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Structure reveals the activation mechanism of the MC4 receptor to initiate satiation signaling

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Obesity is a global epidemic causing morbidity and impaired quality of life. The melanocortin receptor 4 (MC4R) is at the crux of appetite, energy homeostasis, and body-weight control in the central nervous system and is a prime target for anti-obesity drugs. Here, we present the cryo-EM structure of the human MC4R-G_s signaling complex bound to the agonist setmelanotide, a cyclic peptide recently approved for the treatment of obesity. The work reveals the mechanism of MC4R activation, highlighting a molecular switch that initiates satiation signaling. In addition, our findings indicate that Ca²⁺ is required for agonist but not antagonist efficacy. These results fill a gap in understanding MC4R activation and could guide the design of future weight management drugs.

Obesity is a global epidemic, with more than 40% of the adult population considered overweight or obese (1). Obesity is a major risk factor for chronic diseases, including type 2 diabetes, coronary artery disease, and cancer, and constitutes an important cause of morbidity and impaired quality of life (2). Over the years, various weight-loss-targeting drugs have been developed, yet most of these were ultimately removed from the market due to questionable efficacy, lack of target specificity, or severe adverse effects. This highlights the need for a better understanding of the molecular mechanisms governing weight regulation and its related pathologies.

Melanocortin 4 receptor (MC4R) is a member of the melanocortin receptor family (MCRs), a class-A G-protein coupled receptor (GPCR) subgroup consisting of five subtypes (MC1R-5) that mediate multiple physiological effects in humans (3). MC4R is abundant throughout the mammalian central nervous system (CNS) and is expressed in particular within the paraventricular nucleus (PVN) of the hypothalamus, a region critical for energy homeostasis (4–6). MC4R has been shown to play a key role in modulating satiety in pharmacological and genetic studies in animal models and patients (7–9). Loss-of-function mutations in MC4R have been implicated in ~6–8% of early-onset severe obesity cases and are considered the most common form of monogenic obesity (7, 10), while gain-of-function mutations have been linked to low body mass index (BMI) (9). Beyond its critical role in the regulation of food intake and energy expenditure, MC4R also

functions in other CNS regions with clinical implications in pain sensation, sexual function, anhedonia, and blood pressure regulation (11–13).

MC4R is an unusual GPCR as it has both an endogenous agonist and an endogenous antagonist (14, 15). For other GPCRs, with the exception of the close MC4R homolog MC3R (3), only endogenous agonists have been described. Specifically, MC4R is activated by the alpha-melanocyte-stimulating hormone (α -MSH), a proopiomelanocortin (POMC) derived peptide, which mediates anorexigenic effects, and is antagonized through binding of the agouti-related peptide (AgRP), which promotes appetite (14). A recent crystal structure of MC4R bound to a potent antagonist provided a snapshot of the inactive receptor conformation (16), but the molecular architecture of an active complex with cognate signaling partners remained unknown. We determined the structure of the active human MC4R bound to the cyclic peptide agonist setmelanotide and in complex with the G_s heterotrimer at 3 Å resolution by cryo-EM. Setmelanotide (Imcivree) is a promising weight control drug shown to induce weight loss in patients suffering from POMC deficiency (17, 18), leptin receptor deficiency (19) and other forms of severe genetic obesity, and was recently registered by the FDA for the treatment of these conditions (20–23).

The molecular architecture of the native MC4R signaling complex

The setmelanotide-MC4R-G_s complex was assembled by

supplying agonist to cells co-expressing heterotrimeric G_s and full-length MC4R and was stabilized using the single-chain variable fragment scFv16 (24). ScFv16 was originally developed for the stabilization of the $G_{i/o}$ heterotrimer and has been used to determine several $G_{i/o}$ and G_q GPCR complex structures (25–29). We replaced the αN helix of $G\alpha_s$ (residues 1–38) with that of $G\alpha_{i1}$ (residues 1–31) to enable complex recognition by scFv16 ($G\alpha_{s-iN}$) (24). This strategy yielded a stable G_s heterotrimer that coupled to activated MC4R (fig. S1).

