

60 YEARS OF POMC

Regulation of feeding and energy homeostasis by α -MSH

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Abstract

The melanocortin peptides derived from pro-opiomelanocortin (POMC) were originally understood in terms of the biological actions of α -melanocyte-stimulating hormone (α -MSH) on pigmentation and adrenocorticotrophic hormone on adrenocortical glucocorticoid production. However, the discovery of POMC mRNA and melanocortin peptides in the CNS generated activities directed at understanding the direct biological actions of melanocortins in the brain. Ultimately, discovery of unique melanocortin receptors expressed in the CNS, the melanocortin-3 (MC3R) and melanocortin-4 (MC4R) receptors, led to the development of pharmacological tools and genetic models leading to the demonstration that the central melanocortin system plays a critical role in the regulation of energy homeostasis. Indeed, mutations in MC4R are now known to be the most common cause of early onset syndromic obesity, accounting for 2–5% of all cases. This review discusses the history of these discoveries, as well as the latest work attempting to understand the molecular and cellular basis of regulation of feeding and energy homeostasis by the predominant melanocortin peptide in the CNS, α -MSH.

Key Words

- ▶ α -MSH
- ▶ POMC
- ▶ melanocortin
- ▶ MC4R
- ▶ food intake

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Cloning of the melanocortin receptors

The cloning of the melanocortin receptors (MCR) in 1992 significantly advanced our understanding of the physiological roles and sites of action of α -melanocyte-stimulating hormone (α -MSH) (Mountjoy *et al.* 1992). The first two receptors reported corresponded to the previously characterized melanocyte-stimulating hormone receptor (MSHR or MC1R) and adrenocorticotrophic hormone receptor (ACTHR or MC2R). Ultimately, five MCR were cloned, and referred to as MC1R–MC5R. The latter three had no known physiological roles at the time, and therefore were referred to as

melanocortin-3 (MC3R), melanocortin-4 (MC4R), and melanocortin-5 (MC5R), respectively.

MCRs are members of the rhodopsin-like, class A branch of the seven transmembrane-spanning domain G protein coupled receptor (GPCR) superfamily. They couple to, and cause dissociation of the heterotrimeric G protein complex. The G α subunit types activated by ligand-bound MCRs are G $_{\alpha s}$, G $_{\alpha q}$, and G $_{\alpha 11}$. MC3R–MC5R have relatively short N- and C-termini, and intracellular and extracellular loops, placing them among the shortest GPCRs.

All MCRs except for MC2R, bind melanocortin peptides containing the conserved heptapeptide core 'MEHFRWG', found in α -MSH, while the ACTHR further requires a peptide motif C-terminal to the 13 amino acids found in α -MSH (Gantz *et al.* 1993a). Each MCR mediates diverse physiological responses to α -MSH, or ACTH, in the case of MC2R. The MC1R is expressed in skin melanocytes and hair follicles and regulate pigmentation. α -MSH binds to this receptor stimulating the synthesis of eumelanin (brown-black pigments). Agouti (agouti signaling protein) binds to the receptor, competitively inhibiting the binding of α -MSH, to stimulate the synthesis of pheomelanin (yellow-red pigments). MC2R binds α -MSH with lower affinity than other members of the family, but instead, is activated specifically by ACTH and it is exclusively expressed in the adrenal cortex. Activation of this receptor regulates cell proliferation and production of glucocorticoids in the adrenal cortex.

The MC3R is expressed mainly in the brain, including high expression in the arcuate nucleus (ARC), ventromedial nucleus of the hypothalamus (VMH), ventral tegmental area (VTA), central linear nucleus of raphe, with moderate expression in anteroventral preoptic nucleus, lateral hypothalamic area, posterior hypothalamic area, medial habenular nucleus, and paraventricular nucleus of the hypothalamus (PVH) (Roselli-Reh fuss *et al.* 1993, Gantz *et al.* 1993a). Disruption of this gene in knockout mouse models generate a nonhyperphagic increase in adiposity that is associated with reduced lean body mass and increased feed efficiency (Butler *et al.* 2000, Chen *et al.* 2000). The MC4R is widely expressed throughout the CNS (Mountjoy *et al.* 1994) as well as peripheral nervous system (Gautron *et al.* 2010), and in intestinal L cells (Panaro *et al.* 2014). MC4R functions to regulate food intake and energy expenditure, and this role for the receptor has been shown to be evolutionarily conserved in vertebrates from fish to human. MC4R knockout mice as well as human mutants present early onset severe obesity associated with increased fat and lean mass (Huszar *et al.* 1997, Yeo *et al.* 1998). Additionally, MC4R regulates insulin secretion, lipid metabolism, bone mineral density, and body length. MC5R appears to be expressed primarily in exocrine glands. MC5R knockout mice are defective in secretion of multiple exocrine gland products and lack pheromone-induced aggression behaviors (Chen *et al.* 1997, Morgan & Cone 2006).

As the effects of α -MSH on food intake are the focus of this review, we will center our discussion on the physiology, pharmacology, and neuroanatomy of pro-opiomelanocortin (POMC) and agouti-related peptide (AgRP), and their cognate receptors in the CNS, MC4R, and MC3R.

Cloning the MC4R

Historically, the earliest physiological evidence of effects of melanocortin peptides originates before cloning of MC4R, with reports that intracerebroventricular (ICV) injection of ACTH and α -MSH inhibited the feeding drive induced by i.p. injection of a κ -opiate receptor agonist in rats (Poggioli *et al.* 1986, Vergoni *et al.* 1986). Stimulation of food intake by α -MSH had also been reported (Shimizu *et al.* 1989), and thus the characterization of receptors for α -MSH in the brain was ultimately needed to clarify these conflicting findings.

Following the cloning of the MC1R and MC2R, three orphan MCRs were soon cloned as well. Two independent laboratories in 1993 cloned and mapped the human MC4R using homology-based cloning (Gantz *et al.* 1993b, Mountjoy *et al.* 1994). This gene, identified on chromosome 18 (q21.3) in humans, consisted of one large exon with an open reading frame of 1 kb encoding a protein of 332 amino acids. Based on sequence alignment analysis, the closest identified receptor was MC3R, with 58% homology (Gantz *et al.* 1993a, Magen is *et al.* 1994). MC4R couples to G α s protein to activate adenylyl cyclase, resulting in elevation of intracellular cAMP. There is also evidence that this receptor can raise intracellular calcium levels through recruitment of G α q and inositol trisphosphate production in heterologous overexpression systems (Konda *et al.* 1994, Mountjoy *et al.* 2001, Kim *et al.* 2002).

Discovery of the role of α -MSH in feeding behavior and energy homeostasis

When expression of MC4R was mapped in the CNS by *in situ* hybridization, the distribution suggested a role in neuroendocrine and autonomic control (Mountjoy *et al.* 1994). However, the first breakthrough in understanding the MC4R physiological function came from discoveries made in MC1R physiology and pharmacology (Lu *et al.* 1994).

Agouti, a 132-amino acid protein that is produced in the hair follicle, was demonstrated to be a high-affinity ligand of MC1R, competitively blocking α -MSH binding and inhibiting cAMP production (Lu *et al.* 1994). This finding correlated with observations *in vivo* that agouti blocked eumelanin production. Strikingly, agouti was also found to be a high-affinity competitive antagonist of α -MSH action at MC4R, but not other MCRs (Lu *et al.* 1994).

As agouti gene mutations were found to result from gene rearrangements that produced ectopic expression

of agouti (Yen *et al.* 1994), it was inferred that the inhibition of MCRs in the brain by agouti underlie the obesity and metabolic syndrome observed in the yellow (*A^y*) mouse (Lu *et al.* 1994). The development of the first MC4R antagonist (Li *et al.* 1996), and the creation of two different genetic mouse models would ultimately confirm this hypothesis (Fan *et al.* 1997).

