G-protein-independent coupling of MC4R to Kir7.1 in hypothalamic neurons

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The regulated release of anorexigenic α-melanocyte stimulating hormone (α-MSH) and orexigenic Agouti-related protein (AgRP) from discrete hypothalamic arcuate neurons onto common target sites in the central nervous system has a fundamental role in the regulation of energy homeostasis. Both peptides bind with high affinity to the melanocortin-4 receptor (MC4R); existing data show that α-MSH is an agonist that couples the receptor to the Gs, signalling pathway, while AgRP binds competitively to block α-MSH binding and blocks the constitutive activity mediated by the ligand-mimetic amino-terminal domain of the receptor. Here we show that, in mice, regulation of firing activity of neurons from the paraventricular nucleus of the hypothalamus (PVN) by α-MSH and AgRP can be mediated independently of Gs, signalling by ligand-induced coupling of MC4R to closure of inwardly rectifying potassium channel, Kir7.1. Furthermore, AgRP is a biased agonist that hyperpolarizes neurons by binding to MC4R and opening Kir7.1, independently of its inhibition of α-MSH binding. Consequently, Kir7.1 signalling appears to be central to melanocortin-mediated regulation of energy homeostasis within the PVN. Coupling of MC4R to Kir7.1 may explain unusual aspects of the control of energy homeostasis by melanocortin signalling, including the gene dosage effect of MC4R and the sustained effects of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing.

To better understand the diametrically opposed regulation of food intake by α-MSH and AgRP, we sought to identify mechanism(s) by which these peptides control firing activity of MC4R neurons in the paraventricular nucleus of the hypothalamus (PVN), a brain nucleus in which MC4R is known to control food intake. Using electrophysiology with murine hypothalamic slice preparations in which MC4R PVN neurons are labelled with green fluorescent protein (GFP), α-MSH increases the frequency of action potential firing in PVN MC4R neurons recorded from loose patches (Fig. 1a), depolarizing these cells on average by ~8 mV through action on postsynaptic MC4R (Fig. 1b). α-MSH had no effect on neighbouring non-GFP-labelled neurons (Fig. 1c). AgRP hyperpolarized PVN MC4R neurons (Fig. 1d), inhibiting their firing activity.

We next examined if α-MSH depolarized neurons through activation of the Gs,–adenyl cyclase-cAMP-PKA pathway. PKAi (20 μM intrapipette), a peptide inhibitor of PKA, failed to abolish the α-MSH-induced increase in firing frequency in PVN neurons during whole cell recording, (not shown) or to block α-MSH-induced depolarization of membrane potential (Fig. 1e). Inhibition of adenyl cyclase with SQ22536 (25 μM) similarly failed to block α-MSH-induced depolarization of membrane potential (Fig. 1f). Finally, we examined whether blocking G protein signalling can inhibit MC4R-mediated depolarization by loading cells with the inhibitory GDP analogue, GDPβS. To verify that GDPβS blocked G-protein function, we examined effects of activation of D1 dopamine receptor, known to depolarize neurons via activation of Gs, Activation of D1 dopamine receptor by the D1 agonist SKF83822 (5 μM) depolarized PVN neurons (Fig. 1g). GDPβS (5 mM) blocked D1-mediated depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing depolarization, but failed to block α-MSH-induced depolarization of PVN MC4R neurons (Fig. 1g). Several other inhibitors of components of G protein signalling were also ineffective in blocking neuronal firing.
or depolarization of PVN MC4R neurons by γ-MSH (Extended Data Fig. 1a–d), including GTP;S (a non-hydrolysable GTP analogue, 1.5 mM), gallein (a G protein blocker, 25 μM), or U0126 (a MAPK inhibitor, 1 μM). Together, these findings support a novel hypothesis: a G-protein-independent pathway for MC4R-mediated depolarization of PVN neurons.

The currents underlying MC4R-mediated depolarization of PVN neurons were then characterized with current–voltage (I–V) analysis in external 20 mM K+ using whole-cell recording from voltage clamped neurons pre-treated with 0.5 mM tetrodotoxin (TTX), 200 μM picrotoxin (PTX) and 1 mM kynurenic acid (KYN), current responses of PVN MC4R neurons to voltage ramps (−120 to −20 mV for 2 s) were used to generate the I–V relationships of the γ-MSH-induced response (Fig. 2a–c). γ-MSH significantly (33%) decreased cell membrane conductance from 5.7 ± 1.0 nS in control to 3.8 ± 0.8 nS (Fig. 2g), with current generated by γ-MSH being linear from −120 to −60 mV that rectified inwardly at membrane potentials negative to its reversal of polarity. Its reversal potential was near −48.1 ± 3.4 mV in the presence of 20 mM external K+, with an estimated reversal potential of K+ near −53.5 mV (Nernst equation). These results, in addition to those performed in 3.1 mM external [K+] (estimated ErevK+ = −98 mV, not shown), suggest that γ-MSH generates an inward current by closure of a steady-state K+–mediated inward rectifier current, as in hypothalamic arcuate (ARC) neurons.

We next tested the hypothesis that AgRP hyperpolarizes cells by activating K+–mediated currents. Current responses of PVN neurons to voltage ramps (−110 to −50 mV for 2 s) were used to generate I–V relationships of the AgRP-induced response in 20 mM external K+ (Fig. 2d–f). Application of AgRP (50 nM) significantly increased membrane conductance (75%, Fig. 2g), and the AgRP-activated current displayed a reversal of polarity (−46.7 ± 3.0 mV) and rectification properties similar to γ-MSH, suggesting that AgRP increases the density of an inward rectifying K+ current.

A hallmark of Kir channel–mediated current is the relative blockade of K+ outward current by intracellular polyamines and Mg2+ at membrane potentials positive to the ErevK+ (ref. 9). To further characterize the MC4R-regulated current, we examined whether the amplitude of K+ outward current affected by γ-MSH is greater in Mg2+–depleted intracellular solution. Recordings from PVN neurons voltage clamped around −55 mV indicated that the amplitude of the γ-MSH regulated current was approximately ten times greater when intracellular Mg2+ was depleted compared to control (Fig. 2h–j). Another characteristic of Kir channels is sensitivity to phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2)10. While this response is attenuated in zero ATP internal solution in which phospholipid synthesis is blocked (Fig. 2k), addition of 100 μM PtdIns (4,5)P2 diC8 (1,2-dioctanoyl) to the internal solution potentiated the γ-MSH-induced response (Fig. 2k).

