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Obesity-associated mutant melanocortin-4 receptors with normal $G\alpha_s$ coupling frequently exhibit other discoverable pharmacological and biochemical defects

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Abstract

Mutations in the melanocortin-4 receptor (MC4R) are the most common cause of early syndromic obesity known. Most of these mutations result in a loss of protein expression, α-melanocyte-stimulating hormone binding, receptor trafficking or coupling to the stimulatory G-protein, $G\alpha_s$. However, approximately 26% of the obesity-associated mutations characterised to date exhibit none of these pharmacological defects. In the present study, we investigated seven of these apparently normal mutant MC4R in more detail and found that the majority (five of the seven) exhibit marked defects including defective binding of another endogenous melanocortin ligand, defective glycosylation, and defective recruitment of β-arrestin. These data provide support for two hypotheses: (i) that the majority of these rare, obesity-associated mutations are likely defective and causative of obesity and (ii) that β-arrestin recruitment is a valuable marker of normal MC4R function. Recent work has demonstrated a statistical correlation between the efficacy of β-arrestin recruitment to the MC4R and body mass index; however, the data reported here demonstrate both decreased and increased β-arrestin signalling in obesity-associated MC4R mutations.

KEYWORDS
α-MSH, POMC, receptors; membrane/nuclear

1 | INTRODUCTION

The central melanocortin system is known to play a pivotal role in the regulation of energy metabolism and food intake. Mutations in the central melanocortin-4 receptor (MC4R) are found in approximately one out of every 1500 individuals and are associated with up to 6% of cases of severe early-onset monogenic obesity.[1,2] MC4R is a class A (rhodopsin-like), seven-transmembrane G-protein-coupled-receptor (GPCR) that is shown to signal primarily through the $G\alpha_s$ > adenylyl cyclase > cAMP pathway. This signalling pathway in MC4R neurones is known to be required for control of thermogenesis, energy balance and glucose metabolism.[3] A classification system was previously proposed[4] sorting obesity-associated MC4R mutations into functional classes described as loss-of-function mutations caused by defective expression (class I mutants), disrupted trafficking of the receptor (class II mutants), decreased binding affinity (class III mutants) or defective coupling to $G\alpha_s$ (class IV mutants).[5-9] Yet, up to 26% of obesity-associated MC4R mutations[10] exhibit normal receptor activity with respect to these four criteria.[11,12] Although it is possible that these "class V" mutations are non-pathogenic, recent studies have suggested that MC4R signalling is quite complex. The MC4R signalling complex appears to involve a number of accessory proteins.[13-19] Furthermore, the MC4R appears to signal differently in different neuronal cell types, coupling to a K_{ATP} channel to hyperpolarise cells in the brainstem,[20] whereas it couples to an inwardly rectifying potassium channel, Kir7.1, independent of G-proteins, mediating α-melanocyte-stimulating hormone (MSH)-induced membrane depolarisation in the paraventricular nucleus.[15,21]

The melanocortin-2 receptor-associated protein 2 (MRAP2) has also been shown to suppress the constitutive activity at the receptor and regulate sensitivity to α-MSH. Deletion of MRAP2 in both zebrafish and mice has resulted in increased adiposity.[13,14] Four potentially pathogenic variants of MRAP2 were also identified in early-onset obese humans.[13] Upon ligand activation, GPCRs are known to recruit β-arrestin and were considered to only lead to receptor desensitisation, internalisation and either recycling of the receptor back to the membrane or downstream degradation.[22] However, recent studies have shown that recruitment of β-arrestin can couple the receptor to intracellular, G-protein independent signalling cascades.[23,24] One study has reported highly variable recruitment of β-arrestin by naturally occurring MC4R variants identified in the UK Biobank, and demonstrated a statistical correlation between the efficacy of β-arrestin recruitment to the MC4R and body mass index (BMI).[25]

Beyond simply elucidating the signalling mechanism(s) of a receptor critical to energy homeostasis, further investigation of this receptor is needed to advance attempts to develop small molecule therapeutics at this receptor, given the finding of a target-mediated pressor response.[26-28] A more detailed pharmacological model of receptor signalling, for example, may allow for the development of biased agonists that stimulate weight loss but not the pressor response. To better understand essential functional features of the MC4R that have not yet been elucidated, we aimed to identify potential biochemical and/or pharmacological defects in class V obesity-associated MC4R mutations reported to have normal cell surface expression, ligand binding and coupling to $G\alpha_s$.

2 | MATERIALS AND METHODS

2.1 | Selection of MC4R variants

The seven MC4R variants chosen for study, aspartate-37-valine (D37V),[5] proline-48-serine (P48S),[5] valine-50-methionine (V50M),[5,29] histidine-76-arginine (H76R),[2,11] isoleucine-170-valine (I170V),[5,29] asparagine-274-serine (N274S)[5] and arginine-305-serine (R305S)[2,11] were identified from the published literature using specific criteria: (i) implicated in the development of obesity in humans; (ii) resulting in a full-length protein upon translation; and (iii) no known molecular defects in terms of G-protein coupling, cell surface expression and ligand binding affinity.

2.2 | Cells, plasmids, mutagenesis and transfection

All experiments were performed in Chinese hamster ovary (CHO-K1) cells (ATCC CC61) maintained in a 37°C tissue culture incubator with 5% CO_2. Growth media consisted of Gibco (Gaithersburg, MD, USA) Dulbecco's modified Eagle's medium/F-12 with 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid, 10% foetal bovine serum and 1% antibiotic/antimycotic. Cells were no longer used for experimental purposes past passage number 15. The human MC4R plasmid was obtained from cDNA.org (catalogue no. MCR040TN00) and contained an N-terminal 3-human influenza hemagglutinin (3HA) tag cloned in pcDNA3.1$^+$ (Invitrogen, Carlsbad, CA, USA) with the MC4R sequence cloned at the *Kpn*I and *Xho*I restriction sites. This plasmid was used as the template to create mutant receptor vectors via site-directed mutagenesis. pSF-CMV-NEO-COOH-3xFLAG plasmid was obtained from Sigma-Aldrich (St Louis, MO, USA; catalogue no. OGS629-5UG). The sequence for wild-type (WT) human MRAP2 was cloned into *Sac*I and *Xho*I restriction sites using a polymerase chain reaction (PCR) and Quick Ligase (New England Biolabs, Beverly, MA, USA; catalogue no. M2200). All transfections were performed transiently with FuGene HD (Promega, Madison, WI, USA; catalogue no. E2312) using a reverse transfection protocol. A cAMP-sensitive luciferase plasmid (pGL4.29), used for cAMP accumulation assays, was purchased from Promega (catalogue no. E8471). Plasmids for the NanoBit assay (Promega N2014) were provided in the starter kit (Promega; catalogue no. N2014). Human β-arrestin 2 coding sequence was cloned into C-terminal SmallBit plasmid at the *Eco*RI and *Sac*I restriction sites. Cloning of mutant human MC4R receptors into N-terminal LargeBit was performed by amplifying mutant MC4R sequence via PCR using corresponding oligonucleotide primers, followed by *Dpn*1 digestion, restriction enzyme digestion, dephosphorylation of open vector, quick ligation and *Escherichia coli* transformation.