In 1997, several studies were published providing direct evidence supporting this hypothesis and establishing a central role of MC4R signaling in regulation of energy homeostasis. ICV injection of melanotan II (MTII), a cyclic analog of α -MSH, was shown to suppress food intake in four different mouse models: fasted C57BL/6J, *ob/ob*, *Ay*, and mice injected with neuropeptide Y (NPY). Conversely, this inhibition was blocked by coinjection of SHU9119, a cyclic peptide antagonist of MC3R and MC4R. Furthermore, ICV injection of SHU9119 alone increased food intake in mice (Hruby *et al.* 1995, Fan *et al.* 1997). These findings supported the hypothesis that hypothalamic POMC expressing neurons releasing α -MSH tonically to inhibit feeding *via* activation of MC4R target neurons, and chronic blockade of this signaling pathway by agouti is ultimately responsible for the obesity phenotype observed in *A^y* yellow mice (Fan *et al.* 1997).

Animal models manipulating the melanocortin system have been pivotal to further advancing our understanding of the role of α -MSH in regulation of feeding and energy homeostasis (Table 1). In 1997, the first animal model testing the role of α -MSH signaling in the brain was created. By targeting the *Mc4r* gene in embryonic stem cells, MC4R knockout mice were made and used to test the hypothesis that deletion of this receptor would recapitulate the agouti obesity syndrome (Huszar *et al.* 1997). Mice lacking both alleles displayed early onset obesity, hyperphagia, increased linear growth, hyperinsulinemia, and hyperglycemia. Loss of a single allele resulted in intermediate phenotypes compared with wildtype and homozygous siblings, indicating involvement of a gene–dosage effect (Huszar *et al.* 1997). These findings support a model in which the primary mechanism by which agouti induces obesity is by chronic antagonism of MC4R, establishing this receptor as central regulator of energy balance.

Subsequently, two research groups conducted two independent cohort screenings of the *MC4R* gene in individuals with childhood obesity and nonobese controls. They identified frameshift mutations in the *MC4R* gene in children with early onset obesity (Vaisse *et al.* 1998, Yeo *et al.* 1998). The majority of MC4R-induced obese individuals were heterozygous comprising

about 6% of obese children. As observed in knockout mice, human MC4R-induced phenotypes exhibited a gene–dosage effect, unusual for other GPCRs. This finding demonstrated the evolutionarily conserved role of MC4R in energy balance in humans, and generated great interest in α -MSH signaling in the CNS in general, and the MC4R as a target for the development of new antiobesity therapeutics.

Another important event in 1997 was the discovery, characterization, and expression mapping of AgRP, an analog of agouti (Fong *et al.* 1997, Graham *et al.* 1997, Ollmann *et al.* 1997). AgRP mRNA is mainly expressed in the ARC of the hypothalamus and its levels are increased during fasting and in *ob/ob* mice, resulting in an increased drive to feed. AgRP binds with high affinity to MC3R and MC4R (Chai *et al.* 2003). Like agouti, it acts as competitive antagonist of α -MSH at these receptors, with low affinity for MC1R (Ollmann *et al.* 1997). A single ICV injection of AgRP increases food intake for up to a week, and coinjection of α -MSH does blunt the orexigenic effects of the former. Thus, AgRP functions as an inverse agonist of MC4R by decreasing cAMP levels produced by the constitutive activity of wildtype or mutant receptors (Haskell-Luevano *et al.* 2001, Nijenhuis *et al.* 2001, Chai *et al.* 2003). This finding substantiated the commonly held Yin–Yang view of feeding regulation, with POMC neurons and α -MSH inhibiting food intake and energy storage, and NPY/AgRP neurons stimulating it in part, through AgRP antagonism of MC4R signaling (Fig. 1).

In agreement, transgenic mice ubiquitously overexpressing human AgRP exhibit obesity but not yellow fur, suggesting that AgRP, unlike agouti protein, is MC3R/MC4R specific, and unable to promote pheomelanin in hair follicle melanocytes (Graham *et al.* 1997, Ollmann *et al.* 1997). As MC4R knockout mice exhibit significant obesity, some expected that AgRP knockout mice might exhibit leanness. However, at first glance, these mice exhibit normal food intake, body composition, growth rates, and responses to starvation (Qian *et al.* 2002). Subsequent work demonstrated that homozygous AgRP knockout mice do exhibit a very modest reduction in body weight at 6 months of age and adiposity with increased metabolic rate, body temperature, and locomotor activity (Wortley *et al.* 2005). These mice also exhibit a blunted fast-induced refeeding response. It is now known that AgRP neurons are GABAergic, and of course express NPY as well, and all three agents regulate downstream MC4R neurons (Wu & Palmiter 2011). These findings underscore the importance of the AgRP neuronal

Table 1 Mouse models used to study α -MSH signaling in obesity.

Yellow lethal (<i>A^y</i>) and related alleles (<i>A^y</i> , <i>A^{sy}</i> , <i>A^{vy}</i> , <i>A^{iapvy}</i>)	Spontaneous mutation resulting in agouti (<i>Asip</i>) ectopic expression, homozygous lethal; unrelated to agouti ectopic expression, moderate hyperphagia, hyperinsulinemia (2–5× normal), late-onset hyperglycemia, lean body mass, ↓ energy expenditure, ↑ sensitivity to stressors, milder obesity syndrome than <i>ob/ob</i> or <i>db/db</i> , yellow fur color	Dickie (1962, 1969), Frigeri <i>et al.</i> (1983), Bultman <i>et al.</i> (1992), Michaud <i>et al.</i> (1993, 1994a,b), Miller <i>et al.</i> (1993), Klebig <i>et al.</i> (1995)
AgRP transgene	Expression of the AgRP under the control of α -actin promoter, same metabolic phenotype as <i>A^{vy}</i> , same fur color as wildtype littermates	Graham <i>et al.</i> (1997)
<i>AgRP^{PDTR/DTR}</i>	Cross of mice harboring a loxP flanked diphtheria toxin receptor with animals with Cre recombinase under control of AgRP promoter Ablation of AgRP/NPY neurons of the arcuate nucleus of the hypothalamus, ↓↓ food intake, ↓ body weight, complete ablation results in starvation and death	Gropp <i>et al.</i> (2005), Luquet <i>et al.</i> (2005), Wu <i>et al.</i> (2008a,b)
<i>POMC^{-/-}</i>	Hyperphagic, ↓ energy expenditure, adrenal insufficiency, develop obesity exacerbated with high-fat diet, lighter than littermates-colored dorsal and yellow ventral fur	Yaswen <i>et al.</i> (1999), Challis <i>et al.</i> (2004)
<i>POMC, AgRP^{-/-}</i>	Hyperphagic, ↓ energy expenditure, α -MSH but not AgRP administration rescues wildtype phenotype, lighter than littermates-colored dorsal and yellow ventral fur similar to <i>POMC^{-/-}</i>	Corander <i>et al.</i> (2011)
<i>MC4R^{-/-}</i>	Hyperphagic, less obese than <i>ob/ob</i> or <i>db/db</i> , early onset obesity (5–7 weeks), ↓ energy expenditure, dosage effect with, +/- animals have intermediate phenotype, increased linear growth, same fur color as wildtype littermates	Huszar <i>et al.</i> (1997)
Targeted deletion of MC4R at the PVH	<i>Vglut2-ires-Cre;Mc4r^{lox/lox}</i> ; targeted deletion of MC4R in glutamatergic neurons: same phenotype as MC4R null mice including ↑ hyperphagia, ↑ body weight, and ↑ linear growth <i>Sim1-Cre;Mc4r^{lox/lox}</i> ; targeted deletion of MC4R in single-minded 1 positive (SIM1+) neurons: intermediate phenotype between MC4R ^{-/-} and <i>Mc4r^{lox/lox}</i> control mice	Balthasar <i>et al.</i> (2004), Shah <i>et al.</i> (2014)
<i>MC3R^{-/-}</i>	Moderate obesity syndrome of late onset (26 weeks), ↑ body weight predominant in females, ↑ adiposity, ↑ energy efficiency, no change in lean mass, no change in linear growth, same fur color as wildtype littermates	Butler <i>et al.</i> (2000), Chen <i>et al.</i> (2000), Sutton <i>et al.</i> (2006)
<i>MC3R, MC4R^{-/-}</i>	Augmented obesity syndrome compared with <i>MC4R^{-/-}</i> or <i>MC3R^{-/-}</i> ; including ↓ energy expenditure, no change in food ingestion after MTH administration, same fur color as wildtype littermates	Chen <i>et al.</i> (2000)
Mahogany (<i>Atrn^{mg}</i>) and related alleles (<i>Atrn^{mg-3j}</i> , <i>Atrn^{mg-L}</i>)	Autosomal recessive mutation reverting the phenotype of <i>A^y</i> including obesity albeit hyperphagia is present, <i>Atrn</i> encodes for attractin, a melanocortin receptor coreceptor. <i>Atrn^{mg}</i> is a defective splice variant allele that results from a 5 kb retroviral insertion in introns 26 and 27 Fur color reflects eumelanin predominance with diminished pheomelanin, darkened ears, and tail (umbrous coat). <i>Atm^{mg-3j}</i> ; a null allele of <i>Atrn</i> , has darker fur	Lane & Green (1960), Dinulescu <i>et al.</i> (1998), Gunn <i>et al.</i> (1999)
Mahogunin, ring finger 1 (<i>Mgrn1^{md}</i>)	<i>Mgrn1</i> encodes an E3 ubiquitin ligaseThe <i>Mgrn1^{md}</i> allele displays a similar phenotype to <i>Atrn^{mg}</i> and related alleles	Dinulescu <i>et al.</i> (1998), Phan <i>et al.</i> (2002)
<i>Mrap2^{-/-}</i>	Melanocortin receptor accessory protein 2 (MRAP2) knockoutLate-onset ↑ hyperphagia, early onset ↑ body weight, ↑ white fat tissue depots ↓ lean mass, same fur color as wildtype littermates	Asai <i>et al.</i> (2013)

