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# Studies on the Physiological Functions of the Melanocortin System

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**The melanocortin system refers to a set of hormonal, neuropeptidergic, and paracrine signaling pathways that are defined by components that include the five G protein-coupled melanocortin receptors; peptide agonists derived from the proopiomelanocortin preprohormone precursor; and the endogenous antagonists, agouti and agouti-related protein. This signaling system regulates a remarkably diverse array of physiological functions including pigmentation, adrenocortical steroidogenesis, energy homeostasis, natriuresis, erectile responses, energy homeostasis, and exocrine gland secretion. There are many complex and unique aspects of melanocortin signaling, such as the existence of endogenous antagonists, the agouti proteins, that act at three of the five melanocortin receptors. However, there is an aspect of melanocortin signaling that has facilitated highly reductionist approaches aimed at understanding the physiological functions of each receptor and peptide: in contrast to many peptides, the melanocortin agonists and antagonists are expressed in a limited number of very discrete locations. Similarly, the melanocortin receptors are also expressed in a limited number of discrete locations where they tend to be involved in rather circumscribed physiological functions. This review examines my laboratory's participation in the cloning of the melanocortin receptors and characterization of their physiological roles. (*Endocrine Reviews* 27: 736–749, 2006)**



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Abbreviations: ACTH-R, ACTH receptor; AgRP, agouti-related protein; AUN, acute unilateral nephrectomy; CCK, cholecystokinin; CNS, central nervous system; DMV, dorsal motor nucleus of the vagus; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; GHS-R, GH secretagogue receptor; GPCR, G protein-coupled receptor; MSH-R, MSH receptor; NPY, neuropeptide Y; NTS, nucleus of the solitary tract; POMC, proopiomelanocortin; PYY, peptide YY; TSH-R, TSH receptor.

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## I. Introduction

MY INTEREST IN G protein-coupled receptors (GPCRs) began around 1987 when Nakanishi's group (1) published the first paper on the cloning of a neuropeptide receptor, the receptor for substance K. Before this discovery, the only GPCR sequences known were rhodopsin, adrenergic, and muscarinic receptors, so the fact that the substance K receptor was related to rhodopsin demonstrated for the first time that GPCRs could bind ligands as diverse as retinal and peptides and suggested that many GPCRs were evolutionarily related and could thus be cloned using a variety of methods such as low-stringency hybridization and polymerase chain reaction using degenerate oligonucleotides. Many laboratories jumped on this opportunity to clone the genes for a wide variety of GPCRs, and I began to focus on cloning receptors involved in endocrine function. The first receptor we isolated and characterized was the thyroid-stimulating hormone receptor, in a collaboration with Deborah Segaloff (2) and Peter Seeburg. The glycoprotein hormone receptors [TSH receptor (TSH-R), LH receptor, and FSH receptor] were unique in that they each have a large (~30 kDa) extracellular motif required for ligand binding. This finding led us to the erroneous hypothesis that large protein hormones, and perhaps even some peptides, would similarly bind related extracellular domains. We collaborated briefly with Hank Kronenberg and Tom Potts, trying to clone the receptor for PTH using low-stringency hybridization with TSH-R probes. Of course, the glycoprotein hormone extracellular motif was not found in the PTH receptor sequence. Nonetheless, this interest in understanding how hydrophilic protein and peptide hormones bind the highly hydrophobic GPCRs, along with the proximity of Jeffrey Tatro and Seymour Reichlin in the Endocrine Division of the New England Medical Center,

were the sole motivating factors leading to our cloning of the melanocortin receptors. Reichlin had a long-standing interest in the biology of α-MSH (3), and Tatro had developed some unique expertise in radiolabeled MSH binding assays and identification of MSH binding sites in both brain and peripheral tissues (4, 5). Using these methods, Tatro and Reichlin had identified a human melanoma sample with high levels of MSH binding sites, and tissue samples such as this were critical key starting materials for cloning of unknown genes in the pregenome era. Although this review is focused on events that occurred after the cloning of the melanocortin receptors, the cloning of the receptors was dependent on the numerous advances in the biology and chemistry of the melanotropic peptides, and this period of history in the field has been extensively reviewed elsewhere (6, 7).

In the early days after the cloning of the melanocortin receptors, colleagues of mine in the endocrine field often questioned the relevance of working on the melanocortin receptors when the TSH-R and its role in thyroid function and disease seemed a more important biological problem at the time. To everyone's great surprise, the study of melanocortin signaling and function has kept my laboratory, and many others, busy with a continuing stream of findings that have demonstrated both novel and important roles for melanocortin signaling in mammalian physiology.