To identify the subtype(s) of Kir channels involved in the γ-MSH–induced depolarization, we used a panel of Kir channel blockers (Fig. 2l and Supplementary Table 1) with previously characterized channel subtype specificity11. Additionally, we used the bee venom tertiapin Q, a blocker of Kir3.3 and Kir3.1 and glibenclamide, a blocker of Kir6.2/SURx. The depolarizing effect of γ-MSH was inhibited ∼80% by 15 μM VU573, a selective blocker of Kir2.3, Kir3.3 and Kir7.1, and blocked by the Kir7.1 blocker VU590, but not VUR5C, an inactive VU573 analogue, tertiapin Q, glibenclamide, or VU591. These data suggest that Kir7.1 generates the current underlying the γ-MSH–induced depolarization in mouse PVN neurons. Kir7.1 is reported to be resistant to external BaCl2 or CsCl, at concentrations that block other Kir channels12, and the γ-MSH–induced depolarization persisted in the presence of BaCl2 or CsCl. The increased amplitude of this response in higher concentrations of BaCl2 (>1 mM) and CsCl (>2.5 mM), may result from non-selective blockade of other voltage-sensitive outward currents (Fig. 2m–o). Additionally, Kir7.1 is more permeable to rubidium than other Kir(s)13, and when potassium gluconate in the patch pipette was replaced with RbCl (RbCl 130 mM and KCl 4 mM), γ-MSH–induced, Rb–mediated depolarization was observed and its magnitude was significantly greater than physiological K+–mediated depolarization (Extended Data Fig. 2). Using dual fluorescence

Figure 2  | γ-MSH depolarizes and AgRP hyperpolarizes hypothalamic PVN MC4R neurons by regulating Kir7.1. a–c, g. Current–voltage (I–V) analysis of MC4R PVN neurons indicates that 250 nM γ-MSH generates depolarizing current by closure of inward rectifying K+ channels. d–g, I–V analysis of PVN MC4R neurons indicates that 50 nM AgRP generates hypopolarizing current by opening of inward rectifying K+ channels. Current responses of voltage clamped PVN neurons to voltage ramps (a, d) were used to generate the I–V relationship of the γ-MSH– and AgRP–induced response in the presence of 20 mM external K+ . The resultant I–V relationships of the γ-MSH– and AgRP–induced responses were significantly different (unpaired t-test). h, j, k, m, o, n, α-MSH-induced depolarization of γ-MSH-stimulated MC4R neurons (n = 25), exhibiting a greater amplitude in the presence of γ-MSH; asterisk indicates a significant increase from control (unpaired t-test). Depolarizing current by closure of inward rectifying K+ channels, generating K+–mediated current, is significantly enhanced by γ-MSH (95% of control, p < 0.001, unpaired t-test). The resultant I–V relationships of the γ-MSH– and AgRP–induced responses were significantly different (unpaired t-test).
in situ hybridization in sections of mouse PVN, we also determined that approximately 90% of PVN neurons expressing MC4R messenger RNA co-expressed Kir7.1 mRNA (Extended Data Figs 3 and 4).

To confirm that MC4R signalling is capable of modulating Kir7.1 function, we transfected MC4R and Kir7.1 channels into HEK293 cells, using an M125R variant of Kir7.1 that exhibits higher unitary conductance than the native channel, previously demonstrated to allow detection of the channel in cell lines\(^5\). Whole-cell recordings were performed 24–48 h after co-transfecting cells with MC4R, Kir7.1(M125R) and GFP expression plasmids to examine effects of \(\alpha\)-MSH on membrane current by examining the I–V analysis of \(\alpha\)-MSH response. \(\alpha\)-MSH significantly decreased the amplitude (>50%) and slope of current responses, indicating closure of Kir7.1 channels (Fig. 3a–c). This effect was reversible, and the current amplitude was reduced to less than 25% of its own control by 2 mM Ba\(^{2+}\), consistent with the increased sensitivity of the Kir7.1(M125R) variant to Ba\(^{2+}\) (ref. 14). Use of HEK293 cells allowed for a more direct assessment of kinetics of the \(\alpha\)-MSH-induced current, indicating a rapid activation time course with an average \(\tau_{\text{rise}}\) of 32 s. Co-transfection of tagged MC4R and Kir7.1 (also known as KCNJ13) genes, followed by immunoprecipitation and western blot analysis showed a quantitative association of the two proteins in this system (Extended Data Fig. 5).

By loading HEK293 cells with a Ti\(^{2+}\)-sensitive fluorescent dye, Thallos, the flux of Ti\(^{2+}\) ions through open K\(^{+}\) channels can be measured\(^6\). The effect of receptor modulation on channel conductance can then be studied by subtracting flux intensity after experimental treatments from levels after vehicle. Using this system, dose–response analysis indicated that \(\alpha\)-MSH mediates a MC4R-dependent closure of Kir7.1 channels with an half-maximal inhibitory concentration (IC\(_{50}\)) of 10\(^{-7.2}\) M (Fig. 3d, e). Conversely, AgRP mediated a MC4R-dependent increase in Ti\(^{2+}\) flux through Kir7.1, with an EC\(_{50}\) of 10\(^{-8.6}\) M (Fig. 3f, g). AgRP did not appear to couple the MC4R to the cAMP inhibitory G protein, G\(_{\text{a}}\)\(^{16}\), or to expressing Kir4.1, and is not in cells expressing Kir2.1 or Kir2.3. j, VUS73 (10 \(\mu\)M) blocks Ti\(^{2+}\) flux in MC4R+Kir7.1 transfected cells. k, VUS73 does not block MC4R–Kir4.1-mediated Ti\(^{2+}\) flux in the transfected cell assay. l, m, Pre-incubation with 100 nM \(\alpha\)-MSH of MC4R-glo expressing HEK293 cells directly depresses a cAMP test response to a second exposure to 100 nM \(\alpha\)-MSH (l). RLUs, relative light units. Pre-incubation MC4R-Kir7.1 HEK293 cells with 100 nM \(\alpha\)-MSH directly increases the amplitude of the response of Kir7.1 to a second 100 nM \(\alpha\)-MSH exposure (m). n, Kinetics of the normalized maximal cAMP (black) and Kir7.1 (red) response to a single dose of 100 nM \(\alpha\)-MSH, calculated from l and m. In all Ti\(^{2+}\) flux and cAMP accumulation assays, coloured traces indicate mean and bars (black) indicate s.e.m.

d–n, Panels show combined data from three or four (d and e only) independent experiments; points are means from the following number of wells. d, e, n = 111; f, g, n = 96; h, i, n = 40–48; j, k, n = 27–28; l, n = 12; m, n = 48.