system, as well as AgRP action on MC4R in the regulation of energy homeostasis.

Neuroanatomy of the central melanocortin system

POMC and AgRP neurons

To truly understand α -MSH action in the CNS, it is critical to recognize the neuroanatomical substrate underlying the

central melanocortin system (Fig. 2). Neurons expressing MCR, and POMC and AgRP neurons collectively constitute the primary neural components of this system.

Within the CNS, AgRP neurons are restricted to the ARC of the hypothalamus (ARC). Most AgRP neurons (~90%) express another potentially orexigenic peptide hormone, NPY. Unlike AgRP, NPY is one of the most abundant and widely expressed neuropeptides in the mammalian brain. Similarly, POMC neurons express another gene coding for an anorectic peptide called

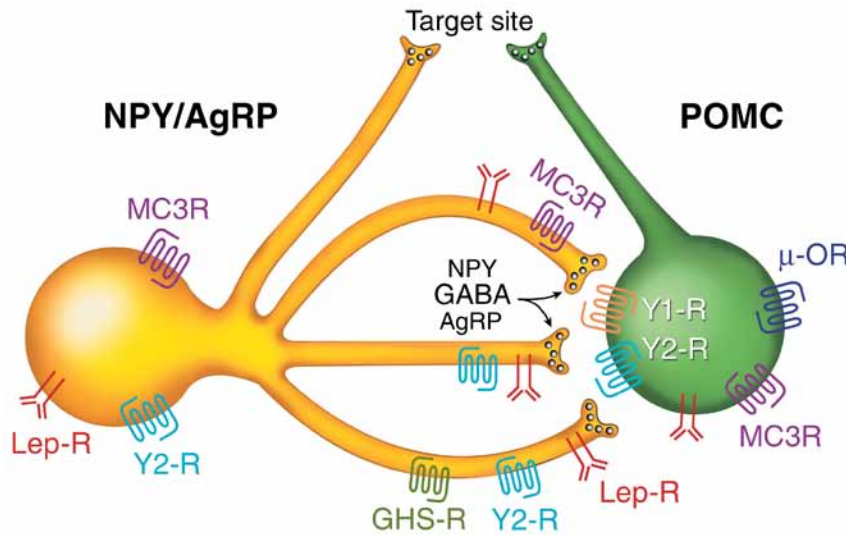


Figure 1

Yin–Yang model of control of feeding behavior and energy homeostasis. NPY/AgRP and POMC neurons within the arcuate nucleus form a coordinately regulated network due to dense NPY/AgRP fibers that project to POMC cell bodies. Some of the receptors for a large number of hormones and neuropeptides known to regulate the network are indicated. These fibers project to many of the same nuclei, where dual release of α -MSH and AgRP were proposed to compete for MC4R binding, to coordinately regulate food intake and energy homeostasis. AgRP, agouti-related peptide; GABA, γ -aminobutyric acid; GHS, growth-hormone secretagogue receptor; Lep, leptin; MC3R, melanocortin 3 receptor; NPY, neuropeptide Y; μ -OR, μ -opioid receptor; R, receptor; GLP-1, glucagon-like peptide 1. Modified, with permission, from Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD & Low MJ (2001) Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature* **411** 480–484.

cocaine- and amphetamine-regulated transcript. NPY/AgRP and POMC neurons are chemically and anatomically distinct; however, approximately 25% of the NPY neurons are derived from the same lineage as POMC neurons during development (Padilla *et al.* 2010). In the rodent ARC, AgRP neurons are expressed homogeneously throughout the rostrocaudal axis, while most POMC neurons are located in the anterior and medial ARC. In rat ARC, POMC neurons are more laterally distributed compared with the mouse (Cowley

et al. 2001). Earlier studies quantifying POMC neurons by β -endorphin immunohistochemistry (Huo *et al.* 2006) or using mice expressing green fluorescent protein (GFP) under the POMC promoter (Cowley *et al.* 2001) have reported around 3000–3500 POMC neurons in rodent ARC. However, a recent report quantifying POMC neurons using an antibody specific to the POMC precursor, estimated around 9000 immunoreactive cells (Lemus *et al.* 2015). On the other hand, the number of NPY/AgRP neurons was estimated to be between

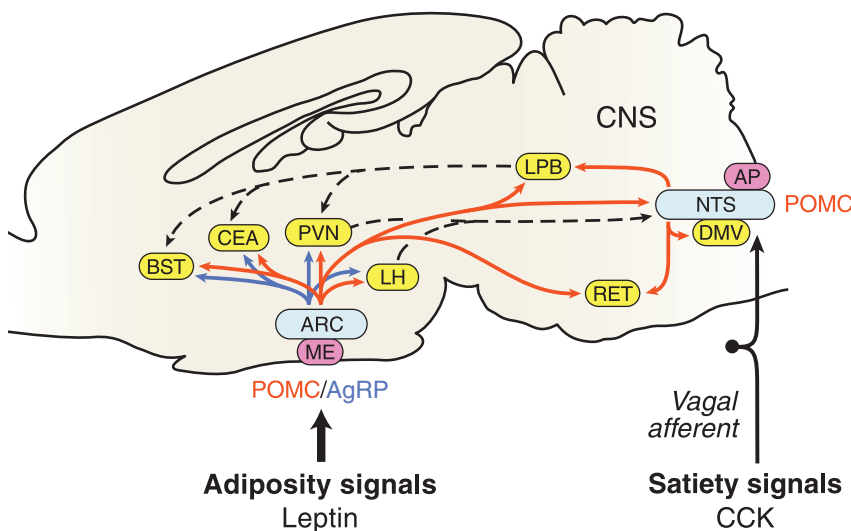


Figure 2

A highly simplified schematic of the central melanocortin system. Receipt of long-term adipostatic signals and acute satiety signals by neurons in arcuate nucleus and brainstem, respectively. Light blue boxes indicate nuclei containing POMC neurons; yellow boxes indicate nuclei containing MC4R neurons that may serve to integrate adipostatic and satiety signals; and pink boxes show some circumventricular organs involved in energy homeostasis. Red arrows designate projections of POMC neurons; blue arrows show projections of agouti-related protein (AgRP neurons). AP, area postrema; ARC, arcuate nucleus; BST, bed nucleus of the stria terminalis; CCK, cholecystikinin; CEA, central nucleus of the amygdala; DMV, dorsal motor nucleus of the vagus; LH, lateral hypothalamic area; LPB, lateral parabrachial nucleus; ME, median eminence; NTS, nucleus tractus solitarius; PVH, paraventricular nucleus of the hypothalamus; RET, reticular formation. For simplicity, only a fraction of the >100 MC4R target sites are shown, and none of the MC3R target nuclei is indicated. Adapted, with permission, from Fan W, Boston BA, Kesterson RA, Hruby VJ & Cone RD (1997) Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* **385** 165–168.

