characterized at the mutant mMC4 receptors reported in this study (Table 3). MTII (DPhe^7) is a full agonist, SHU9119 [DNal(2′)^7] is an antagonist, Nal(2′)^7-MTII is a full agonist, and DNal(1′)^7-MTII is a partial agonist at the mMC4R, Figure 4. These data provide experimental evidence to modify the former hypothesis and to suggest that not only the "bulky" moiety at the 7 position is important for MTII based ligand antagonism, but the stereochemistry of the α-carbon and the position of the naphthyl ring (1′ versus 2′, Figure 3) is also important (*29*). The most significant modifications in antagonist activity of SHU9119 were at the F254S and F259S mMC4Rs as discussed above. However, at both these mutant receptors, similar differences in relative potency were observed for the agonists MTII, DNal(1′)-MTII, and Nal(2′)-MTII, suggesting that these receptor modifications may be important only for the DNal(2′)^7 antagonist ligand. Surprisingly, the mutant mMC4 receptors that discriminated the most between these different MTII substituted analogues were the D114R and D118K mMC4R receptors. Comparison of MTII, DNal(1′)-MTII, and Nal(2′)-MTII at the D114R resulted in a 17-fold difference between MTII and DNal(1′)-MTII and a 30-fold difference between DNal(1′)-MTII and Nal(2′)-MTII (Table 3). However, at the D118K mMC4R, both the DNal(1′)- and Nal(2′)-MTII peptides lost any agonist activity, and radiolabeled SHU9119 was the only peptide to binding this receptor (Table 2). At both the D114R and D118K mMC4 receptors, SHU9119 antagonist activity was not determinable since this relies upon a shift in the EC_{50} value of an agonist and both NDP-MSH and MTII possessed substantial decreased functional activities (Table 3). Both the Asp114 and Asp118 (TM3) receptor residues are hypothesized to interact with the ligand Arg^8 amino acid (Figure 2) (*28*, *32*, *38*−*40*). These D114 and D118 observations suggest the need of a further

hypothesis revision for the mechanism of SHU9119 antagonism at the MC4R, in which not only does the "bulky" addition at the agonist 7 position generate an antagonist, but the topographical orientation (*53*) of the key ligand Arg^8 residue may also be altered by this added "bulky" moiety. Furthermore, the observation of a 74-fold decrease of MTII binding versus only a 3-fold decrease in SHU9119 binding at the E92K mMC4R (Table 2) provides additional experimental evidence to support the hypothesis that substitution at the MTII D-Phe^7 position by "bulky" aromatic groups may be affecting the ligand Arg^8 interactions with the mMC4 receptor.

These data may support an alternative hypothesis for the mechanism of SHU9119 antagonism, where both the stereochemistry and "bulky" naphthyl ring position (2′ versus 1′) are important for positioning of the ligand Arg^8 residue with the corresponding mMC4R amino acids which are speculated to be E92, D114, and/or D118 (Figure 2). This hypothesis can be tested by using two-dimensional NMR techniques to determine if these aromatic ring structures are modifying the Arg^8 side chain in three-dimensional space. Alternatively, in support of the original hypothesis regarding the "bulky" moieties being responsible for antagonism of the melanocortin-based peptides, specific mMC4R amino acids putatively involved in interactions with these "bulky" moieties remain to be identified.