## II. Cloning of the Melanocortin Receptors

Using a cDNA library prepared from the melanoma sample provided by Tatro and Reichlin, Kathleen Mountjoy cloned the human MSH receptor (MSH-R), then used this preliminary sequence data to clone the human ACTH receptor (ACTH-R) (8). Using similar methods, a human MSH-R was also reported by Vijay Chhajlani (9). Because α-MSH consists of the first 13 amino acids of the 39-amino-acid ACTH peptide (Fig. 1), it was not surprising to find that the two receptors shared highly related amino acid sequences. Little MSH-R or ACTH-R mRNA was found in the central nervous system (CNS), yet a large amount of literature had demonstrated effects of melanocortin peptides on learning, behavior, and other central processes. Consequently, degenerate PCR and low-stringency hybridization methods were then used to identify three additional melanocortin receptors, two expressed primarily in the brain (10–13) and one in peripheral tissues (14–19). At that time, the nomenclature of the receptors was determined (MC1-R through MC5-R) based on the order of their cloning, with MSH-R and ACTH-R being renamed MC1-R and MC2-R, respectively (Table 1).

## III. Identification of the *Extension* Locus

The melanin polymers synthesized by the melanocyte can be divided into two major categories, the sulfer-containing yellow-red pheomelanins, and the brown-black eumelanins. The switch regulating the mode of melanin synthesis seems to be linked to the rate limiting enzyme tyrosinase. The primary hormonal stimulator of tyrosinase is α-melanocyte stimulating hormone, which potently elevates intracellular cAMP in the melanocyte via its Gsα-coupled receptor, the MC1-R (20), thus inducing eumelanin synthesis.

Genetic investigations of pigmentation in mice (for review, see Refs. 21 and 22) and a large number of other mammalian species (for review, see Ref. 23) have led to the identification, primarily, of two loci specifically involved in regulation of the eumelanin/pheomelanin switch, agouti and extension. These loci have diametrically opposed actions. Recessive extension alleles result in pheomelanization, or yellow-red coat colors, and dominant alleles result in the "extension" of dark black across the coat of the animal; dominant agouti alleles cause yellow-red coats, whereas homozygosity for null alleles causes dark black coats. Extension alleles act within the hair follicle melanocyte to regulate the eumelanin/pheomelanin switch (24–26), whereas the agouti gene product is made by the surrounding hair follicle cells to regulate the switch both temporally and spatially (27, 28). Thus, when the MC1-R was cloned, it finally became possible to test the hypothesis of Takeuchi (29) that extension might encode the melanocyte-stimulating hormone receptor. This was particularly interesting because many species contained dominant alleles at the extension locus, and no dominant GPCR alleles had yet been reported. The MC1-R coding sequence is found in a single exon, and it was easy to clone and sequence this receptor from genomic DNA from the recessive yellow mouse (*e/e*), and the dark black tobacco ($E^{tob}$), somber ($E^{so}$), and somber-3J ($E^{so3J}$) mice (30). The recessive yellow was homozygous for a MC1-R sequence with a null frameshift mutation, whereas the tobacco and somber animals all had point mutations (30). The tobacco and somber receptors exhibited variable degrees of constitutive activity as a consequence of these changes, thus demonstrating that mutations in GPCRs could mimic the actions of peptide hormones in stabilizing an active receptor conformation. Analysis of extension locus alleles from sheep (31), arctic fox

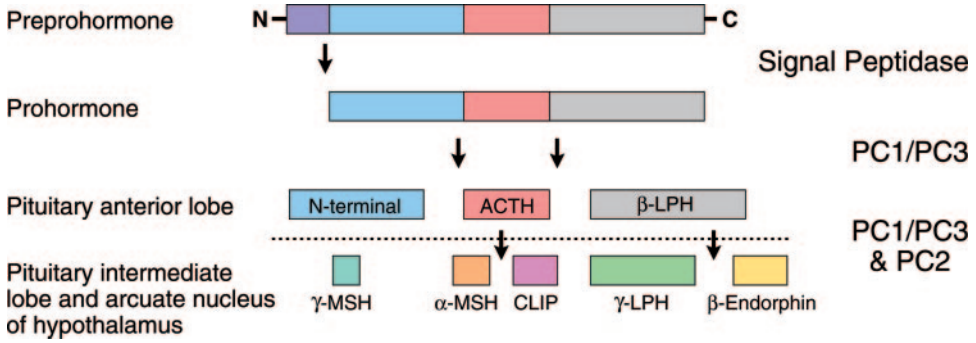


FIG. 1. Structure and processing of the POMC hormone precursor. Graphic shows the processing of the POMC preprohormone into mature melanocortin and β-endorphin peptides in the pituitary and hypothalamus by prohormone convertases. Graphic provided by Drs. Jim Smart and Malcolm Low.