8000 and 10,000 (Betley *et al.* 2013, Lemus *et al.* 2015), analyzed using mice expressing GFP in an AgRP promoter-dependent manner.

Expression of POMC follows a dynamic pattern throughout gestation, and POMC-positive brain regions in the adult mice reveal that POMC expression in many regions in the developing embryo are transient. In adult rodents, POMC mRNA was detected by northern blot in hypothalamus, amygdala, and the cerebral cortex, but was not detectable in midbrain or cerebellar RNA preparations (Civelli *et al.* 1982). A 5' truncated version of POMC mRNA lacking the signal sequence was also detected in amygdala, midbrain, and cortex as well as in several peripheral tissues. However, the role of this truncated version is unclear as it cannot produce active POMC-derived peptides (Clark *et al.* 1990). POMC was detected in the nucleus tractus solitarius (NTS) and lateral reticular formation of the rat brainstem initially by immunohistochemistry against ACTH (Joseph *et al.* 1983, Schwartzberg & Nakane 1983) and later by *in situ* hybridization (Bronstein *et al.* 1992). Most neurons identified as POMC positive by labeling strategies involving POMC-Cre-mediated recombination, which accounts for transient POMC expression that does not persist into adulthood, do not express POMC in the adult brain. In this context, mice expressing GFP under the POMC promoter yielded more satisfactory anatomical data as to ARC POMC neurons as there is almost 100% overlap of GFP and POMC expression in the ARC, but not in other brain regions (Pinto *et al.* 2004). More recent and comprehensive studies comparing POMC-Cre, POMC-GFP, and sensitive *in situ* hybridization techniques have concluded that POMC expression in the adult mouse brain is restricted to ARC and NTS (Padilla *et al.* 2012).

AgRP was identified by its homology to agouti and its expression is restricted to ARC and adrenal gland with very low expression in lung, kidney, testis, and ovaries (Ollmann *et al.* 1997, Shutter *et al.* 1997). AgRP and POMC neurons located in the ARC project to various intra- and extrahypothalamic brain regions, and many reciprocal connections are found. Within the hypothalamus, AgRP and POMC neurons send overlapping projections to many hypothalamic nuclei including PVH, lateral hypothalamus (LH), VMH, posterior hypothalamus, dorsomedial hypothalamus (DMH), and medial preoptic nucleus/area (Bagnol *et al.* 1999). AgRP neurons innervate extrahypothalamic sites such as the bed nucleus of the stria terminalis (BNST), and the lateral parabrachial nucleus (LPB), central nucleus of the amygdala (CEA), and periaqueductal gray (PAG) (Betley *et al.* 2013, Wang

et al. 2015) outside the hypothalamus. ARC POMC neurons innervate many extrahypothalamic regions including BNST, lateral septum, nucleus accumbens, LPB, the periaqueductal gray, and the dorsal motor nucleus of the vagus (DMX). NTS POMC neurons innervate other neurons mostly within the brain stem (Wang *et al.* 2015). Collectively, POMC neurons appear to innervate multiple regions not receiving AgRP innervation, such as the DMX.

AgRP and POMC neurons receive inputs from other hypothalamic nuclei, predominantly from PVH, DMH, VMH, and LH (Wang *et al.* 2015). Extrahypothalamic brain regions innervating POMC and AgRP neurons include lateral septum and BNST. Neurons with cell bodies in the hippocampus, medial mammillary nucleus, and VTA appear to selectively innervate POMC neurons, but not AgRP neurons (Wang *et al.* 2015). NTS POMC neurons receive the majority of inputs from other neurons in the brainstem; however, neurons originating from paraventricular hypothalamic nucleus and amygdala (Wang *et al.* 2015) also innervate NTS POMC neurons.

The large degree of overlap in the regions innervated by both AgRP and POMC neurons also supports the dual regulation of central melanocortin signaling by α -MSH and AgRP (Fig. 1). Besides neuronal inputs, AgRP and POMC neurons are under direct regulation of hormonal and nutrient-related signals. Both neurons express leptin receptors (LepR), while only AgRP neurons express ghrelin receptors. A recent report suggested that the AgRP neurons that project within the hypothalamus do not express LepRb, which was not the case for POMC neurons (Betley *et al.* 2013). Around 30–40% of AgRP neurons respond to leptin with increased STAT3 phosphorylation (van de Wall *et al.* 2008). Virtually no POMC neurons in the NTS exhibit leptin-induced STAT3 phosphorylation or c-Fos expression induction (Huo *et al.* 2006). Nonetheless, about 60% of POMC neurons in the ARC are leptin responsive. In addition, fasting decreases POMC expression in both ARC and NTS, but only ARC POMC expression can be rescued by leptin (Huo *et al.* 2006, Perello *et al.* 2007). Deletion of LepRb from POMC or AgRP neurons results in obesity and hyperleptinemia (Balthasar *et al.* 2004, van de Wall *et al.* 2008) in both genders of mice, and the effect of deletion of LepRb from both neuronal populations is additive (van de Wall *et al.* 2008). Although these results should be evaluated taking into account the difficulties inherent to the developmental problems associated with the Cre lines used, they suggest that POMC and AgRP neurons mediate only part of leptin's effects on energy homeostasis (Padilla *et al.* 2012).

Neuroanatomy of MC3R and MC4R neurons

Downstream of POMC and AgRP, MC3R/MC4R expressing target neurons serve as essential neural nodes for responding to integrated signals of energy status and relaying modulating signals throughout the CNS to maintain energy balance.

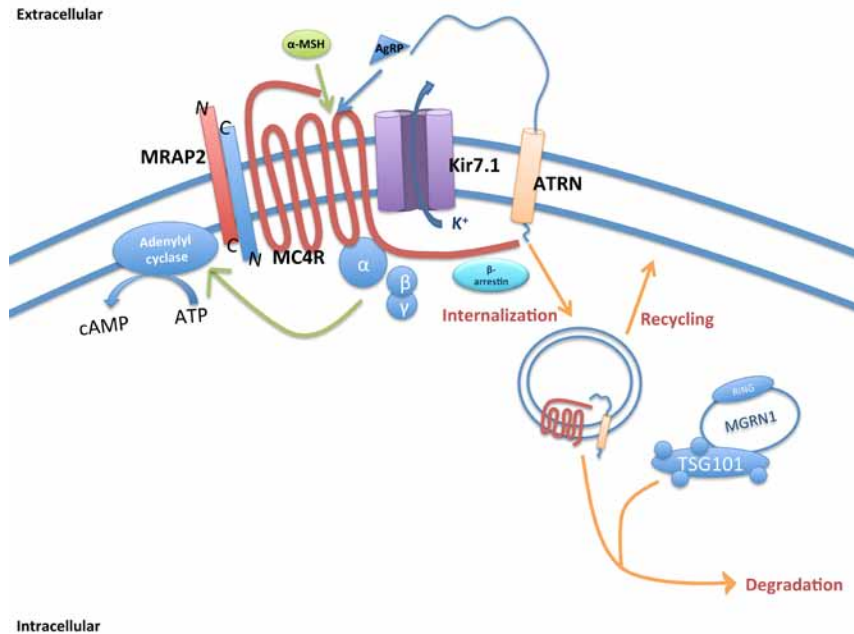
The MC3R was originally identified as the receptor more potently activated by the POMC-derived peptide γ -MSH (Roselli-Rehfuß *et al.* 1993), although the receptor also responds to α -MSH as well. *In situ* hybridization studies delineated the narrow distribution of MC3R within the adult CNS to approximately 30 mapped nuclei, where highest density is found in concentrated subregions of the hypothalamus including the VMH, ARC, anteroventral preoptic area, posterior hypothalamic area, and the medial preoptic area, the limbic system regions of nucleus accumbens (NuAcc) and VTA, and weak signals from a few brainstem nuclei (notably not the NTS) (Begriche *et al.* 2011, Mountjoy 2015). Double-labeled *in situ* hybridization validated before pharmacological studies by showing that a large component of ARC neurons expressing MC3R is also positive for AgRP or POMC mRNA (Bagnol *et al.* 1999). Deletion of the MC3R resulted in a mouse with increased adipose mass, decreased lean mass, and reduced bone density (Butler *et al.* 2000, Chen *et al.* 2000). Further physiological studies suggest MC3R regulates fast-induced refeeding (Renquist *et al.* 2012) and entrainment of anticipatory behavior to nutrient intake (Begriche *et al.* 2011). Recently conducted studies provided evidence for the functional expression of MC3R in the VTA and its role therein as a sexually dimorphic node for regulating the mesolimbic dopaminergic system and reward (Lippert *et al.* 2014). MC3R peripheral expression is detectable in the stomach, duodenum, kidneys, placenta, heart, monocytes, and macrophages; however, the function of the gene at these sites of action has not been well explored (Caruso *et al.* 2014). Nonetheless, it is apparent that one of the central MSH peptides likely mediates some effects on feeding and energy homeostasis *via* the MC3R.