2.3 | Antibodies

Western blotting was performed using the antibodies: HA-tag (6E2) mouse (Cell Signaling Technology, Beverly, MA, USA; catalogue no. 2999); HA-tag (6E2) mouse mAb (horseradish peroxidase [HRP] conjugate) (Cell Signaling Technology; catalogue no. 2367; diluted 1:5000); anti-FLAG M2 mouse (Sigma-Aldrich; catalogue no. F3165); anti-FLAG M2-peroxidase mouse (Sigma-Aldrich; catalogue no. A8592; diluted 1:10 000); GAPDH (D16H11) XP rabbit (HRP conjugate) (Cell Signaling Technology; catalogue no. 8884S; diluted 1:5000); β-actin (8H10D10) mouse mAb (Cell Signaling Technology; catalogue no. 3700S; diluted 1:5000); and anti-mouse immunoglobulin G, HRP-linked (Cell Signaling Technology; catalogue no. 7076S; diluted 1:10 000).

2.4 | In vitro mutagenesis of MC4R

Mutant MC4R receptors were created via site-directed mutagenesis using QuikChange II XL (Agilent Technologies Inc., Santa Clara, CA, USA; catalogue no. 200521) in accordance with the manufacturer's instructions. Following PCR amplification, plasmids were transformed into provided ultracompetent XL-10 gold *E. coli* cells and allowed to propagate in a 37°C rocking incubator for 1 hour before streaking on a Luria broth agar plate containing ampicillin. These plates were incubated overnight at 37°C and single colonies were picked and inoculated in liquid Luria broth media containing ampicillin and allowed to propagate overnight. DNA was then isolated from bacterial cultures using a QiaPrep Miniprep kit (Qiagen, Valencia, CA, USA; catalogue no. 27 106). Mutagenesis was then confirmed via Sanger sequencing provided by the DNA sequencing core at the

University of Michigan (Ann Arbor, MI, USA) prior to use in experimental assays.

2.5 | MSH-induced $G\alpha_s$ coupling and accumulation of intracellular cAMP

CHO cells were plated and transfected in white, flat-bottomed 96-well plates (Corning Inc., Corning, NY, USA; catalogue no. CLS3917) with a cAMP-sensitive luciferase plasmid and corresponding empty vector, variant receptor, and/or MRAP2 plasmids using methods as described above. Transfection was performed with 0.2 μg of DNA per well (0.1 μg of luciferase and 0.1 μg of experimental plasmids). Variant receptor ± MRAP2 (or empty vector) was transfected at a ration of 1:6. Each condition was plated in triplicate. Forty-eight hours after transfection, growth media was removed and 50 μL of Opti-MEM was added to each well (Gibco; catalgoue no. 11 058 021). Then, 50 μL of a serial dilution (starting concentration of 10 pmol L^{-1}, ending concentration of 1 μmol L^{-1}) of α-MSH (Bachem AG, Bubendorf, Switzerland; catalogue no. H-1075), β-MSH (Phoenix Pharmaceuticals Inc., Belmont, CA, USA; catalogue no 043-12) or des-acetyl-α-MSH (Bachem AG; catalogue no. H-4390) was added to corresponding wells, as well as a forskolin (Sigma-Aldrich; catalogue no. F6886) control for each transfection condition. Cells were allowed to incubate at 37°C for 4 hours. Following incubation, all liquid was removed from the cells and 50 μL of room temperature Dulbecco's phosphate-buffered saline (DPBS) (Gibco; catalogue no. 14 040 133) was added to the cells followed by 50 μL of room temperature ONE-Glo EX luciferase substrate (Promega; catalogue no. E8120). The plate was covered with foil and allowed to incubate at room temperature for 10 minutes on an orbital rocker before reading luminescence on an Enspire microplate reader (PerkinElmer, Waltham, MA, USA). Averages of triplicate conditions were calculated using EXCEL (Microsoft Corp. Redmond, WA, USA) and expressed as a percentage of the WT maximum response. Concentration-response curves were generated by pooling data from three independent experiments. Maximal responses (E_{max}) and EC_{50} values were calculated using PRISM (GraphPad Software Inc., San Diego, CA, USA). A one-way ANOVA statistical test was applied to pEC_{50} and E_{max} values to determine significance ($P < .05$), with a Bonferroni post-hoc test for multiple comparisons.

2.6 | Co-immunoprecipitation

CHO cells were transiently transfected in 6-cm poly-D-lysine coated plates using FuGene HD transfection reagent (Promega) and allowed to incubate at 37°C for 48 hours. DNA concentrations were as follows: 5 μg of DNA was transfected per plate. Receptor variant ± MRAP2 or empty vector was transfected at a ratio of 1:3 ratio. Cells were gently rinsed twice with room temperature DPBS followed by cross-linking for 15 minutes at room temperature with 1mmol L^{-1} dithiobis(succinimidyl propionate) (Thermo Scientific, Waltham, MA, USA; catalogue no. PG82081). Reaction was stopped using 10 mmol L^{-1} Tris(hydroxymethyl)aminomethane-hydrochloride solution for 15 minutes at room temperature. Cell lysates were then harvested by adding radioimmunoprecipitation assay buffer (RIPA buffer) to each plate followed by scraping and allowing plate to incubate on rocker at 4°C for 30 minutes. The contents of each plate were then added to a clean Eppendorf tube and centrifuged at 9600*g* for 10 minutes. Supernatants were collected in fresh Eppendorf tubes followed by a Pierce bicinchoninic acid protein quantification assay. Next, 100 μg of lysate in a total volume of 500 μL was incubated overnight with 1 μg of antibody on a rotator at 4°C. The next day, protein G magnetic beads (Thermo Scientific; catalogue no. 88 848) were rinsed and resuspended in RIPA buffer. Then, 30 μL of beads were added to each tube and allowed to incubate on rocker at 4°C for 1 hour. Using a magnetic rack, beads were washed three times with RIPA buffer and eluted in 50 μL of 3× lithium dodecyl sulphate buffer (Thermo Scientific; catalogue no. B0007) with 200 mmol L^{-1} dithiothreitol. Samples were allowed to rest at room temperature for 15 minutes before loading 20 μL on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Blots were imaged on CHEMIDOC (Bio-Rad, Hercules, CA, USA).

