

Melanocortin-4 receptor–regulated energy homeostasis

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The melanocortin system provides a conceptual blueprint for the central control of energetic state. Defined by four principal molecular components—two antagonistically acting ligands and two cognate receptors—this phylogenetically conserved system serves as a prototype for hierarchical energy balance regulation. Over the last decade the application of conditional genetic techniques has facilitated the neuroanatomical dissection of the melanocortineric network and identified the specific neural substrates and circuits that underscore the regulation of feeding behavior, energy expenditure, glucose homeostasis and autonomic outflow. In this regard, the melanocortin-4 receptor is a critical coordinator of mammalian energy homeostasis and body weight. Drawing on recent advances in neuroscience and genetic technologies, we consider the structure and function of the melanocortin-4 receptor circuitry and its role in energy homeostasis.

In mammals, homeostatic maintenance of energetic state is regulated by a sensory feedback system that attempts to preserve stability through the concerted modulation of both energy intake (as caloric consumption) and energy expenditure (as basal metabolism, adaptive thermogenesis and physical exertion). Ultimately, unbalancing of this equation is reflected by alterations in body weight, with a positive (intake > expenditure) or negative (intake < expenditure) energy balance engendering weight gain or loss, respectively. Although acute fluctuations in the relative levels of energy intake and expenditure are tolerated and redressed, chronic destabilization can have severe implications for health¹ and longevity². Furthermore, energy balance regulation biases toward defense against the lower, rather than upper, limits of adiposity. Since survival (rather than viability) is the evolutionary imperative, corpulence represents a ‘fitter’ state than does excessive leanness, which is associated with reduced reproductive function and, potentially, death. Teleologically, it is vital that detection of and response to periods of negative energy balance be safeguarded by a hardwired system that prevents starvation to guarantee survival. Unfortunately, the requisite rigidity of this system, while under strong selective pressure in a world of feast and famine, can be maladaptive in the modern age of caloric abundance, facilitating a chronic positive energy balance and consequent obesity.

Ultimately, this need-detection and response enactment is coordinated by the CNS in reaction to information from the periphery³. Episodic signals, associated with phasic oscillations in short-term energetic state (for example, before, during and after a meal), mediate

the acute consummatory indices of hunger and satiety and adaptive thermoregulatory and metabolic aspects of energy expenditure, while chronic energetic state is reflected in tonic signaling from hormones positively correlated with adiposity^{3,4}. Thus, long-term energetic indicators define a homeostatic tone to which the episodic modulation of acute energy balance is set. Overall energetic state, and hence body weight, is determined by the integration of these afferent signals at discrete sensory nodes within the brain. Located primarily in the hypothalamus and brainstem, such populations represent interoceptive entry points into hierarchical networks that collectively provide an awareness of energetic state and modulate feeding behavior, energy expenditure and metabolism accordingly³.

Deconvolving the circuitry that underlies this control has been a process driven principally by technological advance. The lesion studies of the mid-twentieth century defined the gross neuroanatomical architecture of energy balance networks, while the subsequent genetic era offered insight into the cellular populations and molecular substrates that define overall system functionality. Current understanding of the central melanocortin system epitomizes this evolution and provides a conceptual blueprint for the hardwired control of energetic state. Here we consider the present view of melanocortineric energy homeostasis with a particular focus on second-order neuronal populations defined by their expression of the melanocortin-4 receptor (MC4R).

The central melanocortin system

Central melanocortineric control of energy balance is predicated on an ability to distinguish current and acceptable energetic values, and subsequent rectification of any discrepancy through appropriate physiological and behavioral responses. This sensory function is provided by two counteracting populations of interoceptive neurons in the arcuate nucleus of the hypothalamus (ARC)^{4,5}. ARC neurons expressing agouti-related peptide (AgRP) and coexpressing neuropeptide Y (NPY) and GABA represent the anabolic arm of the melanocortin pathway, robustly stimulated by caloric insufficiency^{6,7} and necessary for driving energy intake, conserving energy expenditure

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and promoting weight gain^{8–10}. By contrast, the catabolic interests of the system are defended by ARC neurons expressing pro-opiomelanocortin (POMC), which, in response to caloric sufficiency, promote a cessation of feeding, increased energy expenditure and weight loss^{9,11,12}. The sensory capacity of ARC^{AgRP} and ARC^{POMC} neurons is contingent on their sensitivity to afferent inputs, both neuroendocrine and neural, that communicate acute and long-term energetic state (for review, see refs. 3,4). In rodents with *ad libitum* access to food, these signals oscillate in accordance with diurnal rhythm, such that the negative energy state fomented over the course of an animal's inactive period (light cycle) is redressed through food consumption during the active period (dark cycle). This flux in energy state is reflected in the *in vivo* electrical activity of genetically identified ARC^{AgRP} and ARC^{POMC} neurons, which exhibit a respective increase and decrease in action potential firing with mounting caloric deficiency¹³. Thus, consistent with their metabolic functions, the activity of ARC^{AgRP} and ARC^{POMC} neurons conveys a real-time awareness of energetic need, which in turn predicts neurotransmitter release and apposite physiological output.

Functionally, both AgRP and the bioactive products of POMC processing, α -, β - and γ -melanocyte stimulating hormone (MSH), act as neuropeptides that engage cognate receptors on second-order postsynaptic targets^{4,5}. Of five G-protein-coupled melanocortin receptors in the mammalian genome¹⁴, only the melanocortin-3 receptor and MC4R are represented in the CNS. A definitive role for the melanocortin-3 receptor in energy balance remains to be fully clarified and will not be discussed here (for review, see ref. 15). The MC4R is activated principally by α -MSH, leading to neuronal depolarization and action potential firing, while AgRP acts as an antagonist or biased agonist promoting neuronal hyperpolarization (see below). Central administration of MC4R agonists promotes satiety, energy expenditure and weight loss. MC4R antagonists increase food intake, energy conservation and weight gain^{4,5}. Thus, the diametric functionality of ARC^{AgRP} and ARC^{POMC} neurons acting at downstream MC4R neurons underscores a bimodal system highly sensitive to fluctuations in energetic state.

Concordant with the catabolic function of α -MSH, genetic *Pomc* and *Mc4r* nullizygosity in mice^{16–18}, and the inactivating *POMC* and *MC4R* mutations in humans^{19–21} result in early-onset obesity defined by hyperphagia and reduced energy expenditure. Genetic ablation of the mouse *Agrp* locus does not engender the expected hypophagic and lean phenotype²², most likely because of developmental redundancy. However, postnatal cytotoxic ablation of ARC^{AgRP} neurons does result in hypophagia and leanness^{23–25}, while postnatal ARC^{POMC} neuron ablation promotes hyperphagia and obesity²⁵. Together, these studies cogently demonstrate that the melanocortin ligands are critical to mammalian energy balance.

Modern neuroscience technologies now enable the real-time manipulation of genetically defined neuronal populations, without the confounding effects of developmental compensation or pharmacological non-specificity²⁶. Chemogenetic^{10,27} and optogenetic^{8,28} activation of ARC^{AgRP} neurons in calorically replete mice rapidly (within minutes) promotes voracious food seeking and consumption and an attenuation of energy utilization, which under chronic conditions drives weight accrual¹⁰. Conversely, chemogenetic silencing of ARC^{AgRP} neurons engenders hypophagia in calorically deplete mice homeostatically motivated to eat^{10,27}. Thus, ARC^{AgRP} neurons are sufficient to drive an energy-saving motor program and are necessary for the enactment of deficiency-related feeding behaviors, including associated risk assessment and food seeking²⁹.

The orexigenic function of inhibitory ARC^{AgRP} neurons is served by the silencing of second-order satiety neurons. Although ARC^{AgRP} neurons inhibit ARC^{POMC} neurons^{9,30}, this is not a mechanism by which they promote acute feeding⁹. Thus, the functional antagonism of these first-order populations is defined by their convergence at common second-order targets. In accordance, neuroanatomical tracing of ARC^{AgRP} and ARC^{POMC} efferents reveals highly comparable projection profiles^{31,32}. Circuit-level use of optogenetics to experimentally isolate discrete ARC^{AgRP} projections demonstrates that these neurons drive feeding through their (noncollateralized) projections to the anterior bed nucleus of the stria terminalis, lateral hypothalamus and paraventricular nucleus of hypothalamus (PVH), and to a lesser extent the paraventricular nucleus of the thalamus²⁸. Pertinently, MC4R expression is closely correlated with these projections to the anterior bed nucleus of the stria terminalis, PVH and lateral hypothalamus^{33–35}.

Although ARC^{POMC} neurons project to the same regions, their explicit wiring architecture, including which circuit-specific efferents are sufficient to promote satiety, is as yet unknown. Furthermore, the rapid temporal dynamics of ARC^{AgRP} neuron-driven feeding contrasted with the far slower effects of ARC^{POMC} neuron-driven satiety. Both chemogenetic¹¹ and optogenetic⁸ stimulation of ARC^{POMC} soma produces a late-onset hypophagic response, evident only after many hours of stimulation, and body weight loss. Similarly, chemogenetic silencing of these neurons augments long-term food intake but has no acute hyperphagic effect⁹. The explanation for the apparent temporal variance in ARC^{AgRP} and ARC^{POMC} neuron-regulated appetite is unclear but is likely attributable to their respective neurotransmitter complement and synaptic organization.