TABLE 1. The melanocortin receptors

| Receptor | Sites of expression | Known physiological functions | Agonists | Antagonists |
|---|---|---|---|---|
| MC1-R | Melanocytes | Pigmentation (regulation of the eumelanin-pheomelanin switch) | $\alpha$-MSH = $\beta$-MSH = ACTH > $\gamma$-MSH | Agouti |
| MC2-R | Adrenal cortex | Adrenocortical steroidogenesis | ACTH | |
| MC3-R | CNS, GI tract, kidney | Energy homeostasis, natriuresis | $\gamma$-MSH = $\alpha$-MSH = $\beta$-MSH = ACTH | AgRP |
| MC4-R | CNS | Energy homeostasis, erectile function | $\alpha$-MSH = $\beta$-MSH = ACTH > $\gamma$-MSH | AgRP, Agouti |
| MC5-R | Exocrine cells | Synthesis and secretion of exocrine gland products | $\alpha$-MSH > $\beta$-MSH = ACTH > $\gamma$-MSH | |

GI, Gastrointestinal.

(32), along with an extensive analysis of mutations induced *in vitro* (33), suggests that, in the case of the MC1-R, constitutive activating mutations may act by stabilizing a charge-charge interaction normally mediated by the charged arginine residue of $\alpha$-MSH. As we were readying this work for publication, constitutively active mutations in rhodopsin were reported (34). Several endocrine diseases, including familial hyperthyroidism, thyroid adenoma, male precocious puberty, Jansen's chondrodysplasia, are now known to result from activating mutations in GPCRs (for review, see Ref. 35).

## IV. Characterization of Agouti

Historically, two allelic series had been characterized in a variety of species to regulate the eumelanin-pheomelanin switch, *extension* and *agouti*. In contrast to extension, recessive alleles at agouti yield dark black coat colors, whereas dominant alleles reduce the amount of eumelanin to produce coats in which yellow to red pheomelanin pigments predominate (Fig. 2). When it was discovered that the extension locus encoded the MC1-R, it seemed natural to propose that agouti might encode an antagonist of the MC1-R (30), particularly because the agouti gene, cloned the same year as the MC1-R, demonstrated the ability to encode a small secreted protein (36, 37). However, it is important to note that in endocrine signaling relying upon hormonal stimulation via Gs-coupled activation of adenylyl cyclase, like the MC1-R, counterregulation could be provided by receptor-mediated activation of Gi to produce inhibition of adenylyl cyclase. Examples of push-pull regulation are found in many hormonally regulated systems, such as heart rate and glycogenolysis. Furthermore, no endogenous GPCR antagonists had been reported. These observations led to the justified comments that "... [Robbins *et al.*] proposes an unnecessarily narrow hypothesis and ignores a more likely one... that agouti protein acts as an agonist on a separate receptor.... The notion of a naturally occurring, physiologically relevant competitive antagonist acting on a cell surface receptor is intriguing, but to our knowledge, without precedence" (38). As pharmaceutical companies began producing recombinant agouti protein, ostensibly for use in cloning the inhibitory agouti receptor, it became possible to test the former hypothesis. Using recombinant agouti protein produced by William Wilkison and colleagues at Glaxo, agouti was indeed found to be a potent competitive antagonist of the MC1-R (39). The activity of the agouti protein at the MC3-R, MC4-R, and MC5-R was also examined in this set of experiments, largely as an internal control for specificity of agouti. Remarkably, although no activity was seen at the MC3-R and MC5-R, agouti was also found to be a potent competitive antagonist of the MC4-R. This finding focused our attention on the other striking phenotype of mice with dominant alleles at the agouti locus, the agouti obesity syndrome. Over the years, five different strains of mice, agouti, tubby, fatty, obesity, and diabetes, had been identified with monogenic obesity; the agouti strain having been identified around the turn of the 20th century (for a review, see Ref. 40). The obesity

FIG. 2. The lethal yellow agouti ($A^Y$) mouse. Photo kindly supplied by Dr. M. L. Lamoreux.