Similarities and Differences of AGRP and the Melanocortin-Based Peptides MC4R Interactions. hAGRP is a 132 amino acid (the mouse homologue is only 131 amino acids) peptide which putatively contains five disulfide bridges (*54*) is the endogenous antagonist of the brain MC4 receptor (*2*). The hAGRP C-terminal fragment consisting of residues 83−132 has been demonstrated to possess the same pharmacological properties as the full-length peptide (*2*, *12*), is commercially available in the synthetic form (Phoenix Pharmaceuticals Inc, and Peptides International), and has been utilized in these studies. It has been proposed that the conserved Arg-Phe-Phe residues found in both AGRP and the agouti peptides may mimic the melanocortin-based agonist Phe-Arg-Trp residue interactions with the MC4R (*22*, *23*). At the mMC4R mutants examined in this study, it appears that the melanocortin-based agonist peptides and AGRP(83−132) may both have interactions with the mMC4 receptors Glu92 (TM2), Asp114 (TM3), Asp118 (TM3), and Phe176 (TM4) amino acids (Table 2). At the E92K mMC4R, significant decreases in agonist binding affinities were observed for NDP-MSH and MTII, but the antagonist SHU9119 was equipotent (within experimental error), compared to the wild-type mMC4R. However, AGRP(83−132) lost the ability to bind to this E92K mutant MC4R (Table 2). At the human E92A mutant receptor, AGRP(87−132) was able to bind with 3.9 nM affinity and α-MSH was able to stimulate this receptor at 4.4 nM potency (*28*). The radiolabeled agonists NDP-MSH and MTII as well as AGRP-(83−132) lost ability to bind the D118K mutant mMC4 receptor, whereas the melanocortin-based peptide antagonist SHU9119 was able to bind this mutant receptor, albeit with 185-fold decreased potency compared with the wild-type. At the D114R, NDP-MSH was unable to bind this mutant receptor; however, 39- and 99-fold decreased binding affinities were observed for [^{125}I]MTII and [^{125}I]SHU9119, respectively, compared with the wild-type mMC4R. Of even more interest is the fact that [^{125}I]hAGRP(83−132) was able to bind to D114R with nearly wild-type affinity (Figure 6), but did not possess any antagonist or agonist activity (data not shown). On the basis of the previously identified homologous mMC1R mutations which resulted in constitutive activation of the mMC1R (*32*), and the proposal that the E92, D114, and D118 (mMC4R numbering) receptor residues may be interacting with the melanocortin ligand Arg residue from the conserved His-Phe-Arg-Trp sequence (*28*, *32*, *39*, *40*), we can speculate that the mMC4R D114 and D118 residues may be interacting with both the agonist Arg residue as well as the hAGRP Arg residue from the Arg-Phe-Phe (111−113) consensus sequence. This is supported by the fact that the Arg 111 antagonist residue (hAGRP numbering) when mutated in both AGRP (*22*) and agouti (*21*) resulted in greater than 130-fold decreased binding at the MC4R. Additionally, the fact that the hAGRP decapeptide Yc[CRFFNAFC]Y was able to stimulate the mMC1R and bind to mMC1R in addition to the brain MC3R and MC4R (*23*) supports the hypothesis that the hAGRP antagonist Arg111 residue may be interacting with the melanocortin receptors in a similar manner as the melanocortin agonist Arg^8 (α-MSH numbering) amino acid. This speculation remains to be unambiguously experimentally verified however. The putative extracellular loop 2 of the MC4 receptor has been identified as being important for AGRP(83−132) binding but not NDP-MSH binding (*25*). Interestingly, our studies characterized F176, juxtaposed to the putative extracellular loop 2, as important for AGRP(83−132) antagonism and melanocortin-based peptide potency. The F176K mMC4R was able to bind AGRP(83−132) with a 3.6 nM binding affinity (Table 2), but no antagonistic activity for AGRP(83−132) was observed (data not shown). These data suggest that F176 is important for functional antagonism of AGRP(83−132) and not molecular recognition of the mMC4R. Mutations of the F176 residue to Lys and Ser also affected the melanocortin-based agonists and antagonists. Interestingly, the F176S mMC4R did not bind the agonist radiolabeled peptides and possessed a 155-fold decreased binding affinity for the SHU9119 antagonist (Table 2), compared to wild-type receptor. Unlike the D118K mMC4R, the F176S mMC4R did not show any stimulatory activity for any of the agonist peptides. The F176K mMC4R possessed 4−6-fold decreased binding affinities of the radiolabeled peptides (Table 2), between 17- and 222-fold decrease in agonist potency (Table 3), and 6- and 40-fold decreased potency for the SHU9005 and SHU9119 antagonists (Table 4), compared to the wild-type mMC4R. These data suggest that the F176 mMC4R amino acid interacts and functions in an important role for both the melanocortin-based peptides and AGRP(83−132). From this study, these appear to be the only mMC4R amino acids that putatively possess common interactions between the melanocortin agonists and the endogenous antagonist AGRP(83−132).