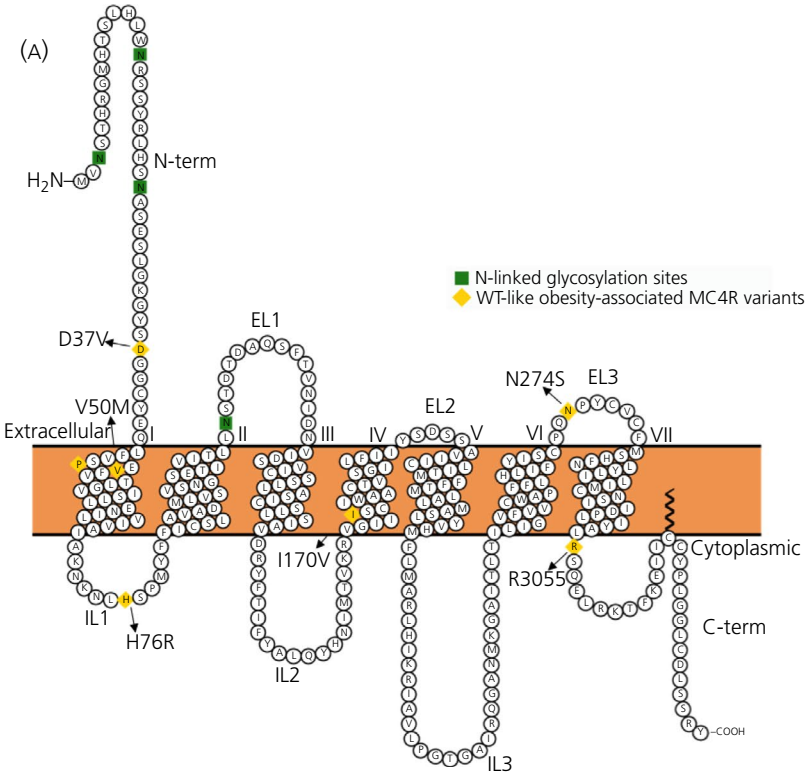
2.7 | Endoglycosidase treatment for confirmation of MC4R glycosylation

CHO cells were plated and transfected in 10-cm poly-D-lysine coated plates in the same manner as previously detailed and allowed to incubate at 37°C for 48 hours. Cells were transfected with 8 μg of receptor variant DNA per plate. Cell lysates were then harvested as described above. To assess the glycosylation pattern, Endo H (New England BioLabs; catalogue no. P0702S) and PNGase F (New England BioLabs; catalogue no. P0704S) were used in accordance with the manufacturer's instructions. Samples were then run on an SDS-PAGE gel and analysed via western blot using CHEMIDOC imager and IMAGE LAB (Bio-Rad).

2.8 | Western blots

Ten to 15 μg of protein from prepared cell lysates was loaded into corresponding lanes on Bolt 4%-12% Bis-Tris Plus precast polyacrylamide gels (Invitrogen; catalogue no. NW04122) at 200V for 30-40 minutes using Bolt Mops running buffer (Invitrogen; catalogue no. B000102). Bolt antioxidant (Invitrogen; catalogue no. BT0005) was added to the first chamber of the mini gel tank (Invitrogen; catalogue no. A25977). Transfer to Immobilon polyvinylidene fluoride membrane (Millipore, Burlington, MA, USA; catalogue no. IPVH00010) was performed using a transblot turbo system (Bio-Rad; catalogue no. 1704150EDU). Membranes were blocked with 5% non-fat dry milk in phosphate-buffered saline with 1% Tween-20 overnight at 4°C. Blots were then washed a minimum of three times for 15 minutes each before imaging using SuperSignal West Dura Extended Detection Substrate (Thermo Scientific; catalogue no. 34 075) using CHEMIDOC TOUCH (Bio-Rad).

(A)



(B)

MC4R variant		Topology
D37V	Asp37Val	N-term
P48S	Pro48Ser	TM-1
V50M	Val50Met	TM-1
H76R	His76Arg	IL-1
I170V	Ile170Val	TM-4
N274S	Asn240Ser	EL-3
R305S	Arg305Ser	C-term

FIGURE 1 A, Schematic representation of melanocortin-4 receptor (MC4R). Amino acids are indicated as a single-letter code within circles. Those affected by mutations and assessed within the present study are represented by yellow diamonds. The corresponding amino acid change is noted for each variant. N-linked glycosylation sites are represented by green squares. The seven-transmembrane domains are indicated by roman numerals. EL, extracellular loop; IL, intracellular loop. Schematic generated using Protter online interactive software. B, Wild-type like, obesity-associated mutations characterised in the present study

2.9 | MSH-induced β-arrestin recruitment

CHO cells were transiently transfected in white, flat-bottomed 96-well plates with WT or mutant-MC4R-LgBit and SmBit-β-arrestin plasmids (0.2 μg of DNA/well at a ratio of 1:1) using FuGene HD transfection reagent and allowed to incubate at 37°C for 48 hours. On the day of experiment, all media was removed from cells and replaced with 25 μL of room temperature DPBS (Gibco) and allowed to equilibrate at room temperature for 10 minutes. Next, 25 μL of room temperature NanoGlo Live Cell Substrate was added to each well, and then the plate was wrapped in foil and gently mixed on an orbital shaker for 15 seconds. Background luminescent signal was measured for 5 minutes using the PerkinElmer Enspire microplate reader. Then, 50 μL of a serial dilution (starting concentration of 10 pmol L^{-1}, ending concentration of 10 μmol L^{-1}) of α-MSH or β-MSH was added to corresponding wells, covered with foil and allowed to incubate at room temperature for 10 minutes before measuring luminescent signal on microplate reader. The optimal time point was chosen as 10 minutes because the signal was observed to peak at this time. Each condition was measured from triplicate wells in each experiment, and each experiment was performed at least three times independently. For data analysis, raw values for background measurement were averaged and used to normalise the MSH response for each corresponding well. The normalised values for the stimulated wells were then averaged. Concentration-response curves were generated by pooling data from three independent experiments. The concentration-response curves for these data were generated in PRISM and analysed to determine E_{max} and EC_{50}. A one-way ANOVA statistical test was applied to pEC_{50} and E_{max} values to determine significance ($P < .05$), with a Bonferroni post-hoc test for multiple comparisons.