**Figure 3** \(\alpha\)-MSH and AgRP couple MC4R to Kir7.1 in HEK293 cells. a–c, Current responses of transfected HEK293 cells to voltage steps (a) and ramps (b) in control, 300 nM \(\alpha\)-MSH, and washout. Bath application of \(\alpha\)-MSH significantly reduced the amplitude (c) and slope of responses to voltage steps and ramps in a reversible manner (bar graph indicates mean ± s.e.m.; \(*P < 0.05, \**P < 0.01, \***P < 0.001, n = 6, paired t-test). d–g, \(\alpha\)-MSH reduces flux (IC\(_{50}\) near 10\(^{-7.5}\) M, d and e) and AgRP increases flux (EC\(_{50}\) near 10\(^{-8.6}\) M, h and i) through Kir7.1 channels in a concentration–dependent manner. Concentration–response curves in e and g are plotted using the maxima from data in d and f. h–k, Specificity of melanocortin receptor–Kir7.1 coupling. h, \(\alpha\)-MSH–induced decrease in Ti\(^{2+}\) flux in MC4R and MC1R expressing cells containing Kir7.1, but not in cells co-expressing Kir7.1 and the MC3R, or expressing Kir7.1 alone. i, \(\alpha\)-MSH–induced decrease in Ti\(^{2+}\) flux via the MC4R is observed in cells transfected with Kir7.1, more weakly in cells expressing Kir4.1, and is not in cells expressing Kir2.1 or Kir2.3. j, VUS73 (10 \(\mu\)M) blocks Ti\(^{2+}\) flux in MC4R+Kir7.1 transfected cells. k, VUS73 does not block MC4R–Kir4.1-mediated Ti\(^{2+}\) flux in the transfected cell assay. l, m, Pre-incubation with 100 nM \(\alpha\)-MSH of MC4R-glo expressing HEK293 cells directly depresses a cAMP test response to a second exposure to 100 nM \(\alpha\)-MSH (l). RLUs, relative light units. Pre-incubation MC4R-Kir7.1 HEK293 cells with 100 nM \(\alpha\)-MSH directly increases the amplitude of the response of Kir7.1 to a second 100 nM \(\alpha\)-MSH exposure (m). n, Kinetics of the normalized maximal cAMP (black) and Kir7.1 (red) response to a single dose of 100 nM \(\alpha\)-MSH, calculated from l and m. In all Ti\(^{2+}\) flux and cAMP accumulation assays, coloured traces indicate mean and bars (black) indicate s.e.m. d–n, Panels show combined data from three or four (d and e only) independent experiments; points are means from the following number of wells. d, e, n = 111; f, g, n = 96; h, i, n = 40–48; j, k, n = 27–28; l, n = 12; m, n = 48.
recruit β-arrestin to the receptor (Extended Data Fig. 6a–c). These data support a G-protein-independent mechanism for the MC4R-mediated regulation of Kir7.1 by AgRP as well. To examine the selectivity of coupling between melanocortin receptors and Kir7.1, we created stable HEK293 cells expressing Kir7.1 (M125R) alone, or Kir7.1 (M125R) plus MC1R, MC3R, or MC4R. α-MSH (100 nM) significantly decreased Tl⁺ flux in Kir7.1 HEK293 cells expressing MC1R or MC4R, but not MC3R (Fig. 3b). α-MSH (100 nM) significantly decreased Tl⁺ flux in HEK293 cells transfected with MC4R plus Kir7.1 or Kir4.1, but not cells expressing Kir2.1 or Kir2.3 (Fig. 3i). The Kir7.1-specific channel blocker VU573 blocked the α-MSH effects on Kir7.1 function in the HEK293 cells (Fig. 3j), as in the slice (Fig. 2l). However, VU573 had no effect on α-MSH inhibition of Kir4.1 in HEK293 cells (Fig. 3k), suggesting the conductance regulated by α-MSH in MC4R PVN neurons primarily involves Kir7.1. PKA activity was also noted for α-MSH-induced closure of Kir7.1 in HEK293 cells (Extended Data Fig. 7a, b), and we also observed a synergistic effect of α-MSH and cAMP on Kir7.1 closure, in this system (Extended Data Fig. 7a–d).

G-protein-coupled signalling of the MC4R exhibits β-arrestin-mediated desensitization. Pre-treatment of MC4R + Kir7.1 expressing HEK293 cells with α-MSH reduced the subsequent magnitude of the G-protein-mediated cAMP response of these cells to α-MSH (Fig. 3i). Supporting the argument that coupling of the MC4R to Kir7.1 is non-G-protein-mediated, the MC4R-dependent inhibition of Tl⁺ flux through Kir7.1 was hypersensitized by prior treatment with α-MSH (Fig. 3m). Thus, while the time course of acute effects of MC4R activation on Kir7.1 was rapid (Fig. 3a), the long term effects on the cAMP and Kir7.1 signalling pathways were highly divergent (Fig. 3n). While cAMP levels peak between 1–2 min after α-MSH treatment, inhibition of Tl⁺ flux by α-MSH treatment continues to increase up to 45 min post-treatment. Low-dose AgRP treatment (1 nM) increased Tl⁺ flux for up to 24 h (data not shown); the apparent reduction in Tl⁺ flux after high-dose AgRP treatment (for example, Fig. 3i) is probably owing to saturation of the fluorescent dye binding substrate.

Additional data support a role for Kir7.1 in regulation of food intake by MC4R in vivo. The AgRP analogue miniAgRP (AgRP87–120, C105A) retains normal affinity for MC4R, and potency in inhibition of MC4R coupling to Gαs (Fig. 4a), yet exhibits 70% reduction in its ability to stimulate food intake in rats. In parallel with loss of orexigenic activity, this peptide has lost its ability to couple MC4R to Kir7.1 (Fig. 4b). Conversely, we have also identified an α-MSH analogue, MC4-NN2-0453 (Novo), that preferentially couples MC4R to Kir7.1 over Gαs in cell culture, and potently depolarizes PVN MC4R neurons (Fig. 4c–f). MC4-NN2-0453 also exhibited biased actions on MC4R in vivo. In a cAMP-mediated response, the peptide was unable to induce intestinal peptide YY (PYY) release in vivo (Fig. 4g), and is a partial agonist ex vivo (Extended Data Fig. 8). In contrast, the peptide potently inhibited food intake at doses equimolar to other α-MSH analogues (Fig. 4h). Knockdown of Kir7.1 gene expression in wild-type and MC4R mutant larval zebrafish, but not Kir7.1 mutant jaguar zebrafish, produced a reduction in linear growth and upregulation of GHRH gene expression, responses reported previously for activation of the MC4R (Extended Data Fig. 9a–e), further supporting the argument that Kir7.1 acts downstream of MC4R.