We used single-particle cryo-EM analysis to obtain a three-dimensional reconstruction of the MC4R- G_s complex at a nominal resolution of 3 Å (Fig. 1, figs. S1 and S2, and tables S1 and S2). The map revealed the architectures of all complex components, as well as the mode of receptor association with the G_s heterotrimer and the orientation of setmelanotide within the orthosteric binding pocket (Fig. 1, C and D). Most notably, MC4R differs from previously reported class-A GPCR- G -protein complexes in the extracellular region, which is relatively open, containing an exposed cavity of 1082 Å³ (Fig. 2, B and C; and fig. S3A). This feature can be attributed to a short extracellular loop 2 (ECL2) that lacks secondary structural elements compared with other class-A members (fig. S3B) (30). As noted previously (16), MC4R lacks a disulfide found in other class-A GPCRs connecting ECL2 with trans-membrane helix 3 (TM3) (31). Instead, the MC4R extracellular region has two other disulfides, which link the N terminus to TM7, and ECL3 to TM6, both of which are clearly visualized in our EM density (C40^{N-term}-C279^{7,30} and C271^{6,61}-C277^{ECL3}; superscript numbers correspond to the Ballesteros-Weinstein nomenclature; fig. S2G). Overall, our structure enables the analysis of the full-length, active MC4R coupled with its cognate cellular effectors and supplies residue-resolution information on the main signaling pathway leading to satiety.

Description of setmelanotide in the MC4R binding pocket

Setmelanotide is a potent mimetic of α -MSH, the endogenous hormone agonist that acts on MC4R to signal satiety and increase energy expenditure (Fig. 2A). Unlike α -MSH, setmelanotide is linked by a disulfide bond between cysteines near its termini. Setmelanotide contains the highly conserved HFRW motif that is shared between all POMC-derived peptides and contributes directly to the activation of receptors of the MCR family (32). However, the phenylalanine in setmelanotide is in the D configuration, whereas it is in the L configuration in α -MSH (Fig. 2A).

In the cryo-EM structure, setmelanotide engages MC4R through extensive electrostatic and hydrophobic interactions with residues in the transmembrane domain (Fig. 3A). These interactions are mostly attributed to the HFRW motif and include hydrogen bonds with S188, π - π stacking interaction

with F51, C-H...O contacts (33) with Y268 and F284, and hydrophobic contacts with T101, I129, L133, I185, I194 and F261 (Fig. 3A). Most of these residues are highly conserved among the MCR family and account for the cross reactivity with POMC-derived peptides. Interactions outside the HFRW motif include contacts between Cys₈ in setmelanotide and Y268, and F284 in the receptor, only the last of which is conserved in members of the MCR family (Fig. 3B and fig. S4). Additional contacts are from Arg₁ and Ala₃ to D122 and I104, respectively, both of which are conserved in MCR family members. Arg₁ in setmelanotide substitutes for a serine in α -MSH and likely contributes to the elevated potency of the drug compared with the endogenous hormone (Fig. 2, A and D).

A notable feature of setmelanotide binding is a Ca²⁺ ion coordinated by both the agonist and the receptor. A Ca²⁺ ion was observed in a similar position in the complex of MC4R with the synthetic antagonist SHU9119 [Fig. 3C and (16)]. In both structures, the receptor contributes the side chains of the acidic residues (D122^{3,25}, D126^{3,29} and E100^{2,60}) to Ca²⁺ coordination, and both setmelanotide and SHU9119 have a direct interaction to the ion using the D-Phe₅ backbone oxygen (D-naphthyl alanine, D-Nal₄ in SHU9119, Fig. 3C). One difference in the coordination of the Ca²⁺ between the antagonist and agonist structures is that SHU9119 contributes the backbone carbonyl of Asp₂ in a backbone-ligand interaction, whereas setmelanotide uses a water-mediated interaction between the backbone carbonyl of D-Ala₃ and the ion (Fig. 3C and fig. S2F). Molecular dynamics simulations suggest that the backbone carbonyl of Cys₂ may alternate with the backbone carbonyl of D-Ala₃ in creating the water-mediated interaction (fig. S5, A and B).