3 | RESULTS

3.1 | Gα$_s$ coupling of MC4R variants in response to α- and β-MSH

In the present study, seven different human obesity-associated class V variants of the MC4R (Figure 1) were characterised in CHO-K1 cells. These seven mutations were previously identified in obese patients and assessed for G-protein coupling, binding affinity and cell surface expression,[30] and categorised as Class V variants, based on normal cell surface expression, ligand binding and coupling to Gα$_s$.[30]

To characterise the coupling of the mutant receptors to Gα$_s$ in response to α-MSH or β-MSH, we used a cAMP-responsive luciferase to measure the accumulation of cAMP upon activation by either ligand. In response to α-MSH (Figure 2A-F), one variant

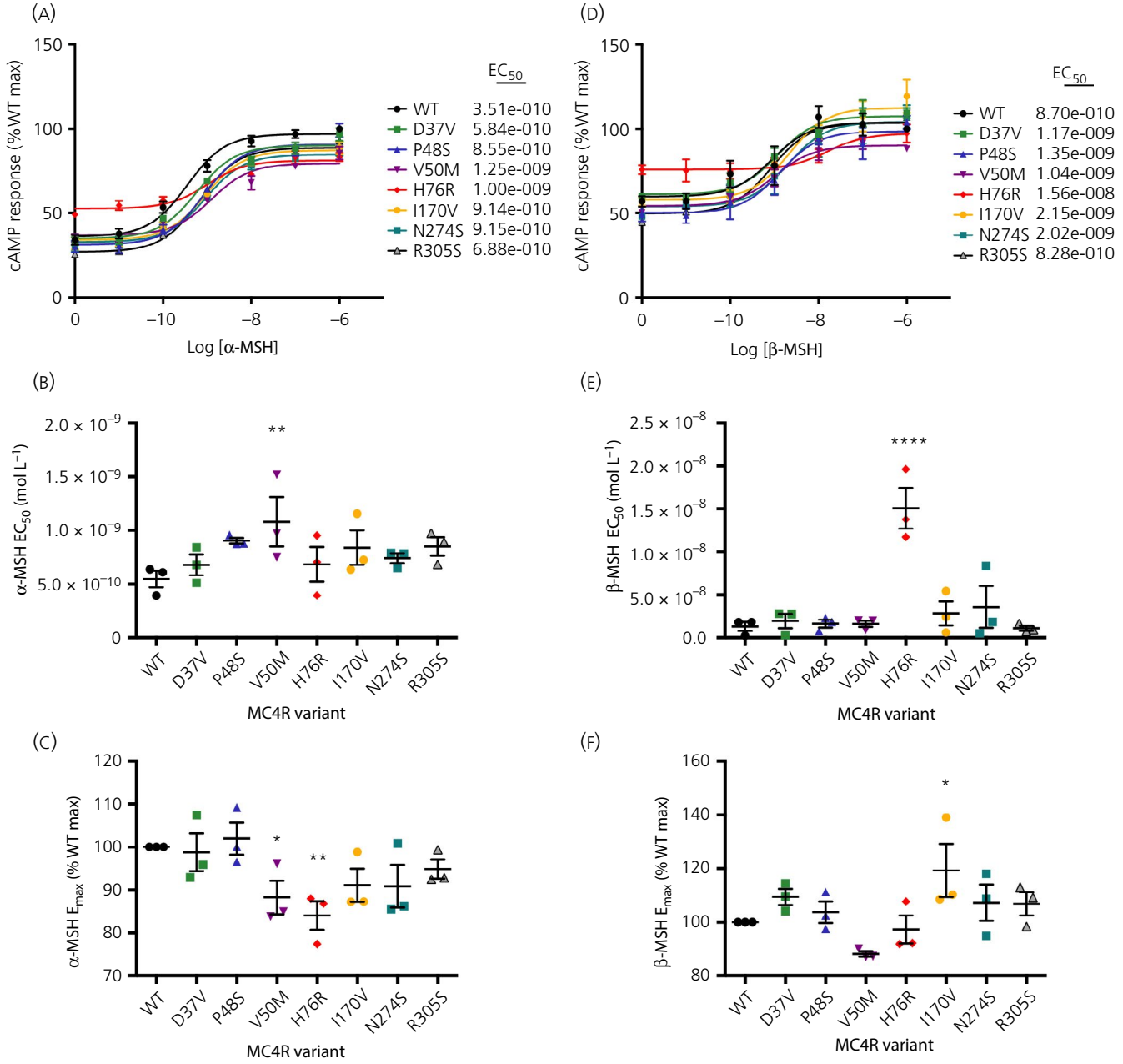


FIGURE 2 Accumulation of intracellular cAMP in Chinese hamster ovary (CHO-K1) cells transiently transfected with wild-type (WT) and mutant melanocortin-4 receptor (MC4R). Transfected cells were treated with α-MSH (A-C) or β-MSH (D-F) and intracellular cAMP was measured using a luciferase-based assay described in the Materials and methods. All experiments were run in triplicate and performed at least three times. Concentration-response curves represent the means of three independent experiments. Results are expressed as the mean ± SEM. *P < .05, **P < .01, ***P < .001, ****P < .0001

(V50M) exhibited a significant increase in EC_{50}, whereas two variants (V50M and H76R) exhibited a significant decrease in E_{max}. In response to β-MSH (Figure 2G-L), one variant exhibited a significant increase in EC_{50} (H76R), whereas I170V exhibited an increase in maximum response. These data suggest that five of these receptor variants exhibit fully normal responses to both α-MSH and β-MSH (D37V, P48S, I170V, N274S and R305S). H76R also exhibited significant constitutive activity in our assay (see supporting information, Figure S1).