The MC4R is more broadly distributed than MC3R, exhibiting expression in cellular nodes in every CNS region with a striking presence in the hypothalamus, NuAcc, and DMX. Mapping via *in situ* hybridization localized MC4R to over 100 distinct nuclei. Hypothalamic nodes of highest concentration include the suprachiasmatic preoptic nucleus, anteroventral periventricular nucleus, supraoptic nucleus, PVH, VMH, DMH, tuberomammillary nucleus, and the lateral hypothalamic area. Extensive brainstem labeling is found in the superior colliculus,

DMX, substantia nigra, raphe, and reticular formation. Following these high expression zones, several regions of the amygdala and isocortex have moderate expression. MC4R may play a role in olfactory response due to its location in discrete cortical nodes. MC4R has also been identified in CA1 and CA2 regions of the hippocampus, throughout the BNST and striatum, and weakly in the thalamus (Mountjoy *et al.* 1992, 1994, Kishi *et al.* 2003, Cone 2005, Tao 2010, Cui *et al.* 2012, Siljee *et al.* 2013, Mountjoy 2015). Localization and distribution of MC4R were further confirmed by studies using a mouse model expressing GFP under control the MC4R promoter (Liu *et al.* 2003). Additional genetic and pharmacological modeling systems inducing or repressing *MC4R* gene function in specific neuronal populations have mapped some neuroanatomical functions of these cells bodies and begun to reveal how the MC4R serves to regulate energy homeostasis. For example, MC4R in the PVH is essential for regulating appetite, while MC4Rs expressed in cholinergic preganglionic parasympathetic neurons are necessary for regulating energy expenditure (Balthasar *et al.* 2005, Rossi *et al.* 2011). Outside of central neurons, MC4R mRNA expression has been detected in astrocytes, spinal cord, heart, lung, kidney, and testis (Caruso *et al.* 2014). One peripheral MC4R-mediated pathway was described after detection of high levels of MC4R expression in enteroendocrine L cells (Panaro *et al.* 2014). The data show the receptor mediates release of L cell products PYY and glucagon-like peptide 1 in either mouse or human, in response to exogenous administration of α -MSH, and of course these peptides have known secondary effects on feeding. The physiological role of MC4R at this site, and the origin of the ligand, likely an MSH peptide, remains unknown.

Pharmacological complexities of α -MSH signaling

The melanocortin signaling system regulates distinct physiological functions on a receptor-subtype specific basis, including energy homeostasis, coat color, hypothalamic–pituitary–adrenal axis, and exocrine gland function, as a result of the unique distributions of expression of each receptor (Vastermark & Schiöth 2011, Cortes *et al.* 2014). In addition, an extended set of coreceptors, signaling partners, and alternate endogenous ligands enrich the tapestry of responses that result from α -MSH stimulation at each receptor (Fig. 3). This functional diversity played an important role in the discovery of many of the components of this system (Cone 2006).

**Figure 3**

The MC4R signaling complex. α -MSH and AgRP signaling at the MC4R involves a multiprotein signaling complex that appears to be highly regulated. In addition to interactions with well-characterized members of the G protein signaling cascade (α -, β -, and γ subunits and β -arrestin), the MC4R appears to interact directly with the transmembrane protein MRAP2, which blocks constitutive activation of the receptor by the amino terminal domain, and enhances sensitivity to α -MSH action. The receptor also appears to interact with inward rectifier channels such as Kir7.1, with α -MSH and AgRP modulating channel activity in a receptor-dependent manner. Attractin and mahogunin are involved in receptor internalization, and attractin may also be involved in AgRP binding. For simplicity, syndecan and defensins are not indicated here.

An example of how variable signaling in the pigmented system advanced research in the field of α -MSH signaling stemmed from studies on the determinants of fur coat coloration in mice and other mammals. As a natural outcome from the analysis of additional mouse coat color phenotypes (Silvers 1979, prepared a compilation of mouse fur color phenotypes also reviewed by Jackson *et al.* 1994), additional modifiers of MCR function were recognized. For example, mutations within the mahogany locus (Lane & Green 1960) rescued the agouti A^y dominant yellow fur phenotype and ameliorated the accompanying obesity syndrome (Dinulescu *et al.* 1998, Miller *et al.* 1997, He *et al.* 2001). Later analysis showed that the products of these genes regulate receptor membrane expression (Overton & Leibel 2011) as discussed below. An additional coat color-related allele was recognized in dogs (Kerns *et al.* 2003, 2004). Characterization of the product of this gene led to the identification of β -defensin 3, an additional endogenous ligand with agouti/AgRP-like activity on MC1R and MC4R (Candille *et al.* 2007). These and recently recognized coat color-unrelated partners for MCR that modulate agonist response or offer alternate signaling pathways are discussed in more detail in the following sections.

Mahogany, attractin-like protein, and mahoganoid

Attractin and mahogunin were originally identified as the suppressors of agouti action in *mahogany* and *mahoganoid* mice. Attractin is a type 1 transmembrane domain protein

that is proposed to be necessary for the action of agouti at both the MC1R and MC4R. It is further suggested that attractin binds to the N-terminal domain of agouti and acts as coreceptor by assisting in the stability of the interaction between MC4R and the C-terminal domain of agouti (He *et al.* 2001). Attractin does not bind directly to MC4R or AgRP, and as such, it was thought to be unlikely that it was involved in the modulation of MC4R activity. However, loss-of-function mutations in attractin as well as mahoganoid (mahogunin ring finger-1) are able to rescue the obesity phenotype in A^y mice by blocking the agouti-dependent degradation of MC4R.

Mahoganoid is an E3 ubiquitin ligase that ubiquitinates (Phan *et al.* 2002, He *et al.* 2003) tumor suppressor gene 101 (Tsg101), which is necessary for the trafficking of the ubiquitinated MC4R from the cell surface to the lysosome for degradation (Kim *et al.* 2007, Jiao *et al.* 2009). Attractin-like protein was found to be another binding partner of MC4R that is believed to play an important role in the receptor trafficking (Haqq *et al.* 2003). These modulators of MC4R activity impact the ability of α -MSH to regulate feeding and body weight.

Syndecans

An unrecognized fact of AgRP pharmacology is that most of the reported data are based on the use of the truncated 83–132 (human sequence) C-terminal peptide (Quillan *et al.* 1998, Nijenhuis *et al.* 2001). Studies with the full-length

prohormone show lesser potency in quenching α -MSH-elicited cAMP responses (Creemers *et al.* 2006, Jackson *et al.* 2006). It is clear then, that any mechanism that acts on relative concentrations of truncated to full-length AgRP will have a discernible effect on α -MSH response and act as coreceptor as seen for the agouti–attractin duo. A provocative model suggests that the charged but amphipathic N-terminal domain of AgRP serves as ‘anchor’ to engage perisynaptic heparan sulfate proteoglycans.

One such heparan sulfate proteoglycan is the membrane-bound, predominantly brain-expressed syndecan-3 (Sarrazin *et al.* 2011). Syndecans are single-membrane spanning glycoproteins that associate three to five heparan sulfate polysaccharide chains (Bernfield *et al.* 1992). Also notable is that at the interface of the heparan sulfate-linked ecto- and the transmembrane domains, a juxtamembrane region that harbors protease cleavage sites is present. This region can potentially be targeted by metalloproteases and the ‘shedding’ of syndecan ectodomains by proteolytic cleavage adds an additional regulatory layer as a mechanism of clearance of bound ligands or paracrine ligand diffusion, as shed ligand-bound heparan sulfates retain activity *in vivo* (Elenius *et al.* 1992, Kim *et al.* 1994).