Ca²⁺ functions as a cofactor required for receptor activation but not inhibition

Molecular dynamics simulations support Ca²⁺ binding by MC4R even in the absence of a ligand, suggesting that Ca²⁺ may have a ligand-independent role or may prime the pocket for ligand binding (Fig. 3D and fig. S5, C and D). While the simulations suggest that Ca²⁺ does not depend on other ligands for binding, MC4R agonists require Ca²⁺ to achieve full efficacy. The EC₅₀ for both α -MSH and setmelanotide decreased by about two orders of magnitude in the presence of physiological Ca²⁺ concentrations (1 mM) in our cellular assays (Fig. 4, A and B; and fig. S6). Indeed, an estimation of a K_B for Ca²⁺ (0.06 μ M, fig. S6M) supports Ca²⁺ binding at physiologic levels to MC4R. Notably, other divalent ions did not have similar effects, nor did we detect allosteric effects on Ca²⁺ binding caused by the presence of other ions (fig. S6, A to J). These results are in accordance with several previous reports that α -MSH as well as synthetic agonists depend on the presence of Ca²⁺ for signaling, with no significant change

in response to other ions (16, 34). We also found that the non-peptide synthetic MC4R agonist THIQ, depends on Ca^{2+} for enhanced activity and binding (fig. S7).

In contrast, we found that physiological Ca^{2+} concentrations do not contribute to inverse-agonist activity of SHU9119, nor to AgRP-induced antagonistic activity. Signaling assays revealed that physiological Ca^{2+} concentrations do not significantly impact the ability of SHU9119 to compete with α -MSH, and that AgRP activity is in fact hampered by the presence of Ca^{2+} (Fig. 4, C and D). This suggests that Ca^{2+} plays a role in modulating receptor susceptibility to endogenous agonists and antagonists.

Notably, one of the residues that was found to coordinate Ca^{2+} positioning within the binding pocket and modulate ligand accommodation in the receptor, D122^{3,25}, corresponds to a conserved cysteine residue in other class-A members, which composes a disulfide bridge missing in MCRs (fig. S8, A to C). These data highlight the possibility that Ca^{2+} serves as a surrogate to the function of the bridge and contributes to the structural integrity of the canonical binding pocket. Given the opposite effect of Ca^{2+} on the endogenous agonist and antagonist, we suggest that Ca^{2+} can provide MC4R sufficient flexibility to modulate the binding pocket to differentially accommodate agonists and antagonists.

Mutation of residues involved in Ca^{2+} coordination reduces receptor activation

Mutational studies were conducted to further explore the function of Ca^{2+} coordination in receptor activity and to assess the implications for human disease. Two of the Ca^{2+} coordinating residues, D122N and D126Y, are naturally occurring mutations associated with obesity (18, 35). An E100A mutation was introduced to probe the function of the third Ca^{2+} coordinating side chain. The D122N mutant displayed a two-log increase in EC_{50} and ~50% decrease in efficacy for α -MSH and setmelanotide under physiological Ca^{2+} concentrations. For the E100A mutant, the impairment was not the same for the natural and artificial agonists. While α -MSH signaling was almost completely abolished by the E100A mutation, setmelanotide was still able to signal, but at half maximum and with a two-log increase in EC_{50} (Fig. 4, E and F). The D126Y mutant resulted in the complete abolishment of receptor function. The more pronounced effect of the D126 substitution can be explained by the fact that D122 and E100 each contribute a single bond to Ca^{2+} coordination, whereas D126 contributes two (Fig. 3C). Both the D122N and E100A mutants remained dependent on Ca^{2+} for signaling for both setmelanotide and α -MSH, suggesting that altering the aspartates at these positions did not eliminate Ca^{2+} coordination (fig. S8, E and F). Flow cytometry analysis indicated similar profiles of surface expression levels for wild-type and mutant receptors, indicating that the observed activity