These data show the MC4R can depolarize or hyperpolarize hypothalamic PVN neurons in response to α-MSH or AgRP, respectively, through a novel G protein independent signalling pathway involving regulation of the activity of Kir7.1. While MC4R also is likely to couple to Gαs, in most cells, cAMP/PKA-dependent activation of Kᵦᵥ7.2 channels, producing α-MSH-induced hyperpolarization, has been demonstrated in MC4R neurons in the brainstem. Thus, while Kir7.1 signalling appears to be essential for depolarization of PVN MC4R neurons by α-MSH, Gαs signalling and elevation of cAMP may be depolarizing or hyperpolarizing, depending on the cellular context (Extended Data Fig. 10).

Direct interactions between GPCRs and K⁺ channels, such as binding and regulation of Kv4.3 by the type 1 angiotensin receptor, or of Kir4.1 and Kir4.2 by the Ca²⁺-sensing receptor have been previously reported. Kir7.1 is a widely expressed inwardly rectifying potassium channel, suggesting the possibility of regulation by other GPCRs. Mutations in Kir7.1 cause pigmentary defects in the jaguar zebrafish. Interestingly, we also find that the MC1R couples to Kir7.1, suggesting that the induction of phoemelin (yellow-red pigments) synthesis in mammals, may involve MC1R–Kir7.1 regulation by agouti.

The discovery of independent agonist activity for AgRP through opening of Kir7.1 also necessitates revision (Extended Data Fig. 10) of the current neuroanatomical model of hypothalamic melanocortin signalling, in which AgRP acts primarily by antagonism of α-MSH. Preliminary data from a limited electron micrographic reconstruction of the PVN, showing dendrites specifically targeted by POMC synapses and cell soma specifically targeted by AgRP synapses, suggests a potential neuroanatomical basis as well for independent action of AgRP and α-MSH. The non-G protein signalling mechanism reported here may
also provide a basis for understanding the persistent action of AgRP on food intake, and the gene dosage effect of MC4R mutations, not typically seen for GPCRs. Furthermore, the pronounced bias for coupling the MC4R to Kir7.1 of α-MSH analogue MC4-N22-0453, shown to potently inhibit food intake in the absence of a presor response, suggests that biased agonists of the MC4R may exhibit useful therapeutic properties.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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1. Mountjoy, K. G., Mortrud, M. T., Low, M. J., Simerly, R. B. & Cone, R. D. Localization of
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2. Ollmann, M. M. et al. Antagonism of central melanocortin receptor in vitro and in vivo by agouti-related protein. Science 278, 135–138 (1997).

3. Srinivasan, S. et al. Constitutive activity of the melanocortin-4 receptor (MC4-R) in neuroendocrine and autonomic control circuits in the brain. Mol. Endocrinol. 8, 1298–1308 (1994).

4. Madonna, M. E., Schurdak, J., Yang, Y. K., Benoit, S. & Millhauser, G. L. Agouti-related protein segments outside of the receptor binding core are required for enhanced short- and long-term feeding stimulation. ACS Chem. Biol. 7, 395–402 (2012).

5. Ghamari-Langroudi, M., Srisai, D. & Cone, R. D. Multinodal regulation of the arcuate/paraventricular nucleus circuit by leptin. Proc. Natl Acad. Sci. USA 108, 355–360 (2011).

6. Smith, M. A. et al. Melanocortins and agouti-related protein modulate the excitability of two arcuate nucleus neuron populations by alteration of resting potassium conductances. J. Physiol. (Lond.) 578, 425–438 (2007).

7. Matsuda, H., Saigusa, A. & Irisawa, H. Ohmic conductance through the inwardly rectifying K+ channel Kir7.1 displays unusual K+ permeation properties in the arcuate nucleus. J. Neurosci. 18, 8625–8636 (1998).

8. Patsy, J. et al. Expression and permeation properties of the K+ channel Kir7.1 in the retinal pigment epithelium. J. Physiol. (Lond.) 511, 329–346 (2001).

14. Döring, F. et al. The epithelial inward rectifier channel Kir7.1 displays unusual K+ permeation properties. J. Neurosci. 18, 8625–8636 (1998).

15. Weaver, C. D., Harden, D., Dworetzky, S. I., Robertson, B. & Knox, R. J. A hallum-sensitive, fluorescence-based assay for detecting and characterizing potassium channel modulators in mammalian cells. J. Biol. Chem. 279, 671–677 (2004).
Data were acquired at 10 kHz using a MultiClamp 700A amplifier (2,000× gain, 3.8 dB filter frequency 5 kHz) and Clampex 10.0.1 software (Axon Instruments, Union City, CA). GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) and Excel 2010 (Microsoft) were used for data analysis. Statistical tests used included the paired t-test, when examining response of the same neurons before and after treatment with a compound, and the unpaired t-test when comparing the responses of different sets of neurons. All studies were approved by the animal care and use committee of Vanderbilt University.

**Thallium assay.** HEK293 cells (ATCC, tested mycoplasma negative) stably expressing human MC4R and Kir7.1 (M125R), or other human receptors (MC1R, MC3R) or channels (Kir2.1, Kir2.3, Kir4.1) as indicated, were grown in MEM supplemented with 10% FBS without antibiotics, suspended in 20 μl of medium plus 1 μg ml−1 tetra-cycline, used to induce expression of the transfected tet-sensitive Kir7.1 gene, and plated in 384-well poly-L-lysine coated optical bottom plates (BD Biosciences) at 20,000 cells per well. Plates were incubated overnight for 22–24 h in the cell incubator at 37 °C, 5% CO2. The following day, each individual cell plate was washed and replaced with 20 μl assay buffer (Hank’s balanced salt solution with HEPES). A thallium−sensitive dye, Thallois (TEFLabs), was diluted with assay buffer to 0.9 mg ml−1, and 20 μl was loaded into each well. Cell plates were kept in the dark for 1 h at room temperature and then washed again with assay buffer to remove residual intracellular dye. Cell plates were then incubated with peptides, drugs and/or channels (indicated for each experiment and only indicated otherwise). Thallium (10 μl of a 0.48 mM stock in assay buffer) was then added and fluorescence generated by thallium influx was recorded for up to 15 min, as indicated, using a Hamamatsu FDS plate reader.

**Zebrafish.** Wild-type Tab 14 or AB strain zebrafish were raised and bred at 26–28 °C, with 14 h light/10 h dark cycle. All zebrafish studies were conducted in larvae unscreened with regard to gender. Larval stage was determined according to ref. 28. The mc4r mutant strain was obtained from the Sanger Institute Zebrafish Mutation Project; the jaguar (G157E) mutant was kindly provided by D. M. Parichy. All studies were approved by the animal care and use committee of Vanderbilt University.