syndrome in mice with dominant agouti alleles, such as the lethal yellow and variable yellow alleles ($A^Y$ and $A^{VY}$), was quite distinct from the syndrome seen in the obese (*ob/ob*) and diabetic (*db/db*) mice. Although *ob/ob* and *db/db* mice were extremely obese, diabetic, hypometabolic, hypothyroid, hypercorticosteronemic, and infertile on the C57BL/6J strain background, $A^Y$ and $A^{VY}$ mice had a milder obesity syndrome with hyperinsulinemia but little diabetes and normal neuroendocrine axes. The finding that agouti was an MC4-R antagonist, along with several additional critical data points described below, led to the hypothesis that ectopic expression of agouti in the CNS and the subsequent antagonism of the MC4-R were responsible for the murine obesity syndrome known for a century to be caused by certain dominant alleles of agouti.

## V. Testing the Melanocortin Hypothesis

Cloning of the MC4-R allowed a further refinement of information regarding possible sites of melanocortin action in the CNS and roles of melanocortins in the CNS. A large body of data on effects of MSH and ACTH in the CNS had suggested exogenous administration these peptides produced activity in learning and memory assays (for a review, see Ref. 41), with a synthetic ACTH derivative called ORG2766 being the most active peptide in these assays. However, pharmacological studies showed that ORG2766 did not bind either of the two melanocortin receptors expressed in the CNS, the MC3-R, or the MC4-R (11, 42). Thus, as described in detail in the review by Adan, the effects of melanocortins on learning and memory are probably largely mediated by cross-reactivity with other receptor systems (41).

Characterization of the distribution of MC4-R mRNA in the rodent CNS demonstrated this receptor to be expressed in many sites involved in autonomic and endocrine function, such as the paraventricular nucleus of the hypothalamus, the dorsal motor nucleus of the vagus (DMV), and the raphe (42). Thus, because dominant alleles of agouti all exhibited ectopic expression, agouti protein was demonstrated to be an antagonist of the MC4-R; and, because the MC4-R was expressed in regions of the CNS involved in energy homeostasis, it became conceivable that the agouti obesity syndrome resulted from chronic blockade of MC4-R signaling. Two approaches were taken to test this hypothesis: 1) development of a melanocortin antagonist and 2) construction of an MC4-R knockout mouse.

### *A. Development of melanocortin antagonists*

At the New York Academy of Sciences Melanotropic Peptides meeting in Rouen, France, in 1993, I met Dr. Victor Hruby, and we agreed to collaborate on the development of MC4-R antagonists. Hruby had conducted structure activity relationship studies on MSH for many years using the frog-skin pigmentation assay and had a collection of hundreds of variant MSH analogs. To be capable of potentially assaying many hundreds of compounds at all of the melanocortin receptors, Wenbiao Chen developed a high throughput colorimetric assay for adenylyl cyclase activity, based on a CRE-$\beta$-galactosidase construct (43). In short, we got lucky; within the first fifty compounds screened, we identified SHU9119 (44) as a potent MC3-R/MC4-R specific antagonist (Fig. 3). The introduction of bulky aromatic amino acids at position 7 of a synthetic cyclic $\alpha$-MSH agonist (Ac-Nle$^4$-c[Asp$^5$,D-Phe$^7$, Lys$^{10}$]$\alpha$-MSH-(4–10)-NH$_2$) led to the activity of these structures as dual antagonists of the MC3-R and MC4-R (44). Two of these compounds, SHU8914 (pI) and SHU9119 (D-Nal(2)), were identified as full agonists of the MC1-R and MC5-R, weak partial agonists of hMC3-R ($EC_{50}$ 1134 $\pm$ 197 nM and 2813 $\pm$ 575 nM, respectively), and subsequently characterized as potent antagonists of the hMC3-R ($pA_2$ = 8.3, each compound) as well as the hMC4R ($pA_2$ = 9.7 and 9.3, respectively). Although unable to unequivocally discriminate between the rodent neural melanocortin receptor subtypes, these antagonists of MC3-R and MC4-R have now been used *in vivo* to define melanocortin pathways that influence physiological control of feeding (45), cardiovascular activity (46), thermoregulation (47), and natriuresis (48, 49).