A link between the melanocortin system and heparan sulfate proteoglycans was first established by studying transgenic mice overexpressing syndecan-1, a predominantly peripherally expressed homolog, under the control of the CMV promoter (Reizes *et al.* 2001). These animals show late-onset obesity, which is additive to the phenotype of the yellow-lethal A^y mouse (Reizes *et al.* 2001). As the predominant form expressed in hypothalamus is syndecan-3, the latter is probably the form implicated in AgRP extracellular concentration regulation (Reizes *et al.* 2001). Further enforcing this hypothesis, fasting animals showed elevated membrane-bound syndecan-3, while refeeding induced proteolytic cleavage and syndecan-3 shedding (Reizes *et al.* 2001). Further evidence was obtained from the analysis of a syndecan-3 knockout mice, which was resistant to diet-induced obesity (Reizes *et al.* 2003, Strader *et al.* 2004, Zheng *et al.* 2010). Taken together, these results support a second-tier regulatory layer for AgRP signaling on MCR, where syndecan shedding might have a role.

Mechanistically, the model of syndecan–melanocortin interaction is contingent on the interaction of the AgRP N-terminus domain with the heparan sulfate moieties on syndecans (Reizes *et al.* 2003). For this to be possible, the full-length hormone, and not the truncated C-terminal domain forms, has to be the predominant species

secreted. Creemers *et al.* (2006) raised the possibility that AgRP is intracellularly cleaved by proprotein convertases eliminating the anchoring N-terminal domain. If in fact, this is the case, the physiological relevance of syndecans in AgRP signaling would be diminished. Further work in this area will be necessary to establish the *in vivo* site of posttranslational cleavage of AgRP in or to settle this controversy.

Defensins

In opposition to mice where the A^y allele that confers a yellow-fur phenotype is dominant, a third allele (A^s) determining black coats was thought to be the dominant form in dogs (Kerns *et al.* 2003). Initially thought to be an *agouti* allele as opposed to an *extension* (i.e. MC1R-linked) allele, analysis of genome sequences in animals with red or black fur phenotypes showed that dominant black coats were conferred by a mutated allele of an unrelated gene that encodes for β -defensin (Kerns *et al.* 2004, 2007, Candille *et al.* 2007). Strikingly, transgenic expression of β -defensin 3 in the yellow-lethal background produced mice with black coats and lean phenotype (Candille *et al.* 2007). Moreover, β -defensin 3 blocked NDP-MSH binding to MC1R and with lesser affinity to MC4R (Candille *et al.* 2007). A later study showed that the human ortholog of β -defensin 3 is a weak partial agonist of MC1R transfected in HEK-293 cells (Beaumont *et al.* 2012) for cAMP accumulation and ERK1/2 phosphorylation, but a different group concluded that defensins might in fact have no intrinsic agonist action on its own (Swope *et al.* 2012).

Defensins are part of the innate immune system in mammals (Ganz 2003). The prospect of defensins serving as links between the immune and energy homeostasis systems is tantalizing. Cachexia is invariably linked to immunosuppression. As strongly charged molecules, defensins might interact with MCR solely through electrostatic interactions explaining their neutral antagonist effect (Nix *et al.* 2013, 2015). There is little doubt that these interactions play a role in determining melanocortin response on MC1R, as defensins seem to block agouti in live animals (Candille *et al.* 2007, Kerns *et al.* 2007). However, data are not as consistent for MC4R, and MC3R is yet to be tested as a potential defensin target. Electrostatic-based binding modalities open the possibility for additional undefined peptidic ligands to emerge as possible ‘modulators’ of AgRP and α -MSH signaling in energy balance (Nix *et al.* 2015).

MRAP1 and MRAP2

Melanocortin 2 receptor accessory proteins (MRAPs) are single-transmembrane proteins that form antiparallel homo- and heterodimers in the cell membrane. MRAP1 is essential for the trafficking, ligand binding, and signaling of MC2R in response to ACTH (Sebag & Hinkle 2007, Metherell *et al.* 2005). Null mutations in MRAP are linked to familial glucocorticoid deficiency type 2 (Metherell *et al.* 2005). MRAP2, a homolog to the former, is expressed in brain and adrenal tissue (Chan *et al.* 2009), interacts with all MCR subtypes, and alters the sensitivity of the MC4R to α -MSH (Sebag *et al.* 2013).

MRAP2 interacts with the MC4R in mammals as well as zebrafish and modulates the pharmacological response to α -MSH by suppressing the constitutive activity of the receptor and increasing sensitivity to α -MSH. In the zebrafish, MRAP2 exists in two isoforms, a and b. *mrapp2a* stimulated growth in the larval zebrafish by suppressing the binding of α -MSH to MC4R, while *mrapp2b* appears to control MC4R activity in the adult by increasing α -MSH sensitivity and suppressing receptor constitutive activity (Sebag *et al.* 2013). MRAP2 is the mammalian homolog of *mrapp2b*, which is not expressed until hatching in the zebrafish. Upon binding, it causes an increase in MC4R expression as well as an increase in α -MSH binding and cAMP generation (Sebag *et al.* 2013). In the zebrafish, neither *mrapp2a* nor *mrapp2b* alters MC3R signaling (Sebag *et al.* 2013) suggesting that the role of MRAP2 in the regulation of feeding and body weight may be specific to its modulation of MC4R. However, other studies have shown that MRAP2 does alter mammalian MC3R signaling (Chan *et al.* 2009, Kay *et al.* 2013). The actual role of MRAP in the modulation of MC3R-regulated food intake and body weight, however, is still up for debate. Recently, MRAP2 has also been demonstrated to play a role in the function of the prokineticin-1 and -2 receptors, suggesting this protein may be a modulator of multiple GPCRs (Chaly *et al.* 2016), and exert effects on feeding and body weight through multifactorial effects.

To further validate the role of MRAP2 in the development of obesity, researchers created mice with whole-body and brain-specific deletion of MRAP2. It was found that at a young age, these MRAP2-deficient mice had a higher cumulative body weight than their wildtype littermates leading to the development of severe obesity (Asai *et al.* 2013). Similarly, in a study of genomes from 500 severely obese humans, one patient was found to have a deletion of one of the MRAP2 alleles and four were MRAP2 deficient, suggesting that, although rare, mutations in

MRAP2 are potentially pathogenic contributors to the regulation of body weight in humans.

Kir7.1 and other ion channels

Based on the finding that AgRP couples MC4R to the pertussis toxin-sensitive Gi/o inhibitory protein in the hypothalamic GT1–7 cell line (Buch *et al.* 2009), it was suggested that MC4R might be coupling to G protein inwardly rectifying potassium channels (GIRKs). This result highlights the ability of MC4R to signal through different G proteins with opposing actions. One is the canonical (for MC4R) Gs α adenyl cyclase stimulatory action activated by α -MSH vs Gi/o inhibitory protein-dependent pathways promoted by AgRP. The ability to signal through Gi/o led to the hypothesis that MC4R could potentially couple to GIRKs, known to be activated by G $\beta\gamma$ binding following release from Gi heterotrimers (Lei *et al.* 2000).

Physiologically relevant data on the mechanism of α -MSH and AgRP signaling through native MC4R derives from the use of electrophysiological slice preparations from mice in which PVH MC4R neurons have been transgenically labeled with GFP (Ghamari-Langroudi *et al.* 2010, 2011). In this preparation, α -MSH depolarizes and AgRP hyperpolarizes MC4R neurons when added to the bath. Notably, by using this experimental setup in the presence of G protein inhibitors such as GDP β S and gallein, the role of GIRKs in α -MSH induced depolarization in MC4R-positive PVH neurons was ruled out. Moreover, Gs α and cAMP signaling were also ruled out, when examined as a potential signaling pathway mediating α -MSH-induced depolarization (Ghamari-Langroudi *et al.* 2015). Current-voltage analyses demonstrated that the conductance involved in α -MSH-induced depolarization was a potassium inward rectifier channel (Kir), and a specific subunit, Kir7.1, was identified using a panel of specific Kir channel blockers.