The acute consummatory effect of ARC^{AgRP} neurons is contingent on the release of GABA and/or NPY^{9,36}. The concerted genetic inactivation of these two ARC^{AgRP} neuron systems (through ARC^{AgRP} neuron deletion of *vGat* (*Slc32a1*) and global *Npy* nullizygosity) abolishes chemogenetic ARC^{AgRP} neuron-driven feeding³⁶, although in isolation neither mutation is functionally sufficient, suggesting that in a constitutive genetic context these two axes exhibit operative redundancy. Indeed, in *Npy*^{−/−} mice ARC^{AgRP}→PVH GABAergic synaptic strength is potentiated, indicating some level of compensatory developmental rewiring⁹. In a developed neural system, however, pharmacological blockade of GABA or NPY signaling specifically in the PVH (at GABA_A receptors and Y1 receptors, respectively) independently abolishes optogenetic ARC^{AgRP}→PVH-driven feeding⁹, implying that both systems are required, and potentially cooperate³⁷, to inhibit satiety-promoting PVH neurons. Unlike the rapid orexigenic effect of centrally infused NPY, AgRP administration is effective only after a number of hours³⁸. In accordance, AgRP, acting at downstream melanocortin receptors, is dispensable for rapid chemo- and optogenetically induced feeding but is required to drive late-onset, longer term consumption^{8,36}. Similarly, the slow-to-build anorectic effect of ARC^{POMC} neurons is contingent on melanocortin receptor availability⁸. Thus, the rapid feeding induced by ARC^{AgRP} neurons is defined by their release of fast-acting neuroactive substances, while AgRP and α -MSH signaling at downstream MC4Rs defines a slower behavioral output.

The absence of a fast-acting neurotransmitter or neuropeptide potentially explains the lack of an acute satiety effect from ARC^{POMC} neuron activation. However, several histological studies have identified GABAergic and glutamatergic subsets of ARC^{POMC} neurons. These studies indicate that approximately 40% of ARC^{POMC} somata are GABAergic, as defined by their expression of the GABA synthesis

genes *Gad65* and *Gad67* (refs. 39–41), although they do not express the vesicular GABA transporter (vGAT)^{40,42}, suggesting that if these cells do release GABA an alternative transporter might be required for vesicular packaging. There is also evidence of a distinct glutamatergic ARC^{POMC} subpopulation, though its reported relative proportion varies from 10% (refs. 40,42) to 50% (ref. 39) depending on the labeling technique employed. At present, the functional significance of these subpopulations remains uncertain and may be defined by both circuit and physiological specificity that has not yet been investigated. As an example, stoichiometric analysis of ARC^{POMC}→PVH synapses revealed approximately 50% to be glutamatergic but only 10% GABAergic⁴³ (although the use of vGAT as a marker of GABAergic terminals may underestimate the number of GABA-releasing synapses; see above). Ultrastructural modeling of melanocortinergergic projections to the PVH demonstrated that, while ARC^{AgRP} neurons form symmetric and predominantly axo-somatic synapses at PVH neurons, ARC^{POMC}→PVH neurons exhibit relatively weak and distal axo-dendritic connections⁴³. Consistent with this, channelrhodopsin-assisted circuit mapping⁴⁴, which serves as the gold standard for the identification of functional fast neurochemical synapses, identified robust monosynaptic GABAergic connectivity from ARC^{AgRP} to approximately 50% of PVH neurons, while ARC^{POMC} derived postsynaptic currents were limited to only 5% of PVH neurons (3% excitatory and 2% inhibitory)⁴³. Thus, in contrast to ARC^{AgRP} neurons, which demonstrate relative homogeneity, a cofunctional and consistent peptidergic and GABAergic nature and robust synaptic organization (at least in the PVH), ARC^{POMC} neurons exhibit neurochemical heterogeneity, a paucity of functional fast neurochemical connections and weak synaptic inputs (at least in the PVH). While these observations likely explain the slow-to-build effect of ARC^{POMC}-driven satiety, it may also suggest the involvement of a more complex neural mechanism underlying melanocortinergergically regulated satiety. For instance, it is possible that α -MSH release from ARC^{POMC} neurons promotes synaptic restructuring and the modulation of fast transmitter inputs, such as has been observed in other melanocortinergergic projections^{45,46}. Furthermore, while ARC^{POMC}-neuron fast neurotransmitter synapses may not be required for the acute regulation of appetite, it remains to be investigated whether they play a more substantive role in other aspects of melanocortinergergic physiology, such as autonomic outflow or glucose homeostasis.

The melanocortin-4 receptor

Mouse models of MC4R function. Concordant with its neuroanatomical distribution^{33–35} and myriad pharmacological observations^{4,15}, ablation of the mouse *Mc4r* locus produces a profound dysregulation of energy homeostasis, leading to overt early-onset obesity¹⁶ (Table 1). *Mc4r* insufficiency promotes hyperphagia, reduced energy utilization, increased fat mass, perturbed glucose homeostasis and sympatho-suppression^{16,47,48}. The *ad libitum* hyperphagia engendered by *Mc4r* nullizygosity is defined by exaggerated dark-cycle consumption⁴⁹ and correlated with an increased motivation to attain food, suggesting enhanced goal-directed behavior⁵⁰. The fidelity of the diurnal eating pattern and the normal response of *Mc4r*^{−/−} mice to periods of calorie restriction⁴⁷ imply that sensory processing of hunger cues, likely at ARC^{AgRP} neurons, remains intact in these mutants. Thus, consistent with later studies^{8,36}, the acute drive to consume food in response to energetic depletion is independent of AgRP→MC4R signaling and is maintained in these mutants presumably as a result of the continued efficacy of ARC^{AgRP} neuron NPY and GABA release. Protracted within-meal consumption is therefore likely to be explained by a failure of α -MSH induced satiety. In agreement,

Pomc deficiency^{17,18} recapitulates most of the *Mc4r*^{−/−} phenotype, including obesity, increased axial length and hypersensitivity to an obesogenic diet. Furthermore, human null mutations in either *MC4R*^{19,20,51,52} or *POMC*²¹ lead to syndromic obesity that mirrors the respective mouse phenotype, cogently highlighting the phylogenetic conservation of this system.

While these global knockout lines demonstrate the importance of MC4R signaling to the control of energy balance, the specific neural populations accountable for the pleiotropic nature of the *Mc4r*^{−/−} phenotype remained unknown. The generation of two conditional Cre recombinase-dependent *Mc4r* alleles was fundamental to the neuro-anatomical dissection of MC4R function (Fig. 1 and Table 2). To assess functional sufficiency, a reactivatable *Mc4r* null allele was generated through the insertion of a Cre-dependent loxP-flanked transcriptional blocking sequence (*Mc4r*^{loxTB/loxTB})⁵³. Necessity was addressed with a complementary *Mc4r*^{loxP/loxP} inactivation allele⁵⁴ (Fig. 1). In the presence of an appropriate Cre-expressing driver line, these alleles are reactivated or deleted, respectively, in a molecularly defined fashion (Tables 1 and 2). As expected, mice homozygous for the disrupted *Mc4r*^{loxTB/loxTB} allele phenocopy the global *Mc4r*^{−/−} knockout⁵³. However, neuron-specific reactivation of the *Mc4r*^{loxTB/loxTB} allele completely ameliorates obesity, indicating a central site of action for energy balance regulating MC4Rs⁵³. More specifically, this effect is attributable to the expression of MC4Rs on glutamatergic neurons, since re-expression of the *Mc4r*^{loxTB/loxTB} allele using a *vGlut2*-(*Slc17a6*)-ires-Cre line completely rescues body weight, adiposity, hyperphagia, energy expenditure, axial length and glucose homeostasis⁵⁴. Correspondingly, while *Mc4r*^{loxP/loxP} mice are phenotypically normal, deletion of this allele from vGLUT2 neurons promotes a metabolic phenotype comparable to global *Mc4r* nullizygosity⁵⁴. Re-expression or deletion of MC4Rs on GABAergic neurons has no discernible phenotypic effect⁵⁴. Thus, whatever the identity of the MC4R neurons responsible for the pleiotropy of the *Mc4r*^{−/−} mouse, they are most likely glutamatergic.

Although CNS MC4Rs have been the predominant focus for energy balance research, recent work has highlighted the contribution of MC4Rs in enteroendocrine L-cells of the intestine. These have been demonstrated to (i) regulate luminal electrolyte secretion, to potentially reduce gastric motility and enhance between-meal satiety, and (ii) promote release of the episodic satiety factors glucagon-like peptide-1 (GLP1) and peptide YY (PYY), potentially enhancing centrally mediated MC4R satiety⁵⁵. The functional significance of these observations remains unknown, but is noteworthy given the correlation between MC4R signaling and the weight-loss efficacy of bariatric surgeries^{56,57}.

Energy intake. Pharmacological studies have been critical in directing genetic dissection of the melanocortin system. While MC4Rs in numerous sites are neuroanatomically positioned to promote satiety, at least when pharmacology probed^{38,58,59}, it is the PVH that has garnered the most attention. Highlighted by the importance of the PVH to energy homeostasis⁶⁰ and robust MC4R expression^{35,53}, micro-injections of α -MSH or AgRP into this region engender satiety and hunger, respectively, at levels beyond that seen at any other site⁶¹, suggesting that PVH^{MC4Rs} represents a point of functional convergence for counteractive ARC^{AgRP} and ARC^{POMC} neurons.