A specific mMC4R mutation was identified that appeared to affect hAGRP(83−132) molecular recognition-receptor interactions but not melanocortin-based peptide molecular recognition-receptor interactions. The F253S (TM6) mMC4R was able to bind to the melanocortin-based agonists and antagonists (Figure 5), but hAGRP(83−132) was not able to bind this mutant receptor (Table 2). At the F254S and F259S mMC4 mutant receptors, increased agonist activity

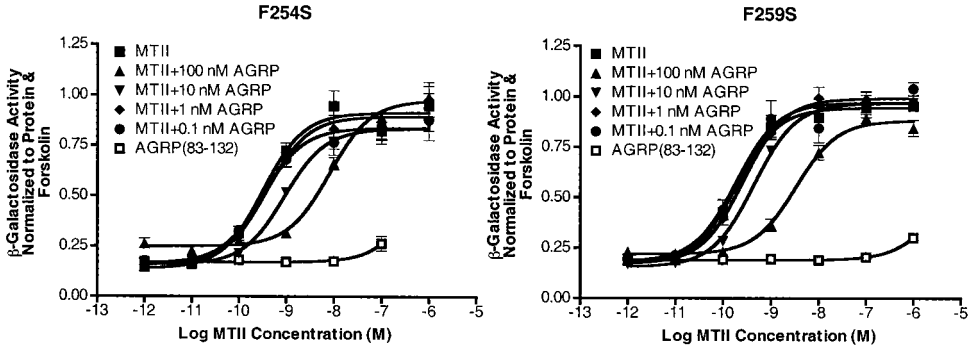


FIGURE 12: Pharmacology of the mMC4R AGRP(83−132) antagonist at the F254S and F259S mMC4Rs where AGRP(83−132) does not possess any significant agonist activity (open square, solid line), compared with the SHU9119 antagonist at these mutant receptors, Figure 7.

was observed for the SHU9119 and SHU9005 antagonists (Figure 7). The AGRP(83−132) antagonist was examined at these receptors to determine if the 50 amino acid antagonist would have similar increased activity at the melanocortin-based peptide antagonists. At both the F254S and F259S, AGRP(83−132) lacked any significant increase in agonist activity (Figure 12).

Since AGRP(83−132) is a 50 amino acid multi-cyclic peptide antagonist and the melanocortin-based peptide antagonists SHU9119 and SHU9005 are 7 and 13 amino acids, respectively, Table 1, the AGRP(109−118) decapeptide Yc[CRFFNAFC]Y was evaluated at the F176K, F254S, and F259S mMC4 receptors. At the F176K mMC4R AGRP-(109−118) was able to competitively displace [^{125}I]MTII and, therefore, bind to this mutant receptor (Figure 9) and similarly to AGRP(83−132) did not show antagonist or agonist functional activity (Figure 9). At the F254S and F259S mutant mMC4 receptors, unlike the melanocortin-based peptide antagonists, AGRP(109−118) did not possesses any agonist activity (Figure 10). These data provide evidence that the increased agonist activity of the antagonists SHU9119 and SHU9005 at the F254S and F259S mutant receptors is specific for the melanocortin-based peptide antagonists and not for AGRP(83−132) or AGRP(109−118).

Constitutively Active mMC4 Receptors. Receptor mutations of the mMC4R (M71K, E92K/R, D114R, and D118K) were performed similar to those identified at the mMC1R which resulted in the generation of constitutively active receptors (*32*, *40*). Unfortunately, constitutively active mMC4 receptors did not result from any of these mutations. However, generation of the M192F mMC4R, based upon the comparative analysis of the primary amino acid sequences of the melanocortin receptors, did result in a constitutively active mMC4R. In the putative TM5 of the mMC1R and the other murine MCRs, a shift of 1 residue for a FF motif was observed (Table 5). The Met residue present at the 192 position (mMC4-R numbering) was observed to be conserved in all the MCR subtypes except the MC1R. Substitution of this Met residue with a Phe was constructed in order to examine potential receptor interactions with the aromatic melanocortin-based ligand DPhe^7 or Trp^9 amino acids. Surprisingly, no significant changes in the ligand binding affinities [NDP-MSH, MTII, SHU9119, and AGRP(83−132), Table 2], agonist EC_{50} values [α-MSH, NDP-MSH, MTII, DNal(1′)-MTII, and Nal(2′)-MTII, Table 3] or antagonistic pA_2 values [SHU9119, SHU9005, and AGRP(83−132), Table 4] resulted. However, an increased basal