A recent study found that des-acetyl-α-MSH was the predominant form of MSH in the adult human brain.[31] Previous studies in which α-MSH was employed to perform molecular assays used acetylated α-MSH, as in the present study. To determine whether or not the acetylation status of MSH might have affected these results, we ran the cAMP accumulation assay and compared findings using WT and two variant receptors (D37V and V50M) (see supporting information, Figure S2). No significant differences were found when comparing EC_{50} values in response to α-MSH vs des-acetyl-α-MSH at the WT and variant receptors tested.

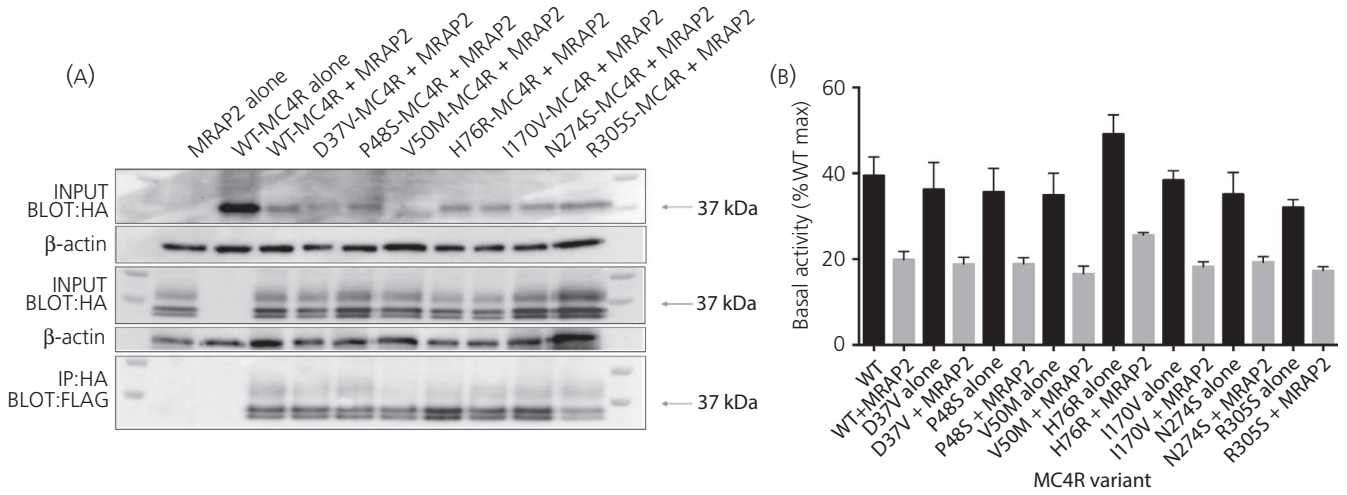


FIGURE 3 A, Melanocortin-2 receptor-associated protein 2 (MRAP2) (C-terminal 3xFLAG tag; anticipated MW: 28 kDa,) protein interaction with WT and mutant melanocortin-4 receptor (MC4R) (N-terminal 3HA tag; anticipated MW: 37 kDa) was assessed using co-immunoprecipitation and western blot techniques described in the Materials and methods. Single blots are shown representative of three individual experiments per set of variants. B, Effect of MRAP2 interaction on constitutive activity of wild-type (WT) and mutant MC4Rs was assessed using a luciferase-based assay as described in the Materials and methods. Bar graphs represent the level of cAMP accumulation when Chinese hamster ovary (CHO-K1) cells were transiently transfected with MC4R ± MRAP2. Results are expressed as the mean ± SEM of pooled data from three independent experiments

3.2 | MRAP2 interacts with and is able to suppress constitutive activity of all variants

In the present study, we aimed to test whether class V obesity-associated MC4R receptors associated with obesity have defects in signalling that are not detected by a conventional $G\alpha_s$ coupling assay. MC4R exhibits a significant degree of constitutive activity, and MRAP2 has been shown to inhibit this activity, as well as increase responsiveness to α-MSH.[14] Thus, mutations enhancing the suppression of MC4R activity by MRAP2, or compromising MRAP2 effects on responsiveness to ligand, might be expected to reduce MC4R activity, which could be expected to cause obesity. To characterise the protein-protein interaction between MRAP2 and MC4R variants, we first performed co-immunoprecipitation from cell lysates collected from CHO-K1 cells that were transiently transfected with WT or mutant MC4R (N-terminal 3HA epitope tag; anticipated MW: 37 kDa) with or without co-transfection of MRAP2 (C-terminal 3xFLAG epitope tag; anticipated MW: 28 kDa). According to previous studies, MRAP2 pulls down as a doublet representing unglycosylated and glycosylated forms of the protein. The band observed above this doublet has been suggested to be a more highly glycosylated form of MRAP2, although this is yet to be confirmed. We found that each MC4R variant was able to pull down MRAP2 in a manner comparable to WT MC4R (Figure 3A, bottom row).

To assess the ability of MRAP2 to suppress the constitutive activity of each variant, we performed a luciferase-based assay measuring the basal levels of cAMP in CHO-K1 cells transiently transfected with WT or mutant MC4R with or without MRAP2 (Figure 3B). We further tested whether MRAP2 had any effect on the WT or mutant receptor's response to α-MSH or β-MSH and found that there no significant differences (see supporting information, Figure S3). Taken together, these results show that there were no defects in these class V variants with respect to suppression of constitutive activity, or alteration in ligand responsiveness by MRAP2.

3.3 | V50M variant exhibits a putative defect in N-linked glycosylation

We next examined the glycosylation state of each variant MC4R protein. Using cell lysates from transiently transfected CHO-K1 cells, we performed western blots to assess the size and banding pattern of the WT and mutant MC4Rs. Interestingly, MC4R V50M exhibited an altered migration pattern compared to WT and this pattern was similar to a variant of MC4R (N26Q) in which one of four N-linked glycosylation sites had been mutated (Figure 4A). We used the Endo H and PNGase F endoglycosidases to confirm the glycosylation of WT MC4R and also to confirm the defect in receptor glycosylation by the V50M mutation. PNGase F is able to cleave all glycans, core and complex, from a protein, whereas the Endo H enzyme is only able to cleave core glycans. Therefore, if a protein has complex glycosylation, it will be resistant to Endo H but not PNGase F, and no band shift will be observed. The results from these experiments (Figure 4B) suggest that WT MC4R undergoes core but not complex glycosylation. V50M again exhibits an altered migration pattern compared to WT MC4R and similar to the N26Q mutant. Both of these mutations are shown to be glycosylated, although not to the same extent as WT; thus, the absence of a single glycosylation event in the V50M mutant is consistent with the data.