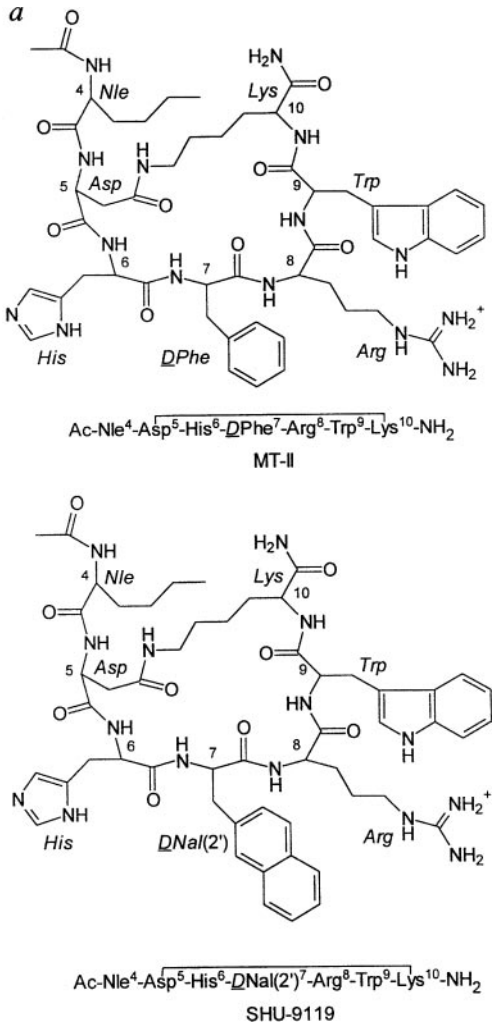


FIG. 3. SHU9119, the first synthetic MC3-R/MC4-R antagonist. The cyclic lactam hexapeptides MTII and SHU9119 differ only by the presence of the amino acids D-phenylalanine (MTII) or the bulkier D-naphthylalanine (SHU9119) at position 7. The addition of the bulkier side group converts the structure from a potent agonist to a potent antagonist of the central MC3-R and MC4-R. Graphic provided by Dr. Victor Hruby.

Melanocortin peptides had been examined in a wide variety of behavioral assays before the cloning of the receptors (for review, see Ref. 41). Data on feeding effects were equivocal. Some showed inhibition of feeding by administration of exogenous α-MSH (50, 51); others showed stimulation of feeding and even weight gain (52, 53). Central administration of SHU9119, a specific antagonist for the central receptors, allowed the demonstration that inhibition of endogenous melanocortin tone stimulated food intake. MTII, a molecule nearly identical to SH9119 with a single amino acid difference (Fig. 3), is a full agonist of the MC3-R and MC4-R and potently inhibited food intake. These data inferred that the central proopiomelanocortin (POMC) circuits acted to inhibit food intake tonically and supported the notion that chronic blockade of the MC4-R could cause an obesity syndrome.

### *B. Characterization of the MC4-R knockout*

Characterization of the MC4-R knockout mouse demonstrated that chronic absence of MC4-R signaling recapitulated the agouti obesity syndrome (54). The animals were obese, hyperphagic, and hyperinsulinemic. Importantly, they exhibited the endocrine profile seen in the obese agouti strains, which is quite distinct from the syndrome seen in the $Lep^{ob}$/ $Lep^{ob}$ or $Lepr^{db}$/ $Lepr^{db}$ mice, resulting from the absence of either leptin or leptin receptor. Perhaps the most remarkable finding resulting from characterization of this mouse was the gene dosage effect. In the initial study, it was reported that animals heterozygous for the receptor were intermediate to wild type and MC4-R null mice in growth rate, serum leptin concentrations, linear growth, and fasting serum insulin concentrations (Fig. 4). This gene dosage phenotype is unusual for GPCRs and also suggested that central melanocortin signaling acts like a rheostat on food intake and energy storage. Several additional model systems demonstrate that a variety of perturbations of melanocortin signaling seen in the $A^{Y}$ and $A^{VY}$ mice, and the MC4-R knockout reproduce the melanocortin obesity syndrome, including overexpression of agouti-related protein (AgRP) (55, 56) or ablation of POMC (57). Targeted destruction of AgRP/neuropeptide Y (NPY) neurons (58, 59) also demonstrates the importance of these neurons for maintenance of body weight.