Table 5: Primary Sequence Comparison in TM5 of the Mouse Melanocortin Receptors

Receptor	TM5 (extra- to intracellular)
mMC1R	TAVLLCLVV**FFL**AMLALMAILYAHM
mMC3R	KMVIVCLIT**MFF**AMVLLMGTLYIHM
mMC4R	SAVIICLIS**MFF**TMLVLMASLYVHM
mMC5R	KYVIICLIS**MFF**TMLFFMAVLYIHM

[a] The putative TM5 amino acid composition has been derived from the corresponding residues of rhodopsin determined by the 2.8 Å resolution structure (*27*).

functional activity was observed (Figure 8A). Based upon putative ligand−receptor interactions, we hypothesize that the M192F mutant receptor may be modifying the receptor conformation to a structure similar to the structure necessary for G-protein coupling in the presence of ligand as a consequence of the interaction of the Phe192 with other aromatic residues in the putative receptor binding pocket (Figure 2). This hypothesis is supported by a previous similar finding for the human luteinizing hormone receptor (*55*). It is possible that this change is mimicking the affect of the Phe^7 aromatic residue in the ligand pharmacophore, His-Phe^7-Arg-Trp, known to be critical for ligand-induced receptor activation (*51*, *56*, *57*). This hypothesis may explain why the M192L and M192W mMC4Rs are not constitutively active (Figure 8B). It is also tempting to hypothesize that the displacement of this aromatic residue in the MC1R makes this receptor more sensitive to the affects of the positive charge in the ligand pharmacophore, thus explaining the sensitivity of the MC1R to constitutive activation by positive charge insertion in the TM2 or TM3 domains (*32*).

Residues Important for mMC4R Stimulation. Both the P252G and F253S mMC4Rs were able to bind to the radiolabeled peptides NDP-MSH, MTII, and SHU9119 with high affinities (Table 2); however, these mutant receptors only resulted in partial cAMP generation by the agonists examined (Table 3, Figure 5), as compared to the nonreceptor dependent induced cAMP generated by forskolin. The Pro 252 mMC4R residue is conserved in most GPCRs (*27*), and when the respective conserved Pro residues were mutated in the m3 muscarinic receptor (P505A) (*58*) and the lutropin/choriogonadtropin receptor (P562F) (*59*), no significant differences in ligand affinity or efficacy were observed. Explanations for this mMC4R P252G and F253S partial agonist observation could be that the receptor is not expressed in high levels or that structural changes in the receptor are a result of the amino acid substitution. However, the fact

that the ligands bind the P252G mutant receptor with nanomolar affinities identical to the wild-type receptor (Table 2) and that the total specific binding (Figure 5E) of the three radiolabeled peptides NDP-MSH (1761 cpms), MTII (3798 cpms), and SHU9119 (7724 cpm) discard these explanations. These observations allow us to speculate that mutation of this highly conserved G-protein coupled receptor Pro residue in TM 6 is important for mMC4R receptor activation and signal transduction, but not for ligand affinity or structural integrity of this receptor.

SUMMARY

In vitro mutagenesis of the mouse melanocortin-4 receptor (mMC4R) has been performed, based upon homology molecular modeling and previous melanocortin receptor mutagenesis studies that have identified putative ligand−receptor interactions. The pharmacology of several melanocortin-based ligands and, at selected mutant receptors, was further evaluated using the endogenous antagonist hAGRP-(83−132) molecule. These studies identify mMC4R amino acids important for melanocortin-based peptide ligand affinity, potency, efficacy, and differentiation of agonism versus antagonistic activity. Additionally, overlapping and distinct melanocortin-based peptide and AGRP(83−132) interactions are identified. A mutation has also been identified that results in a constitutively active mMC4R. Furthermore, significant pharmacological and perhaps physiologically important differences between the human and mouse forms of the MC4R have been identified.

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