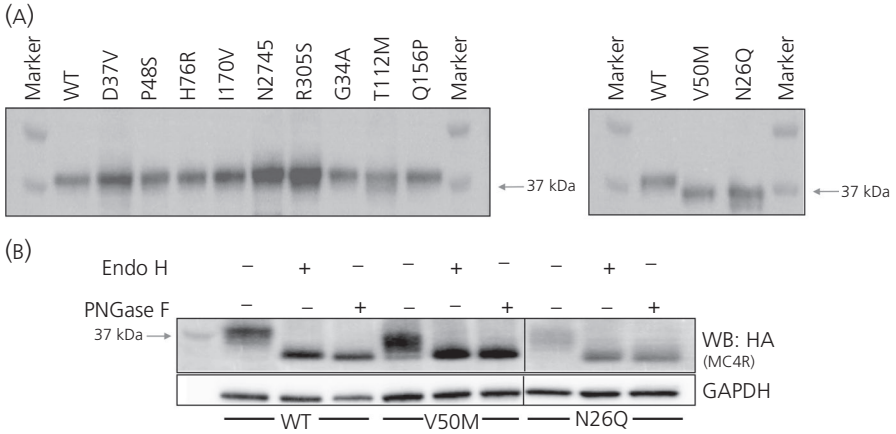


FIGURE 4 A, Cell lysates from Chinese hamster ovary (CHO-K1) cells transiently transfected with wild-type (WT) or mutant melanocortin-4 receptor (MC4R) were collected and analysed for glycosylation pattern, seen as a shift in migration compared to WT. N26Q is a mutation of one of the known glycosylation sites of MC4R. B, Confirmation of glycosylation was performed using EndoH and PNGase F glycosidases and is described in Materials and Methods. Boxes depict where the membrane was cut

3.4 | Five of seven variants tested have altered β-arrestin recruitment in response to α- or β-MSH

Upon activation, GPCRs are known to recruit β-arrestin, which leads to downstream intracellular events such as receptor internalisation, or to novel pathways of intracellular signalling. To assess the recruitment of β-arrestin to WT and mutant MC4Rs in response to α-MSH or β-MSH, we utilised the NanoBit luciferase-based complementation assay (Promega). WT or mutant receptors were fused to the "large bit" of a Nano-luciferase enzyme at the C-terminal end, whereas β-arrestin was fused to the "small bit" at the N-terminal end. These constructs were then transiently co-transfected into CHO-K1 cells and treated with α-MSH or β-MSH. To determine whether the fusion of the "large bit" affects the "normal" signalling of the receptor, we compared the 3HA-WT-MC4R construct with the LgBit-fused receptor at coupling to Gs via the cAMP accumulation assay described previously. We found that the fusion of the "large bit" did suppress the constitutive activity of the receptor and altered the EC_{50} values compared to the un-fused receptor but did not affect its ability to respond normally to ligand (see supporting information, Figure S4A-D). To confirm that MC4R-arrestin interaction was specific, we co-transfected CHO-K1 cells with HaloTag protein fused to the "small bit" (Promega), and MC4R wt and mutant constructs, fused to large bit. The HaloTag protein is expressed throughout the cell and should not interact with "large bit" fused proteins in a concentration-dependent manner. We found this to be true in our experimental set-up (see supporting information, Figure S4E-H), therefore suggesting that the β-arrestin recruitment, as well as any observed defects, are specific to ligand-induced MC4R activation. Five of the seven variants tested had either reduced or excessive β-arrestin recruitment in response to α-MSH or β-MSH (Figure 5 and Table 1). P48S, V50M, H76R, I170V and N274S had either or both altered EC_{50} or E_{max} for β-arrestin recruitment compared to WT in response to α-MSH, whereas P48S, V50M and H76R had either or both altered EC_{50} or E_{max} compared to WT in response to β-MSH. Data comparing EC_{50} values for coupling to Gαs with EC_{50} values for β-arrestin recruitment may be seen in Table 1. These data suggest that the interaction between MC4R and β-arrestin is an important pathway in the regulation of MC4R signalling and its normal function with respect to maintaining body weight.

4 | DISCUSSION

The melanocortin-4 receptor is one of the most well-validated drug targets for the treatment of obesity. However, many peptide and small molecule drugs targeting MC4R have failed in clinical trials as a result of a target-mediated increase in blood pressure.[26-28] The growing interest in biased signalling in GPCR drug discovery highlights the urgency to explore novel signalling pathways when considering target-specific therapeutics.[32-36] Interestingly, a subset of MC4R mutations in humans is associated with the development of obesity, yet these have been reported to couple normally to $G\alpha_s$. Upon MC4R agonist binding, $G\alpha_s$ signalling leads to activation of adenylyl cyclase and elevation of intracellular cAMP that may go on to activate cAMP-responsive element-binding protein.[37] Some data suggests that $G\alpha_s$ signalling is critical for MC4R signalling and regulation of body weight because deletion of the GNAS gene encoding $G\alpha_s$ in MC4R neurones recapitulates the entirety of the obesity syndrome following MC4R deletion.[3,38] However, deletion of cAMP-responsive element-binding protein in the paraventricular nucleus has no influence on the ability of MC4R agonist, MTII, to inhibit food intake.[39] These data, along with the complexity of MC4R signalling and multiple MC4R accessory proteins, further highlight the likelihood that multiple signalling pathways are important for the function of MC4R and its regulation of food intake and body weight. Being able to identify receptor variants with specific pathway defects would allow further insight into the complexity of MC4R-mediated regulation of energy homeostasis and potentially aid in the development of therapeutics for the treatment of obesity without the side effect profile. Activation of extracellular signal-regulated protein kinases 1