## VI. Identification of Physiological Functions of the MC5-R

Several groups independently cloned the MC5-R from mice, rats, sheep, and humans based upon homology with the other melanocortin receptors (14–19). The most striking observation about the MC5-R was that it was widely expressed at low levels in a variety of peripheral tissues, but not the CNS. As such, this distribution of expression did not seem to correlate immediately with any of the reported peripheral actions of the melanocortin peptides in pigmentation, adrenocortical function, inflammation, or exocrine gland function (60). Construction of the MC5-R knockout mouse resolved this question (61). Wenbiao Chen created and then studied this knockout, but found no changes in the antiinflammatory, neuroregenerative, or antianalgesic actions reported for peripherally administered α-MSH. However, when performing a forced swim as part of the stress-induced analgesia assay, Chen made the astute observation that, in between the swim and the paw-retraction assay performed to assess analgesia, "the knockout mice were wetter." Chen quickly determined the molecular basis for this. The knockout mice absorbed more water in their coats and were missing a particular species of sebaceous lipids. Furthermore, MC5-R mRNA was found to be expressed in sebaceous glands, and very high levels were found to be expressed in a variety of exocrine glands, including Harderian, lacrimal, and preputial glands. Chen went on to demonstrate that melanocortin agonists are capable of regulating the synthesis and secretion of diverse products from a variety of exocrine glands. More recent data show that the MC5-R is likely to play a role in mammalian behavior by regulating the synthesis and/or release of pheromones from tissues such as the preputial gland (62–64). These data provided a molecular basis for an earlier series of studies demonstrating effects of pituitary and exogenous melanocortins on sebaceous and preputial gland function in the rodent (146).

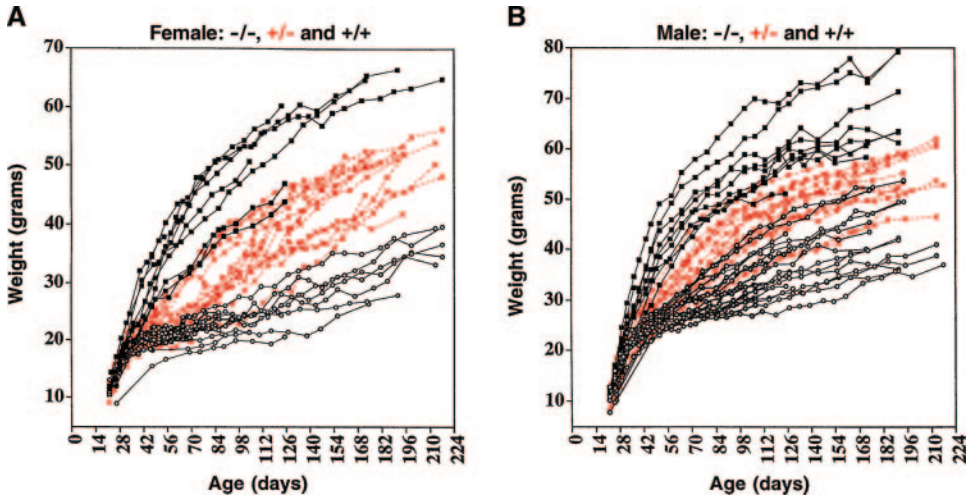


FIG. 4. The gene dosage effect of the MC4-R. Graphs show the growth rate of individual male or female littermates that are wild type (+/+), heterozygous (+/−), or homozygous (−/−) for deletion of the MC4-R. Growth curves of the heterozygous animals are indicated in *red*. [Adapted from D. Huszar *et al.*: *Cell* 88: 131–141 (54) copyright 1997, with permission from Elsevier.]

## VII. Identification of Physiological Functions of the MC3-R

The MC3-R remains the most enigmatic of the melanocortin receptors with regard to its physiological functions. The receptor is expressed both in the CNS and in multiple tissues in the periphery, and is an inhibitory autoreceptor on POMC neurons, yet causes an obesity syndrome when deleted from the mouse genome (65, 66). The obesity syndrome in the MC3-R null is unusual and poorly understood. Deletion of the MC3-R produces a moderate obesity syndrome, with increased weight observed primarily in females, but increased adipose mass observed in both sexes. Compared with the MC4-R null, little hyperphagia is observed in the MC3-R null, even on high-fat chow, suggesting that obesity in this model, in contrast to the MC4-R null, results from increased energy efficiency (67, 68). Furthermore, no increase in lean mass or linear growth is observed in the MC3-R null mouse. Remarkably, despite similar degrees of adiposity in the two models, there is much less insulin resistance and steatosis in the MC3-R null (67).