The ontology of the PVH is defined by the transcription factor Single-minded 1 (Sim1). Developmental haploinsufficient *Sim1*^{+/-} (refs. 62,63) and postnatal *Sim1*^{−/−} (ref. 64) mutant mice exhibit profound obesity, hyperphagia and increased axial length, in a manner reminiscent of the global *Mc4r*^{−/−} mutant, but retain normal

Table 1 Energy homeostasis in *Mc4r* mutant mice

Mouse line	Body weight	Energy intake	Energy expenditure	Glucose homeostasis	Autonomic outflow	Other phenotypes	Ref.
<i>Mc4r</i> ^{-/-} Mixed background	Increased body weight from 5 weeks	Hyperphagic. 46% increase in average daily consumption	NR	Hyperglycemic and hyperinsulinemic (more pronounced in males)	NR	Increased axial length. Normal serum corticosterone levels.	16
<i>Mc4r</i> ^{-/-} Mixed background	Pair-fed male mice show normal body weight. Pair-fed female mice show intermediate body weight.	Non-pair-fed mice hyperphagic at 14 weeks of age (not at 10 weeks)	Reduced basal VO ₂ consumption at 7 weeks (precedes obesity). Non-pair-fed males hypolocomotive during dark cycle. Normal basal body temperature and response to cold exposure	Non-pair-fed mice hyperglycemic and hyperinsulinemic. Pair-fed mice normal glucose and insulin levels	Insensitive to leptin-mediated BAT Ucp1 induction	NR	48
<i>Mc4r</i> ^{-/-} Congenic BL/6 (3–5 generations)	Increased body weight. Exaggerated weight gain on HCD.	Hyperphagic. Normal body weight response to caloric restriction. Normal feeding response to overnight fasting. Increased consumption on high-fat diet.	Reduced basal VO ₂ consumption. Reduced exergonic response to HCD. Increased respiratory exchange ratio on HCD. Normal basal body temperature and response to cold exposure.	Pre-obese mice are normoglycemic and normoinsulinemic. Obese mice show hepatic insulin resistance.	Reduced exergonic response to HCD not due to reduced SNS outflow to BAT	Normal body weight and feeding response to cold exposure. Reduced circulating triglycerides. Increased hepatic fatty acid synthesis.	47, 81, 91
<i>Mc4r</i> ^{-/-} Mixed background	Increased body weight from 5 weeks	Hyperphagic from weaning	Pre-obese mice exhibit increased metabolic rate	NR	NR	NR	134
<i>Mc4r</i> ^{-/-} Mixed background	NR	NR	NR	NR	NR	Increased circulating high-density lipoprotein	93
<i>Mc4r</i> ^{-/-} Mixed obesity-resistant background	Increased body weight and adiposity from 5 weeks	Hyperphagic. Pre-obese mice responsive to anorectic effect of leptin.	NR	NR	Insensitive to leptin-mediated BAT <i>Ucp1</i> induction	NR	135
<i>Mc4r</i> ^{-/-} Mixed background	Increased body weight from 5 weeks	Insensitive to anorectic effects of MTII	Insensitive to exergonic effects of MTII. Hypolocomotive during the dark cycle.	NR	NR	NR	136
<i>Mc4r</i> ^{-/-} Mixed background	NR	Insensitive to anorectic effects of MTII. Pre-obese mice responsive to anorectic effect of leptin. Obese mice unresponsive to anorectic effect of leptin. Mice sensitive to orexigenic effects of NPY.	NR	NR	NR	NR	137
<i>Mc4r</i> ^{-/-} Mixed background (females)	NR	Insensitive to anorectic effects of CCK	NR	NR	NR	NR	138
<i>Mc4r</i> ^{-/-} Mixed background	NR	Higher breakpoint in a progressive-ratio lever-pressing assay	NR	NR	NR	NR	50
<i>Mc4r</i> ^{-/-} Mixed background	NR	NR	NR	NR	NR	No antisecretory response from VIP-stimulated intestinal L-cells. Reduced PYY release.	55
<i>Mc4r</i> ^{-/-} Congenic BL/6	Increased body weight from 7 weeks and increased adiposity	Hyperphagic	NR	Hyperglycemic. Hyperinsulinemic.	NR	Obese mice normotensive, bradycardic, normal hemodynamic response to high salt diet. Obese and pair-fed mice insensitive to hypertensive effects of leptin.	86, 139
<i>Mc4r</i> ^{loxTB/loxTB} Mixed background	Increased body weight from 4 weeks	Hyperphagic. Insensitive to anorectic effects of MTII.	Reduced VO ₂ consumption	Hyperglycemic and hyperinsulinemic from 5 weeks of age	Normotensive	Increased axial length	53, 82

(continued)

Table 1 Energy homeostasis in *Mc4r* mutant mice (continued)

Mouse line	Body weight	Energy intake	Energy expenditure	Glucose homeostasis	Autonomic outflow	Other phenotypes	Ref.
<i>Mc4r^{loxTB/loxTB}</i>	NR	NR	NR	Exaggerated fasting glucose levels and glucose intolerance during the dark cycle. Constant light conditions improves glycemia.	NR	NR	140
<i>Mc4r^{loxTB/loxTB}</i> Congenic 129 and mixed background	Increased body weight from 7 weeks	NR	NR	NR	NR	Reduced sensitivity to Roux-en-Y gastric bypass	56
<i>Zp3-Cre::Mc4r^{loxTB/loxTB}</i> Mixed background	Normal body weight	NR	NR	NR	NR	NR	53
<i>Nestin-Cre::Mc4r^{loxTB/loxTB}</i> Mixed background	Normal body weight	NR	NR	NR	NR	Normalized axial length	53
<i>Sim1-Cre::Mc4r^{loxTB/loxTB}</i> Mixed background	60% reduction in body weight and reduced fat and lean mass	Normalized food intake. Re-sensitized to anorectic effect of MTII	Reduced VO ₂ consumption. Hypometabolism precedes hyperphagia. Insensitive to exergonic effects of MTII.	Mildly improved serum glucose and insulin levels at 5 weeks. Normalized glucose levels and at improved insulin levels 12 weeks	Normotensive	Normalized axial length	53, 67, 82
<i>Chat-ires-Cre::Mc4r^{loxTB/loxTB}</i>	10% reduction in body weight and reduced fat mass	Hyperphagic	Normalized VO ₂ consumption	Mildly improved serum glucose and insulin levels. Insulin resistant.	Hypertensive during dark cycle	Increased axial length	69, 82
<i>Phox2b-Cre::Mc4r^{loxTB/loxTB}</i>	No improvement in body weight or fat mass	Hyperphagic	No improvement in VO ₂ consumption	No improvement in serum glucose levels. Normoinsulinemic and not insulin resistant	NR	Increased axial length	69
<i>Sim1-Cre::Mc4r^{loxTB/loxTB}::vGlut2^{loxP/loxP}</i>	No improvement in body weight or fat mass	Hyperphagic	No improvement in VO ₂ consumption. Insensitive to exergonic effects of MTII	NR	NR	NR	67
<i>vGlut2-ires-Cre::Mc4r^{loxTB/loxTB}</i>	Normalized body weight	Normalized food intake	Normalized VO ₂ consumption	Normalized serum glucose and insulin levels		Normalized axial length	54
<i>vGat-ires-Cre::Mc4r^{loxTB/loxTB}</i>	No improvement in body weight	NR	NR	NR	NR	NR	54
<i>Oxt-ires-Cre::Mc4r^{loxTB/loxTB}</i>	No improvement in body weight	NR	NR	NR	NR	NR	54
<i>Crh-ires-Cre::Mc4r^{loxTB/loxTB}</i>	No improvement in body weight	NR	NR	NR	NR	NR	54
<i>Pdyn-ires-Cre::Mc4r^{loxTB/loxTB}</i>	No improvement in body weight	NR	NR	NR	NR	NR	54
<i>Avp-ires-Cre::Mc4r^{loxTB/loxTB}</i>	No improvement in body weight	NR	NR	NR	NR	NR	54
<i>Drd1-cre::Mc4r^{loxTB/loxTB}</i>	Mild improvement in body weight and fat mass	Mild improvement in hyperphagia on chow. Reduction in meal size. Partial restoration of MTII sensitivity.	No improvement in VO ₂ consumption	NR	NR	NR	49
<i>Mc4r^{loxP/loxP}</i>	Normal body weight	NR	NR	NR	NR	NR	54
<i>Ell1a-Cre::Mc4r^{loxP/loxP}</i>	Increased body weight from 5 weeks. Increased fat and lean mass.	Hyperphagic	NR	NR	NR	Increased axial length	54
<i>Sim1-Cre::Mc4r^{loxP/loxP}</i>	Increased body weight from 6 weeks. Increased fat mass.	Hyperphagic	NR	NR	NR	Increased axial length	54
<i>Chat-ires-Cre::Mc4r^{loxP/loxP}</i>	Increased body weight from 8 weeks. 40% increase by 20 weeks. Increased fat and lean mass. Increased sensitivity to HCD.	Normal feeding behavior. Hyperphagic on HCD. Reduced <i>Ucp1</i> induction on HCD.	Reduced energy expenditure, intermediate to <i>Mc4r^{ΔΔ}</i> . Reduced diet-induced hypermetabolism. Normal body temperature	Hyperglycemia and hyperglucagonemia comparable to <i>Mc4r^{ΔΔ}</i> . Intermediate hyperinsulinemia. Insulin resistant.	Reduced thermogenic response to cold exposure. Reduced <i>Ucp1</i> induction on cold exposure or high-calorie diet.	Normal axial length	70
<i>Phox2b-Cre::Mc4r^{loxP/loxP}</i>	No effect on body weight or composition. Normal sensitivity to HCD.	Normal feeding behavior on HCD	Normal exergonic response to a HCD	Normoglycemic and normoglucagonemic. Hyperinsulinemic.	Normal <i>Ucp1</i> induction	NR	70