changes were not due to poor cell surface availability (fig. S8D). These results are in agreement with previously reported work in which mutations at positions 122 and 126 in MC4R resulted in impaired ligand binding properties (18, 35–38). The differential activation profile of the D122N variant by setmelanotide compared with α -MSH, highlights setmelanotide as a potential therapeutic path for individuals harboring a D122N mutation. Discussion of common mutations in MC4R is further provided in fig. S9.

Identification of a mechanical activation switch in MC4R

Comparing the inactive and active states of MC4R, the outward displacement of TM6 is clearly evident (Fig. 5A and fig. S3C). This structural rearrangement is a hallmark of GPCR activation, allowing the C-terminal $\alpha 5$ helix of the $\text{G}\alpha_s$ subunit to engage the receptor core and initiate signaling. In MC4R, binding of the agonist setmelanotide partly resembles the binding of the antagonist SHU9119 (16) (Figs. 2 and 3), raising the question of how the structural change and GPCR activation are induced selectively by the agonist. A noticeable difference between the binding modes of the antagonist and the agonist is that the D-Nal₄ group of SHU9119, which is bulkier than the D-Phe₅ in setmelanotide, penetrates the binding pocket more deeply and interacts with L133^{3,36} (Figs. 2, B and C, and 5B). L133^{3,36} is a key modulator in class-A GPCR activation, as it directly interacts with W258^{6,48}, which plays a crucial role in TM6 displacement during receptor transition to the active state (31). In the inactive state, L133^{3,36} engages the aromatic face of W258^{6,48}, with the two methyl groups facing the π -electrons and maintaining CH- π contacts (Fig. 5B). In the active conformation, L133^{3,36} is oriented, with the two methyl groups facing away from W258^{6,48}. As a result, W258^{6,48} is free to accommodate an alternate state that allows for the conformational rearrangement of TM6 required for G-protein coupling (Fig. 5, A and B). This conformational change resembles the classic toggle switch in other GPCRs, where in the inactive state F^{3,36} stacks upon W^{6,48} to maintain the inactive receptor, whereas in the active conformation F^{3,36} rotates and allows the release of W^{6,48} [e.g., CB1R (26, 39), CB2R (40), and CCR5 (41), Fig. 5C]. Comparison between the agonist- and antagonist-bound structures suggests that while SHU9119 sterically hinders the displacement of L133^{3,36} toward the active state, the phenyl residue in setmelanotide stabilizes the active state conformation (Fig. 5B).

A challenge in targeting MC4R is to avoid undesired side effects due to cross reactivity with other members of the MCR family (e.g., MC3R and MC1R). Thus, identifying distinct features in the activation mechanism of MC4R is important. MC1R is a close homolog of MC4R, and cross-reactivity with MC1R is often observed when agonists and antagonists designed for MC4R are used clinically (18, 22). Further evidence