**β-arrestin recruitment assays.** β-arrestin recruitment was measured using PathHunter mMC4R cells (DiscoverX, Fremont, CA) stably expressing pro-link attached β-arrestin proteins according to the manufacturer’s protocol. Cells were plated in 384-well plates at 5,000 cells per well. The following day, cells were treated with drug and incubated at 37 °C for 90 min. Substrate was added to each well and luminescence values were obtained after 60 min at room temperature using a SpectroMax plate reader (Molecular Devices; Sunnydale, CA).

**Morpholino oligonucleotide injection, and body length measurement.** Morpholino oligonucleotides, described below, were dissolved in nuclease-free water and stored in −20 °C as 1 mM stock. Serial dilutions were made using nuclease-free water to 0.01, 0.05, 0.1, 0.2, 0.3, and 0.4 mM working solution with 20% Phenol Red (Sigma; 0.5% in DPBS, sterile filtered, endotoxin tested). Before the injection, morpholino oligonucleotides were denatured at 65 °C for 5 min and quickly spun to avoid the precipitation of aggregates. Three to five microinjections was loaded in a microinjection machine, and embryos at one or two cell stages were injected with 1–2 nl of a solution containing antisense targeting morpholino or standard control oligo. Each MO oligo injection was repeated at least three times, and doses were adjusted to optimize the phenotype-to-toxicity ratio. Following morpholino injections, embryos were raised in egg water, changed daily, under standard light/dark cycle up to 6 days post-fertilization (dpf). Dead embryos were excluded at 1 dpf. Embryos were assayed for quantitative RT–PCR of GHRH at 5 dpf. Linear body length (forehead to tail fin) was determined using a micrometre at 5 dpf. Embryos were mounted in 2.5% methyl cellulose, and images were taken by AxionVision (Version 3.1) software with a Lumar V12 Stereo Microscope (Carl Zeiss).

**Quantitative PCR.** Embryos were homogenized in a sonic dismembrator (model 100, Fisher Scientific, Pittsburgh, PA). Total RNA was extracted using an RNAeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. To remove genomic DNA, On-Column DNase Digestion was performed using an RNase-Free DNase Set (Qiagen). One microgram of purified total RNA was reverse transcribed with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). qPCR primers were designed by Beacon Designer 7.0 (Premier Biosoft International, Palo Alto, CA) to minimize primer self-dimerization, and primer sequences are indicated below. qPCRs were performed using 2 μl cDNA (20 ng) as template, 5 pmol of each of forward and reverse primers, and 2× Power SYBR PCR mix (Applied Biosystems, Carlsbad, CA) with nuclease free water (Promega, Madison, WI) to make the final volume to 20 μl in a 96-well plate (Bioexress, Kaysville, UT). qPCRs were performed using an Mx3000P (Stratagene, Santa Clara, CA). The PCR cycle was performed according to manufacturer’s instructions with initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C 20 s, 60 °C 30 s. At the end of the cycles, melting curves of the products were verified for the specificity of PCR products. A standard curve with serial dilutions of cDNA sample was performed.

**Methods**

Hypothalamic slice electrophysiology. MCAR-GFP mice, backcrossed onto the C57BL/6j background, were previously characterized by dual immunohistochemistry and in situ hybridization to validate that GFP-positive neurons in the PVN expressed MCAR RNA. Randomly selected MCAR-GFP male and female mice, 8–12 weeks of age, were deeply anaesthetized with isoflurane before decapitation. The brain was entirely removed and immediately submerged in ice-cold, gassed (95% O2, 5% CO2) artificial cerebrospinal fluid (aCSF), containing (in mM): 126.2 NaCl, 3.1 KCl, 2 CaCl2, 1 MgCl2, 1 NaH2PO4, 26.2 NaHCO3, 10 glucose and 11.2 succinate (350 mosm per kg, pH 7.39 when gassed with 95% O2, 5% CO2 at room temperature). Brain blocks of containing hypothalamic were made by trimming the whole brains while immersed in oxygenated, near-freezing aCSF and glued to a dental-cement cast customized to the size of the block mounted on a plate with adjustable angle. Brain slices of 200-μm thicknesses were then cut at angle range between 44° and 49° in reference to horizontal plane and transferred to a glass beaker containing oxygenated ACSF at 31 °C. After an incubation period lasting at least one hour, a slice was transferred to a recording chamber (~2.0 ml in volume), then submerged and immobilized with nylon strands drawn taut across a C-shaped platinum wire (1 mm outer diameter), and perfused with warmed (31–32 °C) oxygenated ACSF at a rate of 2–3 ml min−1.

EGFP-fluorescent neurons were unambiguously identified and patched using combined epifluorescence and IR-DIC optics. Fluorescent neurons of healthy IR-DIC appearance but of every level of fluorescence brightness were chosen for electrophysiological recordings.

Drugs were either added to aCSF and bath applied to the slice via the perfusion system (for extracellular applications) or to the pipette solution to perform whole cell recordings (for intracelluar applications). The volume of the recording chamber relative to the flow rate assured a complete exchange of solution occurring in less than 1 min. The persisting effects of a peptide were therefore due to prolonged effects rather than a slow wash out.

In this study, whole-cell patch−clamp recordings were used to obtain information about action potential firing activity, and membrane potentials and currents. Unless stated otherwise, whole cell recordings were performed using patch pipettes of 3.4 MΩ to 5 MΩ resistance when filled with a solution containing (in mM): 125 K gluconate, 8 KCl, 4 MgCl2, 10 HEPES, 5 NaOH, 4 Na2ATP, 0.4 Na3GTP, 5 Na2− creatine phosphate, 7 sucrose and 7 KOH which resulted in a pH −7.23 and osmolality of 295–300 mosmol per kg. The permeability of the α7−regulated channels were investigated by replacing K gluconate and KCl with 130 RbCl and used while cells were held around 70 mV. The firing frequency and membrane potential of neurons was measured while cells were held around 70 mV.

To characterize the currents generated by peptides, we compared the I-V relationships of the TTX pre-treated PVN neurons obtained in control with recordings. To establish the current responses of each cell to a depolarizing voltage clamp command, we conducted current−voltage (I–V) relationships of the TTX pre-treated PVN neurons obtained in control with wash out. The averaged current responses elicited under each condition were then plotted as a function of the corresponding values during voltage ramps to obtain individual I–V relationships.

**Tetramethylrhodamine−Ethyl−Mesylate−(TETM)−Rhodamine−payload−injected** zebrafish embryos from the wild type embryos were injected with TETM−loaded nanoparticles intravitreally. The injection was performed at the 1–2 cell embryo stage of zebrafish. Embryos were incubated in 2× embryo medium with 50 μg/ml of TETM−loaded nanoparticles. The fluorescent signal from the injected payload can be observed from the injection day up to 10 days post-fertilization.
on each plate. All measurements were performed in duplicate, and Graph Pad Prism 5.0 was used for the interpretation and analysis of data.