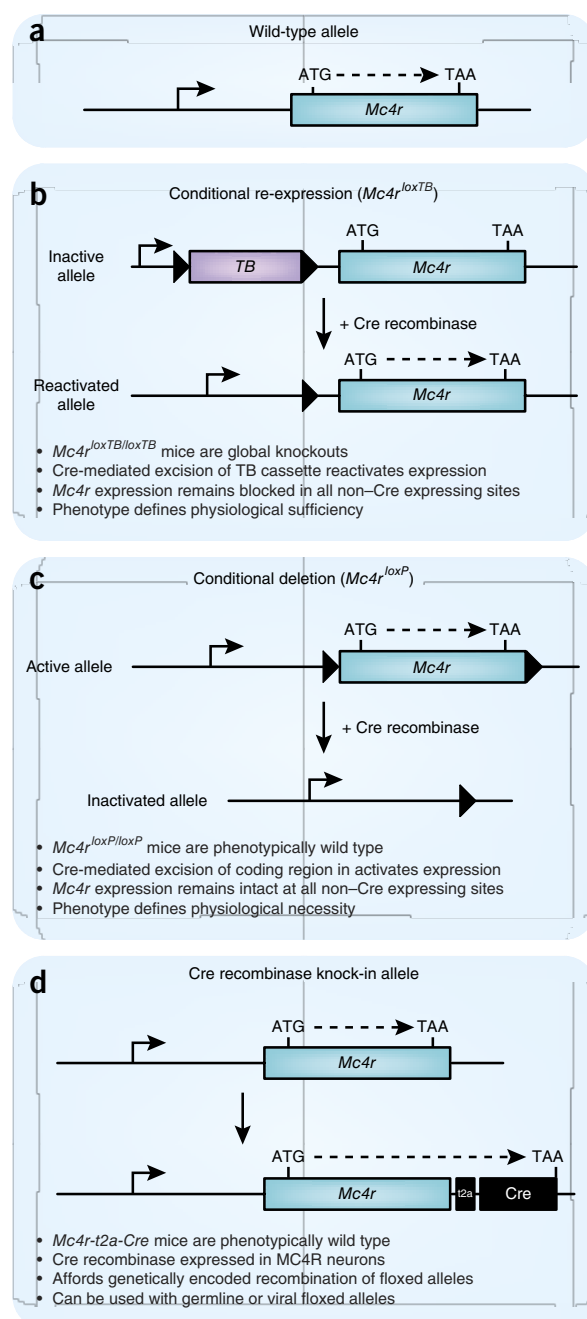
Phenotypes arising from the germline and conditional genetic manipulations of mouse *Mc4r* expression. NR, not reported; CCK, cholecystokinin; HCD, high calorie diet.

Figure 1 Genetically modified *Mc4r* alleles. (a) Wild-type mouse *Mc4r* locus. (b,c) Conditional *Mc4r* alleles for the molecularly defined Cre-dependent re-expression (b) or deletion (c) of the *Mc4r* locus. (b) Conditional re-expression is achieved through the insertion between the transcriptional start site and the *Mc4r* coding region of a transcriptional blocking (TB) cassette flanked by *loxP* sites (triangles). Cre-dependent excision of the region between the *loxP* sites removes the TB cassette and re-establishes *Mc4r* expression in Cre-expressing cells only. (c) Conditional deletion is achieved through the insertion of *loxP* sites flanking the *Mc4r* coding region. Cre-dependent excision of the region between the *loxP* sites deletes the coding region and inactivates the *Mc4r* locus in Cre-expressing cells only. (d) A knock-in *Mc4r-t2a-Cre* driver line was generated by inserting a Cre recombinase cassette downstream of the *Mc4r* stop codon.

energy expenditure. Notably, *Sim1*^{+/-} mice exhibit resistance to the hypophagic, but not exergonic, effects of intra-PVH melanotan II (MTII) administration⁶⁵, suggesting that PVH^{Sim1::MC4R}-expressing neurons are a critical component of MC4R-regulated appetite but not energy expenditure. To address this directly, a BAC (bacterial artificial chromosome) transgenic *Sim1-Cre* line was used to drive molecularly defined recombination of conditional *Mc4r* alleles. Selective restoration of MC4Rs to *Sim1*-expressing neurons (*Sim1-Cre::Mc4r^{loxTB/loxTB}*) on an otherwise *Mc4r*-null background was found to reduce body weight by ~60%, owing primarily to decreased adiposity and rescued axial length⁵³. Remarkably, this reduction in body weight is defined by a complete normalization of feeding behavior, but no improvement in energy expenditure⁵³. *Sim1-Cre::Mc4r^{loxTB/loxTB}* mice also exhibit restored sensitivity to the hypophagic, but not hypermetabolic, effects of MTII⁵³. The deletion of MC4R-expressing *Sim1*-positive neurons (*Sim1^{MC4Rs};* *Sim1-Cre::Mc4r^{loxP/loxP}*) results in reciprocal hyperphagia and obesity⁵⁴. However, these mice were lighter than *vGlut2-ires-Cre::Mc4r^{loxP/loxP}* mice, indicating that other glutamatergic MC4R populations, regulating energy expenditure, contribute to body weight. Therefore, *Sim1^{MC4Rs}* neurons are both sufficient and necessary for the physiological control of homeostatic feeding and body weight, but not energy expenditure, indicating a functional divergence in the energy-balance melanocortineric circuitry.

Although these effects were attributed to MC4R expression in PVH^{Sim1} neurons, allelic recombination in other brain areas demarked by *Sim1-Cre* expression⁵³ or unobserved developmental compensatory mechanisms could contribute to the observed phenotypes. To circumvent these obstacles, postnatal specific recombination was achieved through the stereotaxic injection of a Cre-expressing adeno-associated virus (AAV-Cre) into distinct neuroanatomical structures of adult *Mc4r^{loxTB/loxTB}* and *Mc4r^{loxP/loxP}* mice. Consistent with the *Sim1-Cre::Mc4r^{loxP/loxP}* phenotype, AAV-Cre deletion of PVH^{MC4Rs} results in hyperphagia, increased adiposity and obesity⁵⁴, while PVH-specific *Mc4r* re-expression in *Mc4r^{loxTB/loxTB}* mice reciprocally reduces the obesity and hyperphagia associated with *Mc4r* nullizygosity^{53,54}. In contrast, rescued *Mc4r* expression in the medial amygdala⁵⁴, the lateral parabrachial nucleus (LPBN)⁵⁴, the nucleus of the solitary tract (NTS) and dorsomedial nucleus of the vagus (DMV)⁵⁴, or the lateral hypothalamus⁶⁶ has no effect on feeding behavior. Thus, PVH^{Sim1::MC4Rs} are the main component of MC4R-regulated satiety.

To further refine the neurochemical subtype of energy-balance-regulating PVH^{MC4R} neurons, a battery of knock-in Cre-expressing mice, demarking specific subpopulations of PVH neurons, was used to determine the sufficiency of *Mc4r* reactivation in a neurochemically explicit manner. Re-expression of MC4Rs in neurons expressing corticotropin-releasing hormone (*Crh-ires-Cre*) and/or oxytocin (*Oxt-ires-Cre*), arginine vasopressin (*Avp-ires-Cre*) or pro-dynorphin



(*Pdyn-ires-Cre*) does not reduce body weight⁵⁴, suggesting that MC4Rs expressed on any one of these populations alone does not contribute to energy homeostasis. However, regardless of the uncertain neuropeptidergic identity of PVH^{Sim1::MC4R} neurons, it is likely that their body-weight-regulating function is primarily contingent on their release of glutamate, since re-expression of *Mc4r* and concomitant deletion of *vGlut2* in *Sim1* neurons nullifies the improvement in *Sim1-Cre::Mc4r^{loxTB/loxTB}* phenotype⁶⁷.

As yet, the only other population of MC4Rs to be genetically implicated in the regulation of feeding are those demarked by a dopamine-1 receptor (*Drd1*)-Cre line⁴⁹. *Drd1-Cre::Mc4r^{loxTB/loxTB}* mice exhibit a small reduction in hyperphagia (but no improvement in energy expenditure) that results in a 10% decrease in body weight⁴⁹. While the specific population of *Drd1^{MC4R}* expressing neurons responsible for this phenotype remains to be determined, it is possible that partial

Table 2 Cre driver lines used for *Mc4r* manipulation

Cre driver line	Type	Selectivity	Main sites of neuroanatomical overlap with <i>Mc4r</i>	Ref.
<i>Nestin (Nes)</i>	Transgene	Pan-neuronal	All sites	53
<i>vGlut2 (Slc17a6)</i>	Knock-in	Vesicular glutamate transporter (excitatory neurons)	Most glutamatergic sites	54
<i>vGat (Slc32a1)</i>	Knock-in	Vesicular GABA transporter (inhibitory neurons)	Most GABAergic sites	54
<i>Sim1</i>	Transgene	Single-minded 1	PVH, MeA, NLOT, PH	53
<i>Chat</i>	Knock-in	Choline acetyltransferase (cholinergic neurons)	DMV, IML, LDTg, PPTg	69
<i>Phox2b</i>	Transgene	Paired-like homeobox 2b	DMV, NTS, NG	69
<i>Drd1</i>	Transgene	Dopamine-1 receptor	NAC, PVH,	49
<i>Oxt</i>	Knock-in	Oxytocin	PVH	54
<i>Avp</i>	Knock-in	Arginine vasopressin	PVH	54
<i>Pdyn</i>	Knock-in	Pro-dynorphin	PVH, VMH, DMH, LH, DRN, LPBN	54
<i>Crh</i>	Knock-in	Corticotropin-releasing hormone	PVH	54

Cre driver lines used for manipulation of *Mc4r* conditional alleles. The genetic nature of each Cre line and its neuronal selectivity is provided. The listing of main sites of anatomical overlap defines only the most abundant sites of neuroanatomical coexistence between *Mc4r* and each Cre driver gene and not necessarily cellular coexpression. DMH, dorsomedial nucleus of the hypothalamus; DMV, dorsal motor nucleus of the vagus; DRN, dorsal raphe nucleus; IML, intermediolateral nucleus of the spinal cord; LDTg, laterodorsal tegmental nucleus; LH, lateral hypothalamus, LPBN, lateral parabrachial nucleus; MeA, medial amygdala; NAC, nucleus accumbens; NG, nodose ganglion; NLOT, nucleus of the lateral olfactory tract; NTS, nucleus of the solitary tract; PH, posterior hypothalamus; PPTg, pedunculopontine tegmental nucleus; PVH, paraventricular nucleus of the hypothalamus; VMH, ventromedial nucleus of the hypothalamus.

coexpression (and subsequent reactivation)⁶⁸ in the PVH contributes to this mild improvement in energetic balance.