can be seen in patients treated with afamelanotide, an MC1R agonist used for the treatment of erythropoietic protoporphyria, these patients often suffer from decreased appetite due to cross reactivity with MC4R (42). A puzzling observation is that MC4R antagonists such as SHU9119 act as agonists for MC1R (43). Residues in the orthosteric binding pocket of MC4R and MC1R are almost identical, but L133^{3,36} of the MC4R activation switch is replaced by a methionine, M128^{3,36}, in MC1R (fig. S4). We introduced an L133M mutation into MC4R and confirmed that SHU9119 acts as an agonist for this mutant (fig. S8, G to I). In contrast, we found that AgRP did not activate the mutant receptor, suggesting differences in the binding mode for the endogenous antagonist compared with the synthetic derivative (fig. S8G). Comparing the MC4R structure to structures harboring a 3.36 methionine (e.g., the μ -opioid receptor - MOR1), shows that the rotameric state of L133^{3,36} in an inactive MC4R overlaps with the active state of M151^{3,36} in MOR1 (fig. S8J). Furthermore, superposition of the structure of MC4R bound to SHU9119 with an inactive structure of MOR1 indicates a clash between the naphthyl group in SHU9119 and M151^{3,36} (fig. S8K). These observations suggest why SHU9119 contributes to agonistic activity in the mutant. These experiments also demonstrate the potential for a selectivity window to reduce the cross reactivity with MC1R that contains a methionine at position 3.36. A full analysis of conserved and synonymous residues in the binding pocket of MCR family members is provided in fig. S4.

Exploring MC4R canonical and non-canonical signaling pathways

The conventional G_{α_s} signaling of MC4R is crucial in inducing anorexogenic signaling in the hypothalamus to result in negative energy balance (44–46). However, MC4R can activate other signaling mediators to trigger downstream signaling effects. These include coupling to G_{α_i} , G_{α_q} , and $G_{\alpha_{15}}$, interaction with β -arrestins (47, 48), and through G-protein independent coupling to the inwardly rectifying potassium channel Kir7.1 (49). Our work presents the structural mechanistic properties of MC4R activation by setmelanotide and captures the MC4R- G_s bound state that allows for the delineation of the coupling interface. The MC4R- G_{α_s} interface is mostly composed of interactions between residues in TM3, TM5 and TM6 at the receptor and the C-terminal helix ($\alpha 5$ helix) of G_{α_s} (Fig. 6A and fig. S10). These interactions also include a class-A conserved π -cation stacking between Y370^{H5,23} in G_s , and R147^{3,50} in MC4R, where R^{3,50} composes part of the DRY motif that plays an important role in G-protein coupling (50–54). All residues in MC4R that are involved in G_s interactions are strictly conserved among all MCR family members, reflecting their shared coupling to G_s (fig. S4A) (55). In addition, MC4R mutations in residues involved in G_s recruitment have been associated with impaired signaling

through the G_s pathway and have also been identified in obese individuals (56, 57). Notable examples are A219V and T150I, which demonstrate impaired cAMP production without change in binding affinity to α -MSH compared with the wild-type receptor (9, 35, 58–61). A219^{5,65} is close to the tip of G_{α_s} helix $\alpha 5$. Substitution of Ala with a bulkier Val might create a clash that would disturb MC4R- G_{α_s} binding. Residues that interact with A219^{5,65} (L367^{H5,20}, L372^{H5,25}, and L373^{H5,26}) are also conserved in G_q and G_{15} , both reported to bind MC4R (47, 62), thus mutations at this position, might also interfere with signaling through alternative pathways for this receptor. T150^{3,53} is located at the tip of TM3 and contributes two hydrogen bonds to the binding interface through direct interactions with H366 and Y370. Substitution of Thr by hydrophobic Ile would abolish one of the hydrogen bonds, thus impairing recruitment of the G_{α} subunit.

A recent genetic variant study showed that mutations in MC4R observed in patients with low BMI were associated with MC4R gain-of-function through biased β -arrestin 2 recruitment to the receptor. These studies highlight the importance of targeting preferred pathways by drugs to treat obesity (9). Analysis of mutations associated with gain-of-function or loss of function of MC4R is provided in fig. S9. Comparing setmelanotide and α -MSH ability to recruit G_{α_s} or β -arrestin 2, we saw no bias toward these two pathways (Fig. 6, B and C). Thus our studies suggest that for the wild-type receptor, both setmelanotide and α -MSH will act similarly to activate these pathways.