Evidence for a role for the MC3-R as an inhibitory autoreceptor on the arcuate melanocortin circuit derives from at least four findings: 1) coexpression of the MC3-R mRNA with POMC and NPY in the arcuate (69), 2) inhibition of the spontaneous firing of POMC neurons after application of a MC3-R-specific agonist (70), 3) stimulation of food intake by peripheral administration of a MC3-R-specific agonist (71), and 4) demonstration of an enhanced inhibition of food intake and weight loss after IL-1$\beta$ administration in the MC3-RKO mouse (72).

Given the complexity of the MC3-R, it is useful to provide some background on the pharmacology, expression, and known functions for this receptor. After the successful cloning of MC1-R (MSH-R) and MC2-R (ACTH-R) cDNAs, the MC3-R was the first new member of the melanocortin receptor gene family isolated using PCR and low-stringency hybridization techniques based upon MC1-R and MC2-R sequences (11, 12).

Although unique among the melanocortin receptors in its ability to respond to physiological levels of $\gamma$-MSH, the MC3-R does not show apparent selectivity in its response to stimulation by the various melanocortin peptides $\alpha$-, $\beta$-, $\gamma$-MSH, or ACTH (11, 12). Recombinant human AgRP binds to the human MC3-R and the human MC4-R with high affinity ($IC_{50}$ = 1.1 ± 0.5 nM and 0.5 ± 0.1 nM, respectively) (73) and is a potent antagonist of the human MC3-R and MC4-R (74).

Northern blot hybridization experiments demonstrated that the greatest expression of the MC3-R gene is in the brain, with two mRNA species of approximately 2.0 and 2.5 kb detected in rat hypothalamic poly(A)+ RNA (11). However, using the more sensitive technique of *in situ* hybridization, a thorough examination of MC3-R mRNA distribution in the rat brain demonstrated approximately 35 different nuclei expressing the receptor, with the highest expression seen in the ventromedial hypothalamus, medial habenula, ventral tegmental area, and raphe (11). Not surprisingly, MC3-R mRNA is found primarily in areas of the brain which receive direct innervation from POMC immunoreactive neurons (11). However, the arcuate nucleus contains all of the forebrain POMC expressing neurons and displays moderate levels of MC3-R mRNA, whereas the nucleus of the solitary tract (NTS) containing the other central POMC expressing neurons (75) apparently does not express MC3-R mRNA.

MC3-R expression was also detected in several human gut tissues including the stomach, duodenum, and pancreas, using a combination of RT-PCR and Southern blotting techniques (12). In another study, PCR analysis of human tissues similarly detected MC3-R cDNA in the heart, whereas Southern blotting of amplified cDNA detected expression in the testis, ovary, mammary gland, skeletal muscle, and kidney (76).

The development of specific agonists and antagonists of the MC3-R is important to resolution of the physiological roles of this receptor. More recently, additional work from the Hruby laboratory has led to the creation of peptide agonists and antagonists with specificity for the MC3-R (77–79). Because the MC3-R is thought to function as an inhibitory autoreceptor on POMC neurons, we reasoned that peripheral injections of MC3R-specific agonists would act only within circumventricular organs, or adjacent nuclei like the arcuate to inhibit POMC neurons and thereby stimulate feeding. We were able to test this hypothesis using D-Trp 8-$\gamma$-MSH (78) and, indeed, stimulate feeding via the MC3R when injected peripherally. The work reported in Mayorov *et al.* (79) provides for the first truly useful MC3-R antagonist, c[Nle-Val-D-Nal(2′)-Arg-Trp-Glu]-NH, in that this compound has a 100-fold selectivity for MC3-R over MC4-R.

In addition to both positive and negative effects on energy balance, a variety of other physiological effects have been reported for the MC3-R. Acute unilateral nephrectomy (AUN) induces an increase in both potassium and sodium excretion by the remaining kidney through an adaptive mechanism that is dependent upon intact pituitary function (80) as well as innervation of both kidneys before AUN. Further research demonstrated that, although all of the MSH peptides have some natriuretic activity, an antibody specific to $\gamma$-MSH was able to block the experimental induction of natriuresis by AUN, thereby suggesting a specific role for $\gamma$-MSH in this experimental system (81). The MC3-R null mouse is resistant to the induction of natriuresis by $\gamma$-MSH and is sensitive to high-salt diet-induced hypertension (48). Evidence suggests a role for both central and peripheral MC3-R in this phenomenon (49).