Energy expenditure. The incomplete rescue of body weight in *Sim1-Cre::Mc4r^{loxTB/loxTB}* mice, despite complete normalization of feeding behavior, is underscored by persistent hypometabolism comparable to that in *Mc4r*-deficient mice⁵³. Concordantly, *Sim1-Cre::Mc4r^{loxTB/loxTB}* animals are insensitive to the exergonic effects of MC4R agonism⁵³, confirming that *Sim1*^{MC4Rs} are not responsible for MC4R-regulated energy utilization. Genetic studies have since identified MC4Rs on cholinergic preganglionic sympathetic neurons of the intermediolateral nucleus of the spinal cord (IML) as the critical population^{69,70}. These neurons, like their parasympathetic counterparts in the DMV, are defined by their expression of choline acetyltransferase (ChAT). *Chat-ires-Cre*-mediated recombination of the *Mc4r^{loxTB/loxTB}* allele therefore enables re-expression of MC4Rs on the two efferent arms of the autonomic nervous system (ANS). *Chat-ires-Cre::Mc4r^{loxTB/loxTB}* mice, while still obese, exhibit a 10% drop in body weight that is entirely facilitated by the reinstatement of normal energy expenditure, with no reduction in food intake⁶⁹. To functionally distinguish the relative contribution of IML^{MC4Rs} and DMV^{MC4Rs}, a subtractive genetic strategy used a *Phox2b-Cre* line that labels preganglionic parasympathetic DMV neurons but not preganglionic IML neurons. Unlike *Chat-ires-Cre::Mc4r^{loxTB/loxTB}* mice, *Phox2b-Cre::Mc4r^{loxTB/loxTB}* mice do not exhibit improved energy expenditure, suggesting that it is IML^{MC4Rs} that define this function⁶⁹. Similarly, deletion of MC4Rs from *Chat*-expressing, but not *Phox2b*-expressing, neurons results in decreased oxygen consumption and in subsequent obesity, with no effect on food consumption⁷⁰. Although ChAT^{MC4Rs} undoubtedly contribute to the hypometabolic state of *Mc4r^{-/-}* mice they are seemingly not the totality of energy-expenditure MC4R neurons, since *Mc4r^{-/-}* mice still exhibit lower energy expenditure than *Chat-ires-Cre::Mc4r^{loxP/loxP}* mice⁷⁰. On the basis of pharmacological data, it is possible that MC4Rs expressed in the median pre-optic nucleus and/or DMH may contribute to some elements of melanocortin-regulated energy expenditure⁷¹.

The role of ChAT^{MC4Rs} neurons in energy expenditure is most readily observed in the adaptive response to environmental challenge,

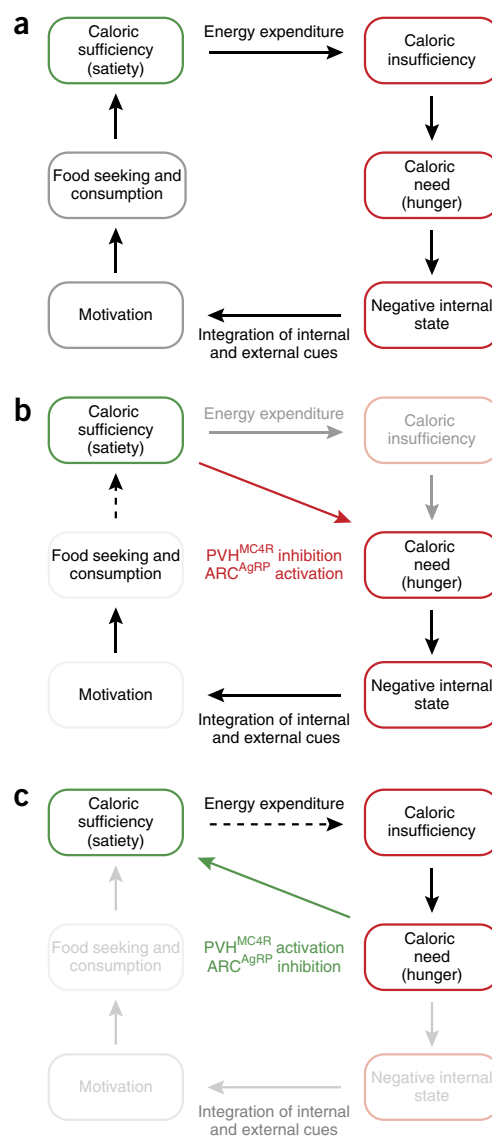
such as exposure to a high-calorie diet (HCD) or a cold environment. Wild-type mice presented with a HCD exhibit hyperthermia and increased energy expenditure that restrain body weight gain; *Mc4r^{-/-}* and *Chat-ires-Cre::Mc4r^{loxP/loxP}* mice, by contrast, fail to enact these adaptive responses^{47,70}. Mechanistically, this elevation in body temperature is mediated by brown adipose tissue (BAT), which is potentially activated by the sympathetic nervous system (SNS) and a vital component of melanocortinergic adaptive thermogenesis^{47,72–74}. SNS outflow to BAT promotes mitochondrial uncoupling, leading to proton leakage and the generation of heat. This process depends on mitochondrial uncoupling protein-1 (UCP1)⁷⁵. Both *Mc4r^{-/-}* and *Chat-ires-Cre::Mc4r^{loxP/loxP}* mice fail to upregulate UCP1 expression in response to a HCD, suggesting that compromised MC4R-mediated SNS outflow to BAT⁷⁰ contributes to their sensitivity to an obesogenic diet. Although UCP1 expression

can also be regulated by the hypothalamic-pituitary-thyroid axis, this system is not a functional target for the MC4R dietary response, since *Mc4r^{-/-}* mice exhibit normal thyroid hormone levels and apposite thyroid responses to a HCD⁷². Acute cold exposure similarly engenders SNS-regulated BAT-mediated thermogenesis that is required for maintaining core body temperature. In this regard it would seem that MC4Rs do not contribute, since two separate studies found *Mc4r^{-/-}* mice to exhibit normal basal body temperature in ambient conditions and apposite thermogenic responses to cold exposure^{47,48}.

Autonomic outflow to white adipose tissue (WAT), the silo for excess caloric energy, is important in regulating lipid metabolism and composition⁷⁶. Sympathetically mediated ‘beiging’ of BAT-like progenitors in WAT has been proposed as an adaptive response to environmental stressors, such as cold exposure and obesogenic diet⁷⁷. This evoked development of UCP1-expressing beige cells elevates body temperature and energy expenditure. Central MC4R-expressing neurons in several sites are anatomically connected to WAT⁷⁸. Notably, cold-exposed *Mc4r^{-/-}* and *Chat-ires-Cre::Mc4r^{loxP/loxP}* mice, but not *Phox2b-Cre::Mc4r^{loxP/loxP}* mice, fail to show evidence of beiging⁷⁰, suggesting that MC4R-regulated SNS outflow to WAT is required for the induction of this adaptive response. Whether a similar mechanism underlies diet-induced beiging remains to be determined. Real-time chemogenetic activation of ARC^{POMC} neurons drives an SNS-dependent beiging effect¹², implicating an ARC^{POMC}→IML^{MC4R} circuit in this process.

Glucose homeostasis. *Mc4r* deficiency in mice is associated with hyperglycemia and hyperinsulinemia that precede the onset of obesity but are exacerbated by fat accrual^{16,48,79}. By contrast, human MC4R deficiency results in euglycemic hyperinsulinemia during childhood but a normalization of insulin levels during adulthood, with no manifestation of diabetes^{51,52,80}. Thus, it would seem that at least the initiating hyperinsulinemic state is conserved in the mouse and human condition, with onset of diabetes in adult *Mc4r^{-/-}* mice potentially defined by subsequent body-weight-dependent insulin resistance. Furthermore, congenic strains of *Mc4r^{-/-}* mice, while still being obese, do not exhibit hyperglycemia or hyperinsulinemia⁸¹, suggesting that there is a role for strain-specific (body-weight-independent)

Figure 2 Melanocortins and the motivational basis of feeding. (a) Caloric insufficiency arising from energy expenditure creates a negative state of tension that represents caloric need and is interoceptively recognized as hunger. This caloric need (hunger) carries a negative valence that provides the motivation to rectify the homeostatic disturbance, which must also take into consideration other internal and external cues before initiating goal-directed food seeking and consumption. Food detection and caloric ingestion relieves the aversive state of hunger, reducing the initiating motivation, and thus satiety carries a positive valence. Experimental manipulation of melanocortinergic neurons bypasses the drive-reduction circuit and artificially creates the states of hunger (b) and satiety (c). (b) A perceived state of caloric need is created upon the activation of ARC^{AgRP} neurons or inhibition of PVH^{MC4R} neurons, artificially enhancing the motivation to consume food despite physiological caloric sufficiency. Consistent with the negative valence of hunger, the activation of ARC^{AgRP} neurons is aversive. (c) A perceived state of caloric sufficiency is created upon the inhibition of ARC^{AgRP} neurons or activation of PVH^{MC4R} neurons, artificially diminishing the motivation to consume food despite physiological caloric insufficiency. Consistent with the positive valence of satiety, the activation of $PVH^{MC4R}(\rightarrow LPBN)$ neurons or inhibition of ARC^{AgRP} neurons is appetitive. Red represents negative valence; green, positive valence. AgRP, agouti-related peptide; ARC, arcuate nucleus of the hypothalamus; MC4R, melanocortin-4 receptor; PVH, paraventricular nucleus of the hypothalamus.



genetic modifiers in the etiology of glycemic dysregulation. It is possible that such genetic nuances also explain the phenotypic differences between *Mc4r* and *MC4R* nullizygous mice and humans.