Also of interest is that AgRP, the endogenous inverse agonist for cAMP responses from MC4R, hyperpolarizes PVH neurons (Ghamari-Langroudi *et al.* 2015). AgRP augmented membrane conductance, suggesting increased potassium channel opening and surface density, leading to hyperpolarization. Cell transfection studies reinforced the finding that AgRP couples the MC4R to Kir7.1 and regulate its opening. The molecular mechanism by which AgRP mediates this effect currently remains unknown and will require further studies to be definitively clarified. Other inward rectifiers such as Kir2.3 and Kir4.1 channels did not couple to MC4R in independent

experiments using cultured cells. As Kir channels form homo- and heterotetramers, it remains to be determined whether MC4R modulates homotetramers of Kir7.1 or heterotetramers with other Kir channel subunits.

Kir7.1 modulation by MC4R also presented new pharmacological paradigms for the receptor, where some ligands have been found to favor the G_{α} vs the Kir7.1 pathway. Ghamari-Langroudi *et al.* (2015) reported changes in potency for well-characterized tool compounds of MC4R, where the MSH analog MC4-NN2-0453, with an EC_{50} of 4.9×10^{-9} M for intracellular cAMP accumulation assays, was found to have an increased potency of 4.9×10^{-9} M in a thallium flux-based assay used to measure

Kir7.1 coupling. Taken together, these findings reveal a novel MC4R signaling pathway, and the potential for the creation of biased ligands that favor the ion channel direct interaction/activation paradigm over classical G protein signaling, making this receptor one of only a handful of GPCRs that were shown to couple directly to ion channels (Zhang *et al.* 2014).

The microcircuitry of α -MSH and AgRP action

The discovery of G protein independent coupling of MC4R to Kir7.1 has suggested that AgRP is both a competitive

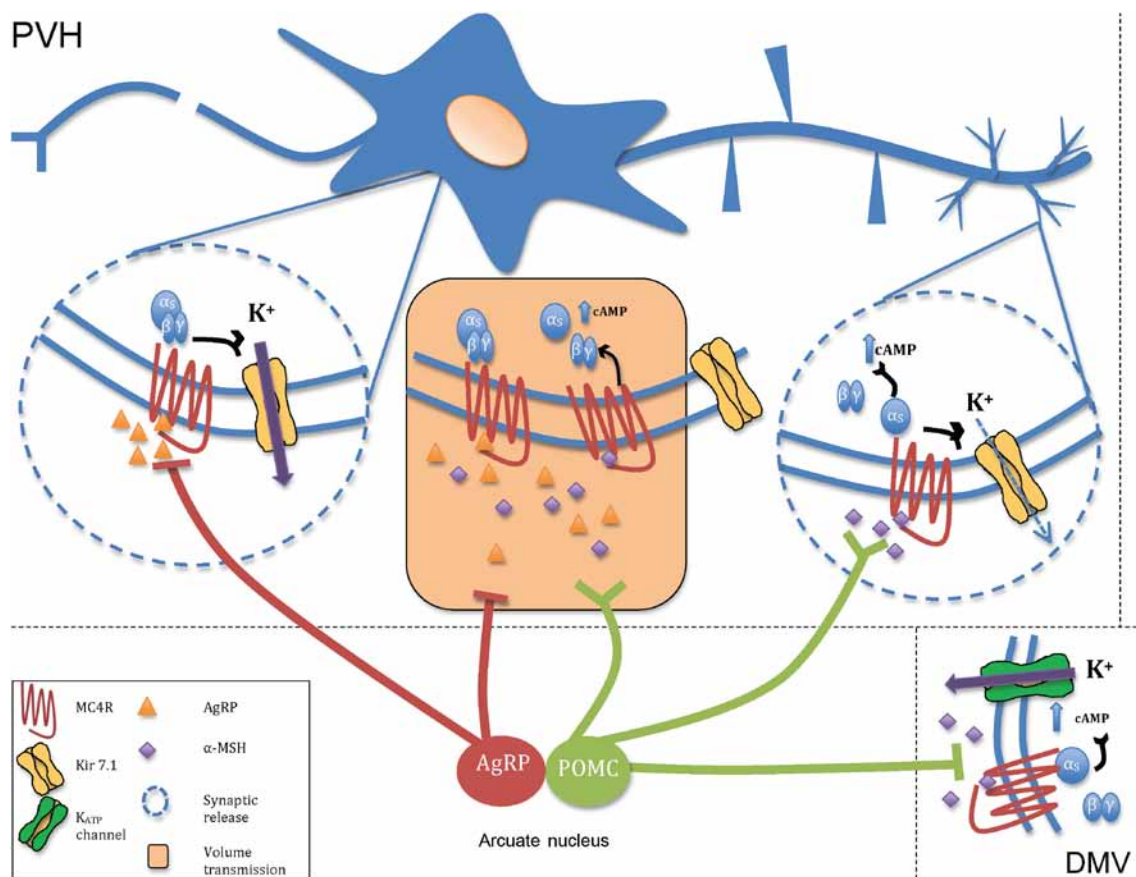


Figure 4

A new model of MC4R microcircuitry. The Yin–Yang model of α -MSH and AgRP action (Fig. 1) suggested competitive binding of these peptides to individual MC4R sites (orange box), and anatomical data suggest in regions where these peptides undergo volume release, that competition for binding to the MC4R may occur. New subcellular anatomical data suggest that in the PVH, AgRP synaptic contacts predominate at cell bodies, while POMC synaptic contacts predominate at distal dendrites. Along with the fact that AgRP immunoreactive fibers are only observed in a subset of MC4R expressing nuclei containing POMC-immunoreactive fibers, α -MSH may thus often act independently of AgRP (right circle). At these sites, α -MSH may be expected to signal through both cAMP, and Kir7.1. 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). Another MC4R signaling pathway, involving cAMP/PKA-dependent activation of K_{ATP} 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). Thus, α -MSH and AgRP utilize a diversity of signaling modalities to regulate feeding and energy homeostasis through the MC4R. Modified, with permission, from Ghamari-Langroudi M, Digby GJ, Sebag JA, Millhauser GL, Palomino R, Matthews R, Gillyard T, Panaro BL, Tough IR, Cox HM, *et al.* (2015) G-protein-independent coupling of MC4R to Kir7.1 in hypothalamic neurons. *Nature* 520 94–98.

antagonist of α -MSH action at the MC4R as well as a biased agonist that can act through MC4R to open Kir7.1 and hyperpolarize neurons (Fig. 4). New neuroanatomical data support both the original Yin–Yang model of α -MSH and AgRP competing for MC4R binding in areas of volume release from POMC and NPY/AgRP neurons (Fig. 1), as well as independent α -MSH and AgRP actions in different brain regions and on different subcellular domains of the target MC4R neurons. Confocal (Bouyer & Simerly 2013) and electron microscopy (Atasoy *et al.* 2008) of PVH neurons suggests that while there are areas of likely volume release of both peptide, PVH cell bodies receive mostly AgRP synaptic contacts, while small distal dendrites receive mostly POMC synaptic contacts. Thus, the microcircuitry is far more complex than originally suggested, in turn having profound impact on models of α -MSH and AgRP action *in vivo* (compare Figs 1 and 4).