**Morpholinol and qPCR oligonucleotides.** Antisense morpholinol oligonucleotide (MO) against the ATG translation initiation site of *agpr*, km13 and 13 standard control MO were designed and synthesized by GeneTools, LLC (Philomath, OR, USA). For morpholinol injection agpr ATG MO: 5’-TTTCCACGCGCGCTGCTGATT TTC-3’. Zebrafish Standard Control MO: 5’-CCCTCTACTCATTCAATATTATAA-3’ km13 MO targeting exon-exon boundary-5’-CAGATGCCATGCGGCGCAG AAAC-3’. For qPCR, grh (growth hormone releasing hormone), forward primer 5’-GTGCTATTATCCGTGGTATAC-3’, reverse primer 5’-ATGTCGACTGACGCTCTATGTT-3’.

**ELISA** (Elongation Factor 1 alpha), with forward primer 5’-GCGAGGCGACGCTCTA AACAT-3’, reverse primer 5’-ATCAAGAAGAATACGGCTAGT GATC-3’. **MC4R-mediated** PYL release assay. Male C57BL/6J mice were ordered from The Jackson Laboratory (Bar Harbour, ME). Experiments were run when the mice reached 12 weeks of age. On the day of the study, the mice were fasted during the daytime for 4 h to reduce plasma PYL levels to baseline. Following the fast, mice were randomly selected to receive an intraperitoneal injection of saline (vehicle), 3 mg per kg of the α-MSH agonist LY2112688 (LY), or an equimolar dose of 6.9 mg per kg of MC4-N2-0453 (NOVO). MC4-N2-0453 corresponds to the previously reported peptide 19-31. After a 15 min equilibration period, the representative intestinal peptide (VIP, 10 nM) was added to stimulate epithelial alpha secretion and 5–10 min later (once the VIP maximum had been achieved) a single concentration of either MC4-N2-0453 (3 nM–3 μM) or α-MSH (300 nM) was administered. Consequent MC4R mediated reductions in *I* were measured for 20 min before a control concentration of PYL (10 nM) was added to reduce *I* levels via epithelial Y1 receptors. All additions were basolateral and changes in *I* were converted to µA cm⁻². MC4R activity and individual peptide responses were pooled and analysed using Graphpad Prism (version 5). All animal care and experimental procedures were approved by the King’s College London Ethical Review Process Committee.

**Fluorescent in situ hybridization for MC4R and Kir7.1 mRNA.** Various mRNA species expressed by PVN neurons were visualized with a variation of FISH (fluorescent in situ hybridization) called RNAscope (ACD; Advanced Cell Diagnostics, Inc., Hayward, CA). RNAscope cDNA probes and detection kits were purchased from ACD and used according to the company’s online protocols. The probe sets directed against MC4R and Kir7.1 mRNA were designed from sequence information from the mouse RefSeq mRNA IDs NM_016977 and NM_00110277, respectively. Four wild type male MC4-GFP littermates were euthanized with an overdose of Nembutal (100 mg per kg, intraperitoneally) and brains were quickly removed and frozen in powdered dry ice. Brains from three MC4R knockout mice were prepared the same way. All brains were stored at −80°C until cut into coronal sections (Leica CM3000). 16-µm sections were adhered to warm Fisher plus slides (Fisher Scientific) and immediately refrozen. Slides were stored at −80°C until fixed with ice-cold 4% paraformaldehyde according to the ACD protocol for fresh frozen tissue. After fixation for 15 min, slides were dehydrated in an ethanol dilution series (50%, 70%, 100%), then immersed in xylene for 10 min, and embedded in paraffin. Two-micron sections from each brain were cut and mounted on glass slides. Sections were incubated with RNAscope probes and a series of signal amplification steps according to the ACD protocol for the Fluorescent Multiplex Kit. In brief, incubation steps included: probe mixtures at kit recommended dilutions for the C1 channel (blank or probes for ubiquitin C mRNA), the C2 channel (MC4R mRNA) and the C3 channel (Kir7.1 mRNA) for 2 h at 40°C, wash twice, AMP-1 FL reagent for 30 min at 40°C, wash twice, AMP-2 FL reagent for 15 min at 40°C, wash twice, AMP-3 FL reagent for 30 min at 40°C, wash twice, AMP-4 FL alt A or B reagent for 15 min at 40°C, wash twice, DAPI reagent for 1–2 min at room temperature, remove standing liquid and immediately cover with slow-Fade Gold (Molecular Probes) or Aqua-PolyMount (Poly-Sciences, Inc.) 4°C incubations were carried out in a HybEZ oven (ACD). Each run of 5 to 10 slides included one positive control (3 probe sets to housekeeping gene mRNA, ACD) and one negative control (3 probe sets for bacterial mRNA, ACD), 3–6 sections of the PVN from each animal, representative of rostral (AP = −0.58 to −0.80 relative to bregma), middle (AP = −0.80 to −1.0) and caudal (AP = −1.0 to −1.2; ref. 30) levels, were processed for imaging. Sections were imaged with a Zeiss 710 scanning confocal microscope using either a LD C-Apochromat ×40/1.0 water lens or a Plan-Apochromat ×63/1.40 oil lens. Z stacks were done on all sections using pin hole settings that resulted in 1.0-µm thick optical sections. Images were opened in Imaris (version 7.6), background subtracted and then contrast enhanced by increasing gamma to 1.5 to 2.3, then exported in tiff format for counting cell mRNA signals. A few numbers of round, fraction delimited spots over and surrounding DAPI-labeled nuclei were manually counted with the aid of Metamorph software. Borders between cells were resolved with the help of grey scale DIC images overlaid on maximum intensity projections of the Z stacks. Negative control sections were used to count fluorescent dots that represented auto-fluorescent signals plus non-specific labeling by the multiplex kit. Sections of WT PVN hybridized with probes against bacterial mRNA were used to count background dots in the Kir7.1 channel and sections from MC4R knockout mice hybridized with the probes against MC4R mRNA were used to count background dots in the MC4R channel. The average number of dots per cell for MC4R and Kir7.1 negative controls were 1.60 ± 0.87 standard deviations and 1.31 ± 0.70, respectively (354 cells from 6 sections for MC4R and 54 cells from 2 sections for Kir7.1; see supplementary figure). For cell counts in PVN sections from wild-type mice, cells were counted as positively labeled for MC4R and/or Kir7.1 mRNA if the number of dots per cell exceeded the mean plus 1.50. Blots were revealed by ECL and film scanned. Densitometry analysis was done using Adobe Photoshop. n = 2.