In summary, this work provides a major leap in our understanding of the molecular basis of MC4R activation and inhibition, ligand specificity, and the contribution of genetic variations to MC4R-related pathologies. Looking forward, this work will set the stage for future efforts to develop novel therapeutic strategies to fight obesity and possibly other weight-regulation disorders.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Figs. S1 to S10
Tables S1 to S5
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MDAR Reproducibility Checklist

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Fig. 1. An overall description of MC4R architecture. (A) Cryo-EM map of the MC4R-G $_s$ signaling complex embedded within an LMNG:GDN micelle. MC4R is in blue, Setmelanotide (SET) in pink and G α_s , β and γ are in yellow, magenta and green, respectively. ScFv16 is in light purple. Bottom map is rotated 90° along the X axis. Map contour level is 0.027. (B) Cartoon model corresponding to map positions presented in (A). (C) A view of MC4R cryo-EM map with setmelanotide in the orthosteric binding pocket. Setmelanotide is in pink, density for Ca $^{2+}$ is presented in light green. Map contour level is 0.027. (D) Setmelanotide in density. Map contour level is 0.02.

Fig. 2. Agonists and antagonists for MC4R. (A) Chemical structures of MC4R agonists α -MSH and setmelanotide (SET) and the antagonist SHU9119. The conserved HFRW motif is in orange. Phenylalanine substitutions in SET (D-Phe₅) and in SHU9119 (D-Nal₄) are highlighted in yellow. (B and C) A view of setmelanotide (B) and SHU9119 (PDB 6W25) (C) at the MC4R orthosteric binding pocket. HFRW motif is in orange with D-Phe₅ (SET) and D-Nal₄ (SHU9119) highlighted in yellow. Active receptor is in blue (B) and inactive in grey (C). Comparing the active and inactive receptor states, the D-Nal₄ moiety of the antagonist SHU9119 penetrates more deeply into the pocket. (D) Dose response curves for the agonists α -MSH (purple) and setmelanotide (pink) measured as cAMP production normalized by % basal activity of the wild-type receptor. Data represented as mean $\pm$ SEM from 3 independent experiments done in triplicate, indicating EC₅₀ values of 3.79 ± 0.87 nM and 0.29 ± 0.04 nM for α -MSH and setmelanotide, respectively. (E) Competition assays of inverse agonists AgRP (blue) and SHU9119 (grey) with α -MSH, calculated as the decrease in cAMP production in the presence of increasing dose of the antagonists. Experiments were conducted in the presence of $1 \mu\text{M}$ α -MSH. Data represented as mean $\pm$ SEM from 3 independent experiments done in triplicate. pIC₅₀ values are 6.67 ± 0.18 and 8.51 ± 0.05 nM for AgRP and SHU9119, respectively.

Fig. 3. Ligand accommodation within the MC4R binding pocket. (A and B) Setmelanotide interactions in the MC4R binding pocket. The receptor is presented in light blue, setmelanotide (SET) in pink, and Ca^{2+} in green. Dashed lines are colored according to the type of interactions, with hydrogen bonds in red, hydrophobic interactions in grey, CH—O in blue, and Ca^{2+} coordination in purple. π - π stacking is indicated by an asterisk. Interactions with HFRW motif are in (A) and with non-conserved residues in (B). (C) A comparison of the Ca^{2+} coordination in the presence of setmelanotide (agonist, pink, left) and SHU9119 (antagonist, grey, right PDB 6W25). Superposition is of the agonist and antagonist is in the middle. The receptor is presented as a cartoon with active conformation in light blue and inactive in grey. Ca^{2+} ion is in green for the active and grey for the inactive structure. (D) Snapshots of the molecular dynamics equilibration steps after 3.7, 4.1 and 9.3 ns (molecular dynamics run with setmelanotide, replica 1) showing the Ca^{2+} ion (green sphere) entering the ion binding pocket (yellow licorice side chains, showing residues D122^{3.25}, D126^{3.29} and E100^{2.60}) of the MC4R receptor (light blue cartoon). Setmelanotide shown in pink, the membrane lipids in cream. In the starting configuration of all the simulations, Ca^{2+} was positioned in the intra and extra cellular water layers, leaving the Ca^{2+} binding site free. Out of a total cumulative simulation time of 2 μs , Ca^{2+} was found in the MC4R-bound position for $\sim 1.8 \mu\text{s}$ and, once binding was established, the ion remained inside the binding pocket throughout the simulations performed (fig. S5, C and D).