Centrally governed glycemia is regulated by the ANS. $Chat^{Mc4r}$ re-expression, including re-expression on preganglionic autonomic neurons, moderately improves hyperglycemia and hyperinsulinemia, as compared to that in *Mc4r^{loxTB/loxTB}* controls⁶⁹. Reciprocally, *Mc4r* deletion in *Chat-ires-Cre::Mc4r^{loxP/loxP}* mice promotes a diabetic hyperinsulinemic phenotype⁷⁰. To dissociate the contributions of the two arms of the ANS, the same measures were taken in *Phox2b-Cre::Mc4r^{loxTB/loxTB}* and *Phox2b-Cre::Mc4r^{loxP/loxP}* mice. *Phox2b^{Mc4r}* re-expression results in normalized insulin levels but persistent hyperglycemia⁶⁹; *Phox2b-Cre::Mc4r^{loxP/loxP}* mice are mildly hyperinsulinemic (although not to the extent of *Mc4r^{-/-}* controls) but normoglycemic⁷⁰. Together these results suggest that that preganglionic parasympathetic *Phox2b^{MC4Rs}* suppress PNS outflow to inhibit pancreatic insulin release, but that this does not influence overall glycemic state or insulin sensitivity, while preganglionic sympathetic $Chat^{MC4Rs}$ do not directly influence insulin release but do set general glycemic tone.

Given that PNS outflow stimulates insulin release, this implies that $MC4Rs$ inhibit rather than activate DMV neurons. Indeed, $MC4R$ agonists cause hyperpolarization at most DMV^{ChAT} neurons⁸² (see below). Thus, global or DMV-specific *Mc4r* deficiency results in exaggerated PNS outflow to the pancreas and hyperinsulinemia. However, the role of $Chat^{MC4Rs}$ is seemingly more complex. Specifically, given the stimulatory effect⁸² of IML^{MC4Rs} , and given that SNS outflow promotes glucose production and excursion, it is surprising that the sympatho-suppressed state of *Mc4r^{-/-}* and *Chat-ires-Cre::Mc4r^{loxP/loxP}* mice actually drives a sympatho-excitatory phenotype (increased glucose excursion and reduced glucose disposal)⁷⁰. It is possible that it is in fact body composition that is the main defining factor in this phenotype, such that the mildly reduced body weight of *Chat-ires-Cre::Mc4r^{loxTB/loxTB}* mice⁶⁹ and the obesity of *Chat-ires-Cre::Mc4r^{loxP/loxP}* mice⁷⁰ explains their improvement and deterioration in glycemic state, respectively. This notion is supported by the normoglycemia exhibited by *Mc4r^{-/-}* mice paired to wild type levels⁴⁸ and *ad libitum* fed *Sim1-Cre::Mc4r^{loxTB/loxTB}* mice⁵³, which have reduced body weight. However, it is likely that the $MC4R$ is involved in least some element of glycemic

control, independently of its body weight effects, since AAV-Cre mediated *Mc4r* re-expression in the lateral hypothalamus mildly improves SNS-dependent glucose tolerance (but not basal glycemic state) in adult *Mc4r^{loxTB/loxTB}* mice, without altering body weight or composition⁶⁶.

Additional autonomic functions. Central administration of α -MSH elevates blood pressure and heart rate in an SNS- and $MC4R$ -dependent manner^{83,84}. This corollary of wholesale $MC4R$ activation has significantly hampered the development of $MC4R$ -based weight-loss pharmacotherapies⁸⁵. Consistent with an $MC4R$ pressor effect, *Mc4r^{-/-}* mice exhibit paradoxical normotension and tachycardia, despite being obese, hyperinsulinemic and hyperleptinemic⁸⁶. However, the re-establishment of autonomic tone in *Chat-ires-Cre::Mc4r^{loxTB/loxTB}* mice unmasks a hypertensive phenotype in accord with their obese state⁸², suggesting that direct $MC4R$ engagement of preganglionic autonomic neurons underlies their regulation of cardiovascular function. In obese $MC4R$ -deficient humans, most of whom are similarly normotensive and tachycardic⁸⁰, there is evidence of both PNS and SNS involvement, consistent with *MC4R* nullizygosity engendering both parasympatho-excitation and sympatho-suppression (for review, see ref. 87). The contribution of DMV^{MC4Rs} to the *Mc4r^{-/-}*

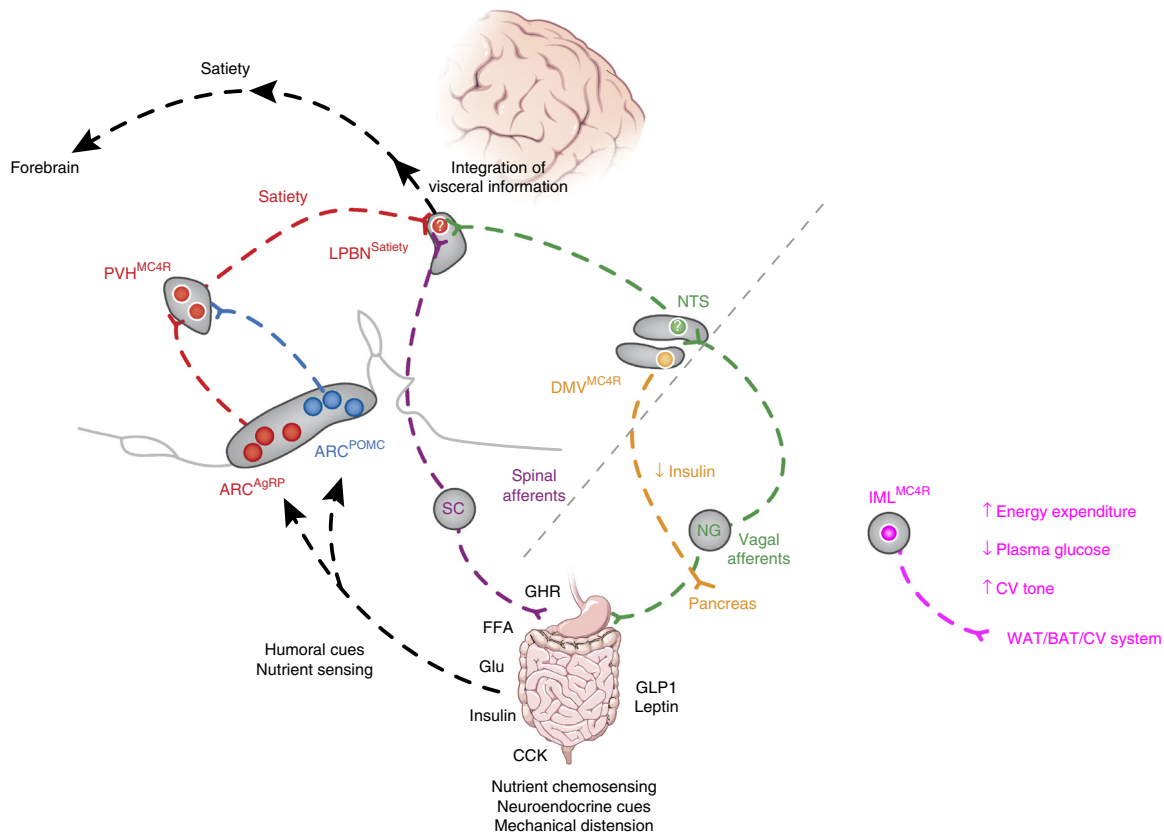


Figure 3 Functional topography of *Mc4r* function. The conditional and AAV-mediated genetic manipulation of *Mc4r* expression has demonstrated a neuroanatomical dissociation of physiological function. PVH^{MC4R} s are the predominant energy-intake-regulating population (and also govern body length) but do not influence energy expenditure. These neurons receive monosynaptic inhibitory input from ARC^{AgRP} neurons and are necessary for the full $ARC^{AgRP} \rightarrow PVH$ feeding phenotype. Real-time optogenetic interrogation identified the LPBN as the site of functional outflow for appetite-regulating PVH^{MC4R} neurons. Furthermore, $PVH^{MC4R} \rightarrow LPBN$ satiety carries a positive valence, contrasting the negative valence associated with hunger-promoting ARC^{AgRP} neurons. $PVH^{MC4R} \rightarrow LPBN$ neurons are depolarized by an MC4R agonist, suggesting that they are activated by upstream satiety-promoting ARC^{POMC} neurons, although this remains to be proven (blue line). A subtractive conditional genetic approach has implicated *Mc4r* expression on preganglionic parasympathetic neurons of the DMV in the tonic inhibition of pancreatic insulin release but not overall glycemic state or energy expenditure. MC4Rs on preganglionic sympathetic neurons of the IML govern overall sympathetic tone, leading to increased energy expenditure, hypertension and decreased glucose output. Red, $ARC^{AgRP} \rightarrow PVH^{MC4R}$ satiety pathway; green, vagal afferents; purple, spinal afferents; yellow, parasympathetic nervous system; magenta, sympathetic nervous system; question mark, neuronal population of unknown identity. AgRP, agouti-regulated peptide; ARC, arcuate nucleus of the hypothalamus; CCK, cholecystokinin; CV, cardiovascular; DMV, dorsal motor nucleus of the vagus; FFA, free fatty acids; GHR, ghrelin; GLP1, glucagon-like peptide-1; IML, intermediolateral nucleus of the spinal cord; LPBN, lateral parabrachial nucleus; NG, nodose ganglion; NTS, nucleus of the solitary tract; MC4R, melanocortin-4 receptor; POMC, pro-opiomelanocortin; PVH, paraventricular nucleus of the hypothalamus; SC, spinal cord.