**Mucosal electrophysiology.** Male C57BL/6J mice descending colon mucosa devoid of overlying muscle was cut into 6 adjacent pieces and each placed between the two halves of an Ussing chamber (aperture 0.14 cm²) and bathed in oxygenated Krebs-Henseleit (KH) buffer (in mM: 117 NaCl, 24.8 NaHCO₃, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 2.5 CaCl₂ and 11.1 D-glucose), 5 ml on both sides. Mucosal was voltage-clamped at 0 mV and the resultant villar transport measured as short-circuit current (Isc), as described above. After 15 min equilibration period, a representative intestinal peptide (VIP, 10 nM) was added to stimulate epithelial alanine secretion and 5–10 min later (once the VIP maximum had been achieved) a single concentration of either MC4-N2-0453 (3 nM–3 μM) or α-MSH (300 nM) was administered. Consequent MC4R mediated reductions in *I* were measured for 20 min before a control concentration of PYL (10 nM) was added to reduce *I* levels via epithelial Y1 receptors. All additions were basolateral and changes in *I* were converted to µA cm⁻². MC4R activity and individual peptide responses were pooled and analysed using Graphpad Prism (version 5). All animal care and experimental procedures were approved by the King’s College London Ethical Review Process Committee.
3× standard deviations of their respective negative controls (5 or more dots for MC4R and 4 or more dots for Kir7.1). Two of the images from wild-type mice were also analysed by an automated cell counting multiplex FISH module from Indica Labs, Inc. (Ahmedabad, India). The automated cell counts agreed with our manual count data with >90% of all MC4R mRNA positive cells also positive for Kir7.1 mRNA in the PVN. All animal care and experimental procedures were approved by Vanderbilt University Medical Center Institutional Animal Care and Use Committee.

27. Liu, H. et al. Transgenic mice expressing green fluorescent protein under the control of the melanocortin-4 receptor promoter. *J. Neurosci.* **23**, 7143–7154 (2003).
28. Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. & Schilling, T. F. Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310 (1995).
29. Cox, H. M. et al. Peptide YY is critical for acylethanolamine receptor Gpr119-induced activation of gastrointestinal mucosal responses. *Cell Metab.* **11**, 532–542 (2010).
30. Franklin, K. B. J. & Paxinos, G. *The Mouse Brain in Stereotaxic Coordinates* (Academic Press, 1997).
Extended Data Figure 1 | G protein signalling is not required for α-MSH-induced depolarization of PVN MC4R neurons. a, b, A MAP kinase kinase inhibitor, 1 μM U0126, fails to block α-MSH-induced increase in firing frequency of PVN MC4R neurons recorded in whole cell configuration. (a, left panel shows a representative trace from one neuron, b, right panel shows mean ± s.e.m. of firing frequency, *P < 0.05, paired t-test). c, GTPγS, a non-hydrolysable GTP analogue, fails to block the depolarization induced by α-MSH, measured in whole-cell configuration. This drug however does block the hyperpolarization induced by μ-opioid agonist, DAMGO (10 μM), (mean ± s.e.m., ***P < 0.001, paired t-test). d, The Gβγ inhibitor gallein fails to inhibit α-MSH-induced depolarization of PVN MC4R neurons (mean ± s.e.m., **P < 0.01, paired t-test), although it blocks the effects of DAMGO (10 μM) on membrane potential. In all electrophysiological studies, each n represents an independent neuron and slice, and no more than two slices were used per animal.
Extended Data Figure 2 | The charge generated by rubidium permeation through MC4R-regulated channels depolarizes PVN MC4 neurons.
a, b, Rb⁺ efflux through MC4R-gated Kir channels can generate greater α-MSH-induced depolarization of PVN MC4R neurons loaded with 130 mM RbCl and 4 mM K⁺ through the recording pipette. Data show the mean and s.e.m. (a, *P < 0.05, unpaired t-test), and a representative trace (b).
Extended Data Figure 3 | Co-expression of MC4R and Kir7.1 in the PVN.

a–d, Detection of MC4R and Kir7.1 mRNA in PVN slices using fluorescent in situ hybridization (RNAscope). Images demonstrate the region of the hypothalamus under study (a; scale bar, 200 μm), colocalization of MC4R (green) and Kir7.1 (red) mRNAs (b, white open arrows, double labelled cells; yellow arrows, Kir7.1 expression only; scale bar, 10 μm), and negative controls (c, MC4R probe with tissue from MC4R knockout mice; scale bar, 10 μm; d, bacterial probe with tissue from wild-type mice; scale bar, 10 μm). Data is representative of four male mice.
Extended Data Figure 4 | Quantitation of MC4R and Kir7.1 RNA in PVN cells. Single-molecule RNA detection in sections was quantitated by counting fluorescent dots associated with individual cells (Extended Data Fig. 3). Background threshold was determined from the number of dots per cell in sections resulting from hybridization using a negative bacterial DNA control, or from hybridization of the MC4R probe to sections from the MC4R knockout mouse (top panel, columns 1 and 2). Threshold-subtracted dot numbers were then used to determine the per cent of PVN cells expressing MC4R or Kir7.1, and the per cent of MC4R cells expressing Kir7.1; cells were considered positive if the number of dots exceeded the mean of the negative controls by 3× standard deviations (bottom panel). Data from the number of cells indicated was collected from multiple PVN sections derived from four male mice.
Extended Data Figure 5 | MC4R and Kir7.1 coimmunoprecipitate from transfected HEK293 cells. **a**, Cells transfected with the indicated genetically flagged proteins were incubated with the reversible crosslinker dithiobismaleimidoethane (DTME) before lysis. Lysates were immunoprecipitated using the indicated antibody (F, Flag; HA, haemagglutinin; X, no antibody), crosslinking was reversed with 100 mM dithiothreitol (DTT), and samples separated by SDS–PAGE. The membrane was blotted with the M2 anti-Flag antibody to detect Kir7.1.