Fig. 4. Ca^{2+} acts as a cofactor for MC4R activation. (A and B) Dose response curves for the agonists α -MSH (A) and setmelanotide (B) in the absence and presence of 1 mM Ca^{2+} . The absence of Ca^{2+} is indicated with a dashed line. Data represented as mean $\pm$ SEM from 3 independent experiments done in triplicate. (C and D) Competition assays of antagonists AgRP (C) and SHU9119 (D) with α -MSH in the absence and presence of 1 mM Ca^{2+} . Assays are calculated as the decrease in cAMP production in the presence of increasing dose of the antagonists. Experiments were conducted in the presence of 1 μM α -MSH. Data represented as mean $\pm$ SEM from 3 independent experiments done in triplicate. (E and F) Mutations in residues involving Ca^{2+} coordination significantly reduce receptor's activation through α -MSH (E) and setmelanotide (F). Dose response curves for the agonists indicate elevation in cAMP production and are represented as mean $\pm$ SEM from 3 independent experiments done in triplicate. Cell cytometry analysis indicating surface expression of wild-type and mutants is in fig. S8D. Mutant dependence on Ca^{2+} is in fig. S8, E and F.

Fig. 5. MC4R activation switch. (A) Superposition of the MC4R active complex (blue) with an antagonist bound receptor (SHU9119, PDB 6W25, grey). Setmelanotide (SET, blue) and SHU9119 (grey) bound at the MC4R canonical pocket differentially interact with L133^{3.36} that composes the switch that controls receptor conformation. The outward movement of TM6 is indicated by black dashed lines, $G\alpha_s$ is in yellow. (B) The bulky D-Nal₄ moiety in SHU9119 stabilizes the inactive switch conformation (grey), by which L133^{3.36} is stacked upon W258^{6.48}. The D-Phe₅ moiety in setmelanotide contributes to an alternative rotamer, releasing W258^{6.48} which enables the outward movement of TM6 to allow the accommodation of $G\alpha_s$ and receptor activation (blue). The arrow indicates switch alternation from the inactive to the active state. (C) Superposition of MC4R activation switch (active - blue, inactive - light cyan) with a classical 'toggle twin switch' of CB1R (PDB 6N4B and 5TGZ, for active - orange, and inactive - light yellow conformations, respectively). The comparison between the two switches indicates a similar conformation of the two switches despite the difference in the identity of the switch residues. In MC4R the switch is composed of L133^{3.36} and W258^{6.48}, whereas the classical switch in CB1R is composed of F200^{3.36} and W356^{6.48}.

Fig. 6. MC4R G-protein coupling and biased signaling. (A) Analysis of residues in MC4R involved in G_s recruitment. Residues in MC4R which are discussed in the main text are highlighted with an asterisk. A full description of interaction is provided in fig. S10. (B) Mini-G_s recruitment upon setmelanotide and α-MSH binding. Recruitment was monitored by nano-luciferase complementation using an MC4R-LgBit and a mini-G_s smBit. (C) β-arrestin 2 recruitment to MC4R. β-arrestin 2 was monitored by nano-luciferase complementation using an MC4R-LgBit and β-arrestin 2-smBit in HEK293 cells and plotted as a change in luciferase over time. For both (B) and (C), Y-axis is presented as a percent of basal as plotted in dose response curves for the agonists indicated and are represented as mean ± SEM from 3 independent experiments done in triplicate. Analysis in the absence and presence of Ca²⁺ is provided in fig. S6, K and L.