knockout cardiovascular phenotype remains unexplored. Despite the preautonomic role of the PVH and the mild pressor effect observed upon intra-PVH agonist infusion^{88–90}, $Sim1^{MC4R}$ re-expression does not cause hypertension. However the non-obese nature of these animals may mean that, even if autonomic tone were partially rescued (which remains unknown), there is no initiating pathology to drive hypertension⁸². Dissociating the neural mechanisms underlying the MC4R pressor effect will be critical for the generation of energy-balance-regulating MC4R compounds devoid of cardiovascular side effects for the safe and effective treatment of obesity.

Further to its effects on WAT composition, MC4R-regulated ANS outflow modulates peripheral lipid metabolism to influence the deposition and circulation of fat. *Mc4r*- and *MC4R*-deficient mice⁹¹ and humans⁹² exhibit elevated respiratory quotients, indicative of a shift from fat utilization to fat storage. This perturbation in substrate utilization exacerbates adipose tissue expansion and weight gain beyond that defined simply by a net positive energy balance. In light of MC4R expression in preganglionic IML neurons⁶⁹, as well as in preautonomic outflow neurons innervating WAT⁷⁸, it is likely that these

phenotypes are defined by generalized sympho-suppression. Indeed, pharmacological blockade of central MC4R signaling engenders an increase in respiratory quotient, WAT lipid content and lipogenic gene expression in an SNS-dependent, but body-weight-independent, manner⁹². Furthermore, MC4R engagement of the parasympathetic nervous system regulates hepatic cholesterol uptake such that genetic or pharmacological inactivation of central murine⁹³ (but not human⁸⁰) MC4R signaling elevates circulating levels of cholesterol and high density lipoprotein:cholesterol complexes. Thus, MC4R-regulated autonomic tone influences nutrient handling and lipid metabolism independently of obesity to contribute to metabolic fitness.

***PVH^{MC4R}* neural circuitry.** Conditional genetic manipulations have been fundamental to defining the neuroanatomical landscape of *Mc4r*-regulated physiology. However, the neural interactions underlying functional outflow can only be resolved through the application of circuit-level cellular technologies. Unlike pharmacological and germline genetic approaches, such tools do not directly address the contribution of an individual molecular component, but rather provide insight

into the significance of an explicit population (as defined by a Cre driver mouse line; **Fig. 1**) expressing that component.

Consistent with role of PVH^{MC4R}s in the regulation of energy intake^{53,54} chemogenetic activation of Cre-expressing PVH^{MC4R} neurons promotes satiety during times of physiological hunger, indicating that they are neuroanatomically positioned to suppress homeostatic feeding³⁵. Activation of PVH^{MC4R} neurons has no effect on energy expenditure, validating prior genetic observations⁵³. Loss-of-function studies further demonstrate that these neurons are necessary for the maintenance of satiety, such that their inactivation during times of caloric sufficiency results in increased goal-directed light-cycle food consumption³⁵. Given the functional reciprocity of the melanocortin ligands, the bidirectionality of PVH^{MC4R}-regulated appetite supports their position as a point of functional convergence for inhibitory feeding-promoting ARC^{AgRP} neurons and excitatory satiety-promoting ARC^{POMC} neurons. Indeed, channelrhodopsin-assisted circuit mapping demonstrates that the vast majority of PVH^{MC4R} neurons receive GABAergic input from ARC^{AgRP} neurons and that this interaction is necessary for ARC^{AgRP}→PVH-driven feeding. However, the rapid feeding effects engendered by ARC^{AgRP}→PVH photostimulation or artificial PVH^{MC4R} manipulations do not necessarily speak to the explicit function of MC4Rs. In this regard, beyond their expression of a cognate receptor and sensitivity to selective pharmacological agonists³⁵, the functional role of endogenous AgRP or α -MSH at PVH^{MC4R} neurons remains to be determined. It will be important to further refine the position of PVH^{MC4R} neurons downstream of ARC^{POMC} neurons, since this is the circuit that ultimately defines satiety.

Sites of functional outflow for PVH^{MC4R} neurons were identified through efferent projection mapping³⁵. Since glutamate release is critical to the function of PVH^{MC4R} neurons^{54,67}, putative postsynaptic cells in efferent sites were assayed for excitatory monosynaptic connections arising from presynaptic PVH^{MC4R} neurons. Despite a broad descending projection profile, glutamatergic connections were identified only in the LPBN and DMV, implicating these site as prospective third-order nodes involved in feeding regulation³⁵. Connected LPBN neurons are located specifically in the central compartment (cLPBN). *In vivo* optogenetic stimulation of PVH^{MC4R}→cLPBN terminals, but not those in DMV or ventrolateral periaqueductal gray, promotes satiety, confirming this structure as site of functional outflow for melanocortin-regulated appetite. In this regard, the role of the LPBN as an interoceptive relay⁹⁴ befits its role in appetite regulation. LPBN lesions induce hyperphagia and obesity⁹⁵, and hypo-responsivity to anorectic neuroendocrine factors⁹⁶. Input from ascending sensory afferents (both vagal, via NTS, or spinal)⁹⁴, in addition to a direct chemosensing capacity^{97,98}, influences LPBN neuron activity in a viscerotopic fashion. Thus, it is possible that a neurochemically defined satiety population or populations integrates both ingestion-related visceral stimuli and hypothalamically derived homeostatic information to jointly determine behavioral and physiological responses to food consumption. The explicit identity of the cLPBN satiety population engaged by PVH^{MC4R} neurons remains unknown, but it is glutamatergic, as LPBN^{vGLUT2} neurons are necessary for the real-time maintenance of satiety and are selectively engaged by PVH^{MC4R} neurons^{35,54}. Furthermore, these satiety cLPBN neurons are distinct from appetite-suppressing calcitonin-gene related peptide (CGRP)-expressing neurons in the external LPBN (eLPBN). eLPBN^{CGRP} neurons inhibit food consumption under conditions in which it is unfavorable to eat (such as sickness)⁹⁹ and carry negative valence when optogenetically activated¹⁰⁰. That satiety-promoting PVH^{MC4R}→cLPBN neurons do not engage eLPBN^{CGRP} neurons

demonstrates how specific neuroanatomical circuits subserve the context-specific regulation of food intake.

In its most simplistic biological form, appetite is a function of energy requirement. The physiological representation of this caloric need is recognized as hunger, the detection of which ensures the motivation to enact behaviors that lead to food consumption (**Fig. 2a**). Recent work has demonstrated that the motivation to engage in these behaviors is driven by the aversive nature of hunger, as represented by ARC^{AgRP} neuron activation¹⁰¹, which promotes a negative physiological state that is alleviated upon caloric intake¹⁰². Thus, the consumption of food, and hence satiety, is reinforcing to ensure the motivation required to respond to periods of caloric deficiency. Supporting this, experimentally induced satiety through optogenetic stimulation of PVH^{MC4R}→LPBN terminals³⁵ (or chemogenetic silencing of ARC^{AgRP} neurons¹⁰¹) encodes a positive valence that hungry mice preferentially seek out, essentially alleviating their hunger through self-stimulation of this neural circuit (**Fig. 2b**). Remarkably, the positive valence encoded by ARC^{AgRP} neuron silencing and PVH^{MC4R} neuron activation in hungry mice is energy-state dependent, such that in calorically sated mice the activation and silencing of these populations, respectively, no longer represents a preferential state (**Fig. 2c**)^{35,101}.

In experimentally distilling the motivational basis of feeding behavior, these complementary studies demonstrate, consistent with early theories concerning need states and their homeostatic rectification^{103,104}, that the motivation to enact the behaviors (food-seeking and consumption) that fulfill an unmet need (caloric insufficiency) is driven by the negative valence of the physiological state (hunger) and that reestablishment of homeostasis encodes a positive valence associated with relief from this state. It is tempting to speculate that the unpleasant feelings associated with hunger, combined with the relief, if not pleasure, that comes from subsequent food consumption, is one of the main reasons why dieting proves so difficult. Specifically, the motivational incentive to respond to periods of hunger (a state exaggerated by dieting) has evolved to be a robust and unerring survival drive, the disregard of which is difficult. Consequently, the therapeutic activation of a system that encodes both satiety and positive valence (such as PVH^{MC4R}→LPBN) could conceivably serve as a potent adjunct to caloric restriction for the treatment of obesity.