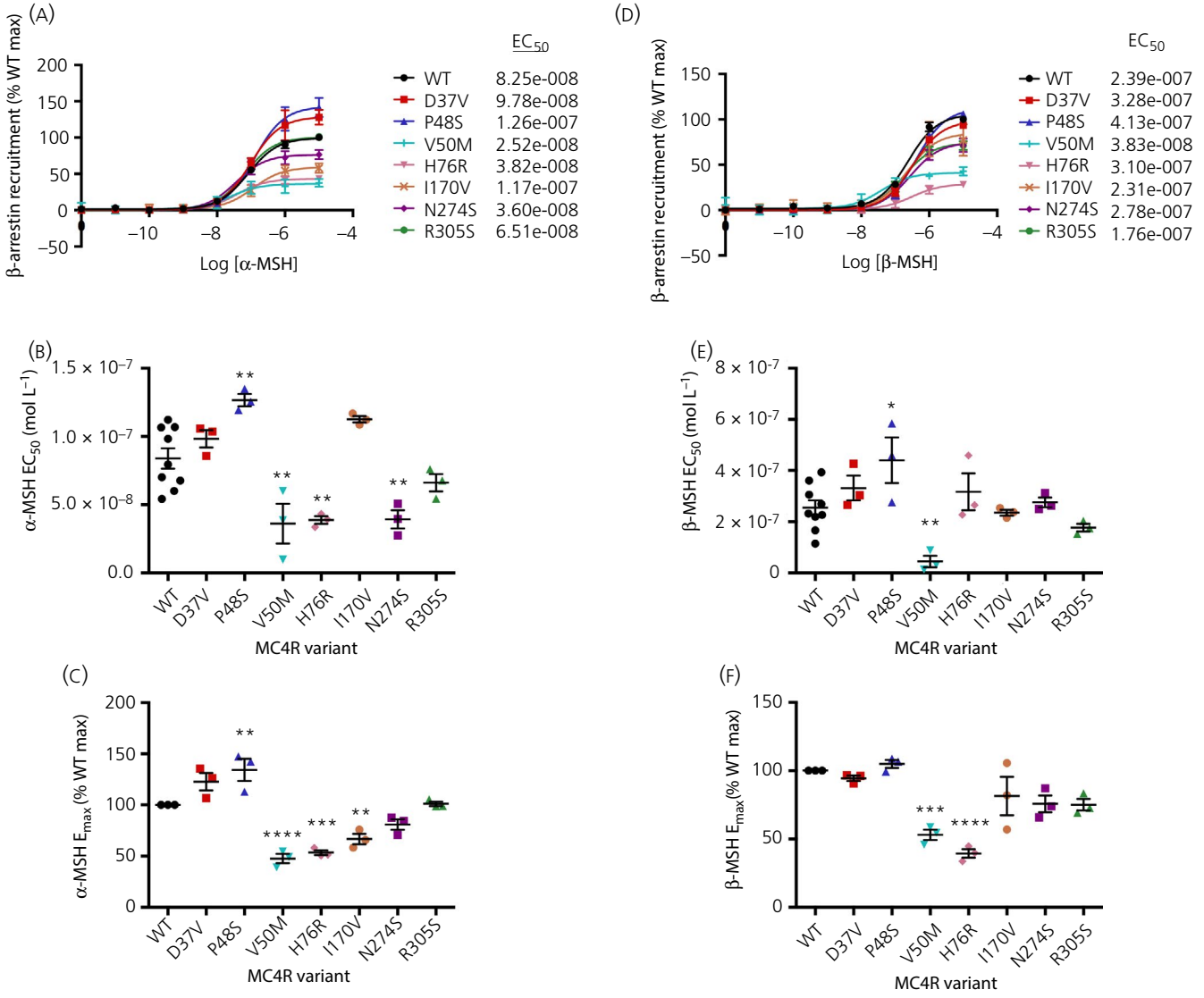


FIGURE 5 Recruitment of β-arrestin in Chinese hamster ovary (CHO-K1) cells transiently transfected with wild-type (WT) and mutant melanocortin-4 receptors (MC4Rs). Transfected cells were treated with α-MSH (A-C) or β-MSH (D-F) and β-arrestin recruitment was measured using a luciferase-based, enzyme complementation assay described in the Materials and methods. All experiments were run in triplicate and performed at least three times. Concentration-response curves represent the means of three independent experiments. Results are expressed as the mean ± SEM. *P < .05, **P < .01, ***P < .001, ****P < .0001

TABLE 1 Log EC_{50} values of WT and mutant MC4R for $G\alpha_s$ coupling and for recruitment of **β**-arrestin. Data are from Figure 2 and 5

	LogEC_{50} (mean ± SEM)			
	α-MSH		**β-MSH**	
	$G\alpha_s$ coupling (cAMP)	**β-arrestin recruitment**	**$G\alpha_s$ coupling (cAMP)**	**β-arrestin recruitment**
Wild-type	−9.45 ± 0.10	−7.08 ± 0.06	−9.06 ± 0.30	−6.62 ± 0.07
D37V	−9.23 ± 0.12	−7.01 ± 0.12	−8.93 ± 0.18	−6.49 ± 0.08
P48S	−9.07 ± 0.12	−6.90 ± 0.10	−8.87 ± 0.27	−6.38 ± 0.07
V50M	−8.90 ± 0.15	−7.60 ± 0.35	−8.98 ± 0.18	−7.42 ± 0.26
H76R	−9.00 ± 0.19	−7.42 ± 0.12	−7.81 ± 0.38	−6.51 ± 0.29
I170V	−9.04 ± 0.10	−6.93 ± 0.18	−8.67 ± 0.21	−6.64 ± 0.26
N274S	−9.04 ± 0.11	−7.44 ± 0.13	−8.70 ± 0.29	−6.56 ± 0.12
R305S	−9.16 ± 0.90	−7.19 ± 0.43	−9.08 ± 0.14	−6.75 ± 0.10

Abbreviation: MSH, melanocyte-stimulating hormone.