Analysis of α -MSH action in the CNS using optogenetics and chemogenetics

Introduction to optogenetics and chemogenetics

The first circuit level genetic manipulation of the POMC and AgRP neurons involved the targeted expression of diphtheria toxin receptor (DTR) in each neuronal subpopulation, followed by administration of DT to ablate the entire neuronal population (Luquet *et al.* 2005). These studies demonstrated that AgRP neurons are essential for food intake, and that loss of POMC neurons in the ARC, but not NTS, results in hyperphagia, decreased energy expenditure, and obesity (Zhan *et al.* 2013). These findings undoubtedly stimulated interest in application of optogenetic and chemogenetic methods to the study of POMC and AgRP neurons. The development of optogenetics and designer receptors exclusively activated by designer drugs (DREADDs) has enabled circuit level manipulation of genetically defined neuronal populations involved in hunger and satiety. Optogenetics (Deisseroth 2011) employs microbial opsins to control neuronal firing properties. The principal opsin used in function studies of feeding circuits is humanized channel rhodopsin, a nonspecific cation channel that can depolarize cells when activated by 470 nm light (Boyden *et al.* 2005). By packaging hCh2R into a Cre-inducible adeno-associated viral vector, this construct can be selectively expressed in target populations via stereotactic injections (Atasoy *et al.* 2008, Sohal *et al.* 2009, Tsai *et al.* 2009). The connectivity and behavioral relevance of these neurons can then be investigated with *ex vivo* channel rhodopsin assisted

circuit mapping (CRACM) and *in vivo* photostimulation (Adamantidis *et al.* 2007, Aravanis *et al.* 2007, Zhang *et al.* 2010). DREADDs (Sternson & Roth 2014) are a related strategy that enables remote control of neuronal activity without the need for specialized hardware (Armbruster *et al.* 2007, Alexander *et al.* 2009). The first-generation DREADDs are molecularly evolved human muscarinic receptors that have minimal endogenous activity and can be activated only by clozapine-*N*-oxide (CNO), an otherwise inert ligand (Armbruster *et al.* 2007). Importantly, DREADDs can be used to induce either Gq (hM3Gq) or Gi (hM4Gi) signaling pathways to depolarize or silence neurons (Sternson & Roth 2014), respectively. Together, these tools have been used to further understand how AgRP, POMC, and MC4R neurons regulate acute and chronic food intake.

Optogenetic and chemogenetic analysis of AgRP and POMC neurons

Initial studies found that 1 h hCh2R stimulation of ARC AgRP neurons induced 0.85 g of food intake in satiated mice (Aponte *et al.* 2011, Atasoy *et al.* 2012). The frequency of stimulation was proportional to the magnitude of food ingested indicating that this effect was presumably due to increased action potential frequency (Aponte *et al.* 2011). Interestingly, the acute phase of ARC AgRP-stimulated food intake was maintained on the *A^v* background and therefore independent of MC4R inhibition (Aponte *et al.* 2011). Similar experiments using DREADDs also found that hM3Gq activation of ARC AgRP neurons resulted in increased food intake and reduced oxygen consumption. hM4Gi-mediated inhibition reduced food intake indicating that ARC AgRP-regulated feeding is bidirectional (Krashes *et al.* 2011). Furthermore, chronic hM3Gq activation of ARC AgRP neurons was found to cause an obesity phenotype that could be reversed following cessation of CNO administration (Krashes *et al.* 2011). By combining ARC AgRP targeted DREADDs with existing KO mouse models, follow-up studies revealed that NPY or GABA was necessary for the acute effects on ARC AgRP neuron activation, while AgRP was sufficient for long-term effects (Krashes *et al.* 2013). More recent experiments have used hCh2R and DREADDs to further define how ARC AgRP neurons encode a negative balance signal for energy depletion (Betley *et al.* 2015) and how they evoke typified energy seeking behaviors even in the absence of food (Dietrich *et al.* 2015). Together, these studies establish a critical role of AgRP neurons in the

regulation of feeding behavior and serve as a model by which to study the role of other genetically defined populations.

Optogenetics and DREADDs have also been used to study the role of POMC neurons in feeding behavior. Chronic but not acute optogenetic stimulation of ARC POMC neurons was found to reduce food intake and cause weight loss (Aponte *et al.* 2011, Zhan *et al.* 2013). Importantly, unlike ARC AgRP-dependent feeding, the satiating effect of ARC POMC stimulation was lost on the *A^y* background and therefore dependent on MC4R signaling (Aponte *et al.* 2011). This finding has been repeated with hM3Dq activation of ARC POMC neurons, whereby animals displayed a 50% reduction of food intake and a 6% drop in their total body mass (Zhan *et al.* 2013). Furthermore, chronic but not acute hM4Di-mediated inhibition of ARC POMC neurons was found to cause hyperphagia, but only after 24 h. Although this finding points toward a role for α -MSH signaling in regulating long-term energy balance, these studies are at odds with pharmacological data that have shown robust acute anorexic effects of MC4R agonists (Fan *et al.* 1997). Although it is possible that supraphysiological MSH dosing paradigms used during pharmacological studies might be responsible for some of this effect, NTS POMC neurons have also been implicated in acute feeding behavior. Indeed, acute hM3Dq stimulation of NTS POMC neurons has been found to cause acute anorexia while chronic activation of this population does not seem to affect food intake (Zhan *et al.* 2013). The mechanism that underlies the discordance between anatomical subsets of POMC neurons remains unknown; however, it is likely a result of their distinct projection fields (Wang *et al.* 2015). Alternatively, acute stimulation of ARC POMC has been shown to promote endocannabinoid evoked feeding, which may be responsible for the delayed response of ARC POMC neurons, but not NTS POMC neurons (Koch *et al.* 2015).

AgRP target sites that mediate acute feeding behavior

Optogenetics and DREADDs also enable the determination of the target sites that are necessary and sufficient and for evoked feeding behaviors. Despite evidence of a functional inhibitory ARC AgRP to ARC POMC projection, coactivation of these two populations did not blunt light evoked ARC AgRP feeding (Atasoy *et al.* 2012, Betley *et al.* 2013). However, site-specific photostimulation of GABAergic ARC AgRP efferents within the PVH was found to be sufficient for acute feeding behavior (Atasoy *et al.* 2012, Garfield *et al.* 2015). This effect was replicated

with hM4Gi-mediated silencing of PVH SIM1 neurons indicating that inhibition of this population is sufficient for feeding behavior. Furthermore, coactivation of PVH SIM1 cell bodies and ARC AgRP efferents did not cause food intake. Initially, PVH OXT neurons (a subpopulation of PVH SIM1 neurons) were thought to be the mediators of ARC AgRP to PVH-induced food intake (Atasoy *et al.* 2012). However, both *ex vivo* and *in vivo* experiments have challenged this finding (Wu *et al.* 2012, Sutton *et al.* 2014, Garfield *et al.* 2015). Using CRACM, ARC AgRP fibers were found to evoke time locked inhibitory postsynaptic currents within 83% of PVH MC4R cells and 0% of PVH OXT expressing cells (Garfield *et al.* 2015). Importantly, MC4R neurons were found to be distinct from OXT neurons as evidenced by immunohistochemistry. Furthermore, costimulation of inhibitory ARC AgRP fibers and PVH MC4R were found to block evoked feeding behavior, while PVH OXT did not. Additionally, PVH MC4R neurons project to and activate the LPB, which evokes a satiety response (Garfield *et al.* 2015). In addition to the PVH, ARC AgRP are known to project to numerous other brain regions. Using a similar efferent projection stimulation strategy, ARC AgRP fiber activation in the BNST, LH, and PVT was sufficient for evoked feeding behavior (Betley *et al.* 2013). This contrasts with ARC AgRP fibers that project to ARC POMC neurons, PBN, CEA, and PAG, which do not evoke feeding. Interestingly, while the BNST and the LH both express MC4R, inhibition of MC4R neurons within these sites does not appear to be responsible for the increased food intake seen with ARC AgRP afferent stimulation (Garfield *et al.* 2015).

Summary

It has been a long road from the discovery 60 years ago of the POMC peptides, to the cloning of the *POMC* gene in 1978, identification of receptors for α -MSH in the brain in 1993, and demonstration of a role of central melanocortin signaling in the control of energy homeostasis in 1997. This year provides another landmark: in January 2016, the US Food and Drug Administration has just awarded orphan drug status to the first α -MSH-based therapeutic for obesity. The α -MSH analog RM-493 (Kievit *et al.* 2013, Chen *et al.* 2015), also known as setmelanotide, was awarded orphan drug status for POMC deficiency and Prader–Willi syndrome. This new innovation will surely generate additional interest in the field, both in terms of basic science aimed at understanding the molecular basis of regulation of energy homeostasis by α -MSH, as well as the development

of therapeutics for obesity and cachexia modeled after the effects of α -MSH and AgRP at MCR in the CNS.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Author contribution statement

All authors contributed equally to the writing and editing of this manuscript. In addition, T G prepared Figs 1 and 4, and L E G produced Table 1.

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