MC4R signal transduction. The physiological functions of the MC4R are ultimately defined by the intracellular mechanisms that transduce ligand–receptor interactions into neuronal excitability. Although originally defined as a G α_s -coupled receptor, recent investigation into the nature of the binding of α -MSH and AgRP to MC4Rs have revealed the diversity of signaling cascades engaged by their orthosteric binding¹⁰⁵. Depending on cellular context, application of MC4R agonists promotes G α_s -dependent cyclic AMP synthesis¹⁰⁶, G α_q -dependent phospholipase-C activity¹⁰⁷ and G-protein-dependent MAPK signaling^{108–110}. However, these studies relied on heterologous *in vitro* systems, and thus their extrapolation to *in vivo* relevance is uncertain. In probing transgenically labeled PVH^{MC4R} neurons in *ex vivo* slice preparations, a recent study revealed that α -MSH agonism, and subsequent neuron excitation, is mediated by the closure of the inwardly rectifying potassium channel Kir7.1, in a G-protein-, β -arrestin- and MAPK-independent manner¹¹¹. However, this mechanism is cell-type specific, as the inhibitory action of α -MSH at DMV^{MC4R} neurons is contingent on G α_s -mediated activation of an ATP-dependent potassium channel⁸². The relevance of these mechanisms to MC4R-regulated physiology remains to be determined, but genetic ablation of the *Gs α* (*Gnas*) coding region in mice, while not specific to

MC4R, leads to profound syndromic obesity that is associated with reduced energy expenditure and sympathetic outflow, independent of hyperphagia¹¹². Targeted deletion from Sim1 neurons promotes only mild obesity with no influence on feeding behavior¹¹³. These observations may suggest that MC4R coupling to $G\alpha_s$ in the PVH is not a functional requirement for the regulation of feeding behavior, potentially identifying a role for $G\alpha_q$ or G-protein-independent Kir7.1 signaling. Together these data indicate that the MC4R is likely to exhibit promiscuous coupling to multiple intracellular mediators in a physiology-specific context.

α -MSH signaling is potentiated by the actions of a conserved family of melanocortin receptor accessory proteins (MRAPs). The murine¹¹⁴ and teleost¹¹⁵ isoforms of MRAP2 sensitize the MC4R to α -MSH binding and augment signal transduction. In mice, the global or Sim1-specific deletion of the sole MRAP2 paralog results in late-onset obesity that is, paradoxically, not defined by hyperphagia or reduced energy expenditure (nor reduced anorectic sensitivity to MTII)¹¹⁴.

The melanocortin system is unique in possessing a second, counteracting ligand in the form of AgRP. Initial pharmacological observations of AgRP function led to its characterization as an MC4R antagonist, serving to block α -MSH agonism^{116–118}. AgRP has also been identified as an inverse agonist, operating independently of α -MSH to promote G-protein uncoupling at constitutively active MC4Rs^{119–122}. However, the physiological significance of constitutive MC4R activity *in vivo* is unclear¹²³. Most recently AgRP has been identified as a biased agonist, directly promoting membrane hyperpolarization independently of constitutive activity or agonist presence. This mechanism has been observed in both a G-protein-dependent and G-protein-independent manner. In a heterologous cell system, AgRP binding selectively recruits $G_{i/o}$, leading to reduced cAMP accumulation¹²⁴, while in *ex vivo* PVH^{MC4R} neurons, AgRP drives G-protein-independent activation of Kir7.1 and subsequent hyperpolarization¹¹¹. Little is yet known of the physiological significance of the MC4R–Kir7.1 interaction, but future genetic studies are likely to provide invaluable insights.

MC4R pharmacotherapy

Driven by the ever more pervasive issue of human obesity, much effort has been directed toward unraveling the neural circuits that coordinate the regulation of energy homeostasis and body weight. In fulfilling a highly conserved physiological function, the central melanocortin system sits at the intersection of obesity pathology and treatment. Nullizygous mutations in human *POMC*²¹ and *MC4R*^{19,20,52} genes engender pleiotropic obesity syndromes that are consistent with their epistatic cofunctionality. Furthermore, *POMC*^{125,126} and *MC4R*^{52,127} haploinsufficiency predisposes carriers to obesity, albeit with varying penetrance that speaks to the importance of environmental factors and genetic modifiers. Indeed, *MC4R* mutations represent the most prevalent form of monogenic obesity (0.5–6% of obese individuals)⁵², and *MC4R* variations are highly correlated with fat mass¹²⁸. However, contrasting this pathological association is the potential of the MC4R as a pharmacotherapeutic target for obesity^{129,130}. Consistent with rodent studies, pharmacological compounds that augment MC4R signaling promote appetite suppression and weight loss in humans¹³⁰. Unfortunately, these drugs influence other MC4R-regulated processes, such as cardiovascular tone, sexual function and pandiculation, limiting their clinical application¹²⁹. At present only one MC4R compound, setmelanotide (RM-493; Rhythm Pharmaceuticals), is undergoing clinical trials, and only for use in the rare obesity conditions Prader-Willi syndrome and *POMC* deficiency. Setmelanotide reduces food intake and increase

energy expenditure in several preclinical models^{131,132}. In obese rhesus macaques¹³² and a small cohort of obese humans¹³³, this drug had no adverse effects on blood pressure; however, the latter study reported sexual side effects¹³³. Alternative strategies such as positive allosteric modulators (PAMs), which augment MC4R signaling upon orthosteric binding of α -MSH, and molecular chaperones, which increase cell surface availability of MC4Rs, are being investigated as a means of enhancing the efficacy of endogenous α -MSH to offer more physiologically relevant temporal specificity¹²⁹.

Basic science efforts to experimentally identify the relevant neuronal populations that underlie the various physiological functions of the MC4R will be critical for the development of new obesity-specific compounds. Such studies will afford insight into the involvement of neuroanatomically discrete MC4R populations and potentially facilitate the identification of differentially expressed targets amenable to pharmacological manipulation. For instance, PVH^{MC4R} neurons are the preponderant agent of melanocortinergic appetite control and thus a foremost target for weight-loss therapies. While explicit pharmacological manipulation of these MC4Rs is as yet impossible and confounded by drug action at MC4R populations governing autonomic outflow, PVH^{MC4R} neurons express other potential targets not expressed in preautonomic MC4R neurons. Transcriptomic profiling will be vital for contrasting neuroanatomical populations of MC4R-expressing neurons and identifying physiology-specific targets. In addition, evidence of promiscuous MC4R intracellular coupling (see above) may also suggest that these receptors employ functionally exclusive signaling cascades. *In vivo* interrogation of these mechanisms may afford understanding of the signal transduction pathways that define the function of satiety-promoting MC4Rs.

Conclusions and future directions

The central melanocortin systems regulates body weight and overall metabolic fitness in a manner defined by its need-based orchestration of physiology and behavior. The initiation of these processes is contingent on the interoceptive function of counter-acting ARC^{AgRP} and ARC^{POMC} neurons that represent a cellular interface between afferent indicators of physiological state and neural circuits governing response enactment. The capacity of the melanocortin system to subserve both catabolic and anabolic modalities of energy balance is determined by their antagonistic engagement of second-order MC4R-expressing neurons. While additional, non-MC4R mechanisms and populations contribute to aspects of ARC^{AgRP} and ARC^{POMC} functionality³⁵, the phenotypic similitude arising from the experimental manipulations of these first- and second-order populations strongly supports MC4R expressing neurons as important mediators of melanocortinergic energy balance.

Conditional genetic manipulations of *Mc4r* expression reveal a functional divergence in the melanocortinergic circuitry, such that the regulation of specific physiologies or behaviors proceeds via neuroanatomically discrete populations of MC4R neurons (Fig. 3). Indeed, although many MC4R populations remain unexplored, experimental interrogation of those in the PVH (feeding behavior), DMV (insulin release) and IML (energy expenditure and glucose metabolism) has demonstrated their synergistic but non-overlapping functions. The rationalization of these phenotypes in the context of the upstream melanocortinergic circuitry has subsequently been facilitated by the application of modern neuroscience technologies. Thus, explicit genetic access to MC4R neurons in combination with these techniques affords understanding of both the up- and downstream circuitry that defines physiological function. In this regard, further deconvolution of the LPBN circuitry underlying the satiety effect of PVH^{MC4R}

neurons is critical to understanding the means by which real-time fluctuations in energy state are translated into volitional behavior. The complex anatomical substructure, neurochemical composition and viscerotopic arrangement of the LPBN suggests that there is likely to be a functionally explicit population of neurons that integrates labeled lines of satiety-related homeostatic and visceral information and informs decision-making processes via its ascending projections, potentially to limbic (central nucleus of the amygdala, lateral hypothalamus, bed nucleus of the stria terminalis and cortico-thalamic structures (paraventricular nucleus of the thalamus and insular cortex). Furthermore, this homeostatic system is likely to be influenced by neural inputs that convey context-specific information to modulate functional output in accordance with the broader state of the animal and its environment. Expansion of this aspect of the melanocortineric circuitry will have important implications for how homeostatic processes—in particular, feeding behavior—can be influenced by factors such as stress, reward and circadian rhythmicity, and provide insight into the complexity of human energy balance.

The rising prevalence of clinical obesity, due primarily to caloric overconsumption, has highlighted the need for more efficacious pharmacotherapies. The ability of MC4R compounds to promote weight loss has long been held as evidence of therapeutic potential, yet the multiplicity of physiologies regulated by this receptor has subverted the generation of a safe and effective drug. To this end, further basic science endeavor in deconvolving MC4R biology—in particular, with regard to the neurons that express these receptors, their circuitry and intracellular cascades that define their function—is critical for streamlining pharmaceutical efforts to specifically target the body-weight-regulating MC4R neurons.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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