TABLE 2 Characterisation summary of variants assessed within the present study

hMC4R variant	$G_s\alpha$-coupling				MRAP2 protein interaction	Effect of MRAP2 on constitutive activity	Glycosylation status	Beta-arrestin recruitment			
	α-MSH		β-MSH					α-MSH		β-MSH	
	E_{max} (% WT)	EC_{50} (nmol L^{-1})	E_{max} (% WT)	EC_{50} (nmol L^{-1})				E_{max} (% WT)	EC_{50} (nmol L^{-1})	E_{max} (% WT)	EC_{50} (nmol L^{-1})
WT											
D37V	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔
P48S	▲	↔	↔	↔	↔	↔	↔	▲▲	▲▲	↔	▲
V50M	▼	▲▲	↔	↔	↔	↔	Loss of single glycan	▼▼▼	▼▼	▼▼▼	▼▼
H76R	▼▼	↔	↔	▲▲▲▲	↔	↔	↔	▼▼▼	▼▼	▼▼▼▼	↔
I170V	↔	↔	▲	↔	↔	↔	↔	▼▼	↔	↔	↔
N274S	↔	↔	↔	↔	↔	↔	↔	↔	▼▼	↔	↔
R305S	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔	↔

Note: ↔: WT-like; ▲: increase (number indicates level of significance); ▼: decrease (number indicates level of significance).
MRAP2, melanocortin-2 receptor-associated protein 2; MSH, melanocyte-stimulating hormone; WT, wild-type.

and 2 is known to occur downstream of both G-protein and β-arrestin signalling; thus, in a previous study, only a small fraction of class V mutants tested (five of 25) exhibited reduced phosphorylation of extracellular signal-regulated protein kinases 1 and 2 following [Nle4,D-Phe7]-α-MSH treatment out of 293 cells transfected with MC4R.[40]

In the present study, we functionally characterised seven class V variants of the MC4R and showed that there are indeed other G-protein-dependent and G-protein-independent signalling pathways defective in most of the receptor mutants in this class (Table 2). Mutations in the region of the pro-opiomelanocortin gene that encodes β-MSH have been shown to result in severe obesity in humans, demonstrating that it is also an important ligand in the central control of energy homeostasis.[40-42] In the present study, we show that some class V mutants, such as H76R, exhibit reduced responsiveness to β-MSH in both $G\alpha_s$ coupling and β-arrestin recruitment assays. This further supports the hypothesis that loss of β-MSH activity at MC4R is important for the normal regulation of energy homeostasis.

The most surprising finding was that a majority of the obesity variants characterised in the present study exhibited alteration in β-arrestin recruitment. During the preparation of this manuscript, a study of a large collection (n = 49) of naturally occurring variants of the MC4R demonstrated a statistical correlation between the efficacy of β-arrestin recruitment to the MC4R and BMI, which is a correlation not found with coupling to $G\alpha_s$.[25] Importantly, many of these variants analysed in the present study are not associated with morbid syndromic obesity. By contrast, the majority of obesity-associated mutations result in defective cAMP signalling. Furthermore, although four of seven obesity-associated mutations characterised here exhibited decreased β-arrestin recruitment, 1 exhibited an increase in maximal β-arrestin recruitment (P48S), whereas a second indicated a trend towards increased β-arrestin recruitment (D37V). In these cases, it is possible that the mutations lead to obesity by increasing internalisation, rather than producing defective β-arrestin signal transduction.

Thus, distinct from the argument in Lotta et al,[25] our interpretation of the data suggests that β-arrestin recruitment is a marker of normal MC4R function, although the data do not yet support a role for β-arrestin signalling in BMI. Lotta et al[25] did not report the EC_{50} values for β-arrestin recruitment. However, it is important to note from the data reported in the present study that, although EC_{50} values are highly assay-dependent, we nonetheless observed that the native ligand α-MSH is more than two hundred fold more potent in $G\alpha_s$-mediated signalling than recruitment of β-arrestin, with fusion of the large bit tag (see also supporting information, Figure S4) only accounting for a 10-fold shift in EC_{50} (EC_{50} for α-MSH-induced coupling to $G\alpha_s$ = 3.4×10^{-10}; EC_{50} for α-MSH-induced β-arrestin recruitment = $8 \times 10^{-8)}$. Further experimentation is necessary to identify the molecular and physiological consequences of β-arrestin recruitment to the MC4R because MC4R signalling through β-arrestin has not yet been demonstrated.

Previous studies have shown that MC4R exhibits constitutive activity and that this is linked to the N-terminal domain of the receptor.[43]

A cluster in the N-terminal domain of the receptor was identified after characterisation of several naturally occurring, obesity-associated mutations of the MC4R. None of the receptor variants that were included in the present study are located in this cluster; however, constitutive activity has also been shown to be regulated by interaction with the accessory protein, MRAP2.[14,44] In the present study, we show that co-expression of the MRAP2 in vitro is still able to suppress the constitutive activity of the seven class V receptors tested here. Expansion of the variants tested could possibly lead to the identification of variants with defects in MRAP2 interaction. The possibility of MC4R coupling to other G-proteins is still debated. It is noted that some studies have claimed that MC4R interacts with the inhibitory G-protein, $G_{i/o}$, which leads to a decrease in cAMP production.[45] Other studies suggest that MC4R couples to $G_{q/11}$ and mediates the mobilisation of intracellular free calcium.[43,46] However, we did not include these experiments in the present study because coupling to these pathways is not a reliable readout in an overexpression system and is highly variable between cell lines.

It is possible that other pathways that have not been tested in the present study could be affected, such as other posttranslational modifications (eg phosphorylation, palmitoylation, etc), receptor dimerisation or interaction with other membrane proteins and/or channels (eg Kir7.1). MC4R has four glycosylation sites but the function of this receptor modification is still unclear. One of the variants tested in the present study, V50M, did exhibit a defect in glycosylation, reduced responsiveness to α- and β-MSH, and reduced the ability to couple to β-arrestin, although it is unclear whether the glycosylation defect contributes to the altered signalling properties. Granell et al[47] suggest that glycosylation status may not affect the function of the receptor.

In conclusion, through careful analysis of pharmacological properties, we demonstrate that five of seven class V receptors tested have marked functional defects detectable in a simple transfection cell system. It is possible that some mutations (eg, the D37V or R305S mutations for which we found no significant phenotypes) are not causally linked to obesity, have defects that are only detectable in a neuronal cell milieu or are defective in yet unknown pharmacological properties of the receptor. A second conclusion from the present study is that a majority of class V MC4R mutations associated with obesity exhibit either decreased or increased β-arrestin recruitment. This finding suggests that MC4R must couple normally to β-arrestin for the normal regulation of energy homeostasis, and that biased ligands, differentiating β-arrestin from $G_s\alpha$ signalling, should be developed and tested to determine the physiological consequences of β-arrestin recruitment to the MC4R.

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SUPPORTING INFORMATION

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