**b**, Relative quantitation of protein immunoprecipitation. Densitometry analysis to measure the amount of immunoreactive Kir7.1 material was performed using Adobe Photoshop. Amount of material immunoprecipitated with the Kir7.1-3X-Flag was set at 100%. Data shows relative amount of Kir7.1 immunoprecipitated using an antibody against the 3HA-MC4R protein; bars indicate range of data from 2 independent lanes. The protein molecular weight of Kir7.1 is calculated at 40 kDa, and the two larger bands represent glycosylated forms of the protein that are absent when the N-linked glycosylation site at position 93 is mutated (data not shown). Data are representative of three independent experiments.
Extended Data Figure 6 | AgRP-induced increase in thallium flux does not involve G, signalling or β-arrestin recruitment. a, b, Subtracted Tl⁺ flux examining effects of 200 nM AgRP indicates that 8 h pre incubation with pertussis toxin of MC4R- and Kir7.1-expressing HEK293 cells fails to block AgRP-induced Kir7.1 regulation (mean ± s.e.m., n = 110, combined data from three independent experiments). c, Addition of α-MSH stimulates β-arrestin recruitment to the MC4R in HEK cells stably expressing MC4R and β-arrestin fused to complementary fragments of β-galactosidase (DiscoverRx PathHunter assay, black line, logEC₅₀ = -7.69). In contrast, increasing concentrations of AgRP are without any effect using the same assay (red line). Individual points show mean ± s.e.m. n = 12, representative of 3 independent experiments.
Extended Data Figure 7 | Role of PKA and cAMP in the α-MSH-induced closure of Kir7.1. 

a, b, Subtracted Tl⁺ flux assay examining effects of 100 nM α-MSH indicate that pre-incubation with 1 μM H89, a PKA inhibitor, fails to block α-MSH-induced regulation of Kir7.1. Data (n = 16) show mean ± s.e.m. of kinetic traces (a) and maxima (b). VHC = vehicle. 

c, d, Subtracted Tl⁺ flux assay examining effects of raising intracellular cAMP by forskolin (FSK, 20 μM) and IBMX treatment, with and without 100 nM α-MSH. Data show kinetic traces (c) and maxima (d). IBMX, 100 μM 3-isobutyl-1-methylxanthine; VHC, vehicle, mean ± s.e.m., n = 64, ***P < 0.01, unpaired t-test. Data representative of 3 independent replicates.
Extended Data Figure 8 | The α-MSH analogue MC4-NN2-0453 (NOVO) is a partial agonist of the MC4R in a murine colon mucosal assay of MC4R activity. The activation of the MC4R inhibits vectorial ion transport across colonic epithelium, measured as reductions in the short circuit current ($I_{sc}$).

**a.** Kinetic response to a sub-maximal basolateral concentration of α-MSH or NOVO, showing more rapid achievement of maximal activity with α-MSH, *P < 0.05*, one-way ANOVA with Bonferroni’s post-test.

**b.** Full concentration-response to MC4-NN2-0453 (NOVO), showing that the compound does not achieve the efficacy reached by a maximal dose of α-MSH (denoted by the dashed line. Full characterization of the MC4R mediated α-MSH response in colonic epithelium is presented elsewhere. Each data point represents the mean of five measurements from independent colon samples, with approximately six samples per animal obtained from 15 mice.
Extended Data Figure 9 | Effects of Kir7.1 and MC4R signalling in larval zebrafish. a–c, Knock-down of the Kir7.1 gene by kcnj13 morpholino oligonucleotide (MO) suppresses the axial growth of larvae in wild-type and mc4r null zebrafish. Sibling wild-type or mc4r-null zygotes were bred and injected with antisense kcnj13 morpholino oligonucleotide at day 0. a, The axial body length was measured at 5 dpf. Each group of 30 fish was harvested for RNA extraction and cDNA synthesis. b, Relative expression of ghrh mRNA was measured and normalized to the housekeeping gene ef1a with qRT–PCR. The wild type fish that were injected with MO against kcnj13 expressed significantly higher copies of ghrh mRNA than those that were injected with control MO. (control MO, n = 9, 1.056 ± 0.116 vs kcnj13 MO, n = 9, 1.935 ± 0.294, unpaired t-test, *P < 0.05). MC4R-null fish that were injected with kcnj13 MO have significantly higher GHRH expression than MC4R-null fish that were injected with control MO (control MO, n = 9, 1.040 ± 0.164 vs KCNJ MO, n = 8, 2.395 ± 0.461, one-way ANOVA, P < 0.05). c, Representative WT fish injected with kcnj13 MO vs control MO. d, jaguar wild-type and null mutant siblings were bred and injected with 7.5 ng non-targeting standard control or 7.5 ng antisense morpholino oligonucleotide targeting agrp or kcnj13. d, Knockdown of AgRP with agrp MO in the absence of Kir7.1 also reduces larval growth (mean ± s.e.m., n = 43, ***P < 0.001, unpaired t-test). e, The deletion of Kir7.1 in jaguar null blocks effects of KCNJ13 MO on MC4R-mediated inhibition of growth (mean ± s.e.m., n = 58, unpaired t-test). Data are representative of three independent experiments.
Extended Data Figure 10 | A model for α-MSH and AgRP signalling at PVN MC4R neurons. Data presented here supports a model in which MC4R may couple to both Ga signalling and regulation of Kir7.1 activity in PVN MC4R neurons. α-MSH results in elevation of intracellular cAMP through activation of Ga, and inhibition of K<sup>+</sup> efflux through Kir7.1, both of which are depolarizing. AgRP lowers the constitutive activity of the MC4R and blocks α-MSH binding, but data here show that AgRP also acts as an agonist to increase K<sup>+</sup> efflux through Kir7.1, producing a strong hyperpolarizing signal. The relative distribution and composition of the MC4R signalling complex in different subcellular compartments of PVN MC4R neurons has not been directly determined. Earlier models of α-MSH and AgRP action suggested competitive binding of these peptides to individual MC4R sites (orange box). Existing neuroanatomical data characterizing POMC and AgRP neuronal projections show that α-MSH may act independently of AgRP at many sites in the central nervous system, since AgRP immunoreactive fibres are only observed in a subset of MC4R-expressing nuclei containing POMC-immunoreactive fibres (right circle, for review see ref. 25). The ability of AgRP to act independently of α-MSH as a potent hyperpolarizing agonist, via regulation of Kir7.1, suggests the likely existence of independent AgRP sites of action (left circle). Recent reconstruction of electron microscopy images of the PVN in which POMC- and AgRP-containing synaptic vesicles have been specifically labelled with a genetically encoded marker provides preliminary anatomical support for this new model<sup>26</sup>. This study demonstrates that 52% of AgRP boutons in the PVN are not found in synapses, potentially supporting volume transmission of AgRP that may lead to competition with α-MSH at synaptic and/or non-synaptic sites. Additionally, the study found the vast majority of AgRP and POMC synaptic sites localized to different subcellular compartments of PVN neurons, supporting the independent action of both peptides. Synaptic release sites on soma were almost exclusively AgRP-containing, while POMC release sites were concentrated on distal dendrites. Another MC4R signalling pathway, involving cAMP/PKA-dependent activation of K<sub>ATP</sub> channels and α-MSH-induced hyperpolarization, has been demonstrated in MC4R neurons in the dorsal motor nucleus of the vagus in the brainstem (bottom right)<sup>21</sup>. Thus, while Kir7.1 signalling appears to be essential for depolarization of PVN MC4R neurons by α-MSH, Ga<sub>s</sub> signalling and elevation of cAMP may be depolarizing or hyperpolarizing, depending on the cellular context.