## VIII. Characterization of Physiological Functions of Central Melanocortin Signaling

Data resulting from the creation of the MC4-R null and the administration of SH9119 showed that a blockade of MC4-R signaling produced the agouti obesity syndrome and stimulated food intake. Although defective MC4-R function caused obesity, it clearly was important to elucidate the normal physiological roles of MC4-R signaling. Earlier studies on the obese $A^{Y}$ and $A^{VY}$ strains had demonstrated that the agouti obesity syndrome involved hyperphagia, but also involved a more efficient utilization of calories because $A^{VY}$ mice pair-fed to wild-type intake levels still become obese.

Further, the very finding that the MC4-R is expressed in up to 150 different brain nuclei (42) implied that the physiological roles for this receptor were complex and varied, and much of our work has been focused on this task.

### *A. Leptin signaling and long-term adipostasis*

Leptin receptor expression in POMC neurons was identified shortly after cloning of the ObR, suggesting that melanocortin signaling might be downstream of leptin's adipostatic signal (Fig. 5). However, although initially highly controversial, several observations supported the notion that melanocortin signaling mediates only a subset of leptin's actions and that melanocortin signaling mediated aspects of energy homeostasis that are independent or additive to leptin. First, of course, is the simple observation that the syndromes resulting from deficient leptin signaling *vs.* deficient MC4-R signaling are strikingly different, as described earlier. Second, the fact that leptin receptor is expressed in many sites other than POMC and NPY neurons argues clearly for melanocortin-independent actions of leptin. In an early experiment, the independent actions of these signaling pathways was tested by simply breeding $A^Y$ and $Lep^{ob}/Lep^{ob}$ mice and characterizing the phenotypes of the resulting strains (82). Remarkably, the $A^Y/Lep^{ob}/Lep^{ob}$ mice were more obese and hyperinsulinemic than the two contributing strains, arguing for leptin-independent actions of melanocortin signaling. In this case, because most POMC and NPY/AgRP neurons express leptin receptor, it is imagined that although leptin acts tonically on POMC and other CNS circuits, other factors may acutely regulate the melanocortin signal while the leptin input essentially is held constant. For example, although altered leptin levels may change CCK responsiveness (83, 84), and thus alter satiety, MC4-R signaling appears to be essential for the acute inhibition of feeding by CCK (85). Thus, the hyperphagia in the MC4-RKO has both a primary component (CCK nonresponsiveness, in part) and a secondary component due to a partial defect in leptin signaling. That the defect in leptin signaling in the MC4-R null is partial was originally argued by Palmiter, who demonstrated that young lean MC4-R null mice remained highly responsive to leptin treatment (86), and by an elegant set of experiments in which the leptin receptor was selectively deleted from POMC neurons (87). These animals exhibit a moderate obesity syndrome relative to the severely obese $Lep^{ob}/Lep^{ob}$ mice. Thus, much of the leptin resistance documented in the older obese MC4-R null mice is undoubtedly due to the common leptin resistance occurring downstream of obesity and the attendant elevation of serum leptin levels.

### *B. Autonomic function*

When the MC4-R was cloned and its distribution of expression in the rat CNS determined by *in situ* hybridization (42), it became apparent that the receptor was likely to be involved in the regulation of autonomic outflow. This was clear from its expression in sites such as the paraventricular nucleus, the raphe, and the DMV. Later, when MC4-R blockade was determined to be the cause of the agouti obesity syndrome, it became important to examine the effects of the receptor on autonomic control of functions relevant to energy homeostasis, thus we chose to examine the effects of melanocortin signaling on metabolic rate and the central control of serum insulin levels. These studies demonstrated that the MC4-R null mouse becomes hyperinsulinemic before becoming obese and that intra-PVH administration of a melanocortin agonist suppressed fasting serum insulin levels, increased tissue insulin sensitivity, and increased basal metabolic rate as measured by indirect calorimetry (88). Pharmacological blockade of sympathetic signaling blocked the ability of MTII to lower fasting serum insulin levels. MTII administration was also seen to increase sympathetic nerve activity to brown fat, renal, and lumbar nerves (89). Additional data show that the central melanocortin may affect other autonomically regulated functions, including erectile response (90), blood pressure, and heart rate (91). For example, the bradycadia and hypotension elicited upon injection of $\alpha$-MSH into the dorsovagal complex can be blocked by co-injection of the antagonist SHU9119, implicating MC4-R densely expressed in the DMV (91).

### *C. Acute regulation of energy intake*

*1. Nutrient sensing and response.* The MC4-R null mice were found to be obese and hyperphagic, but what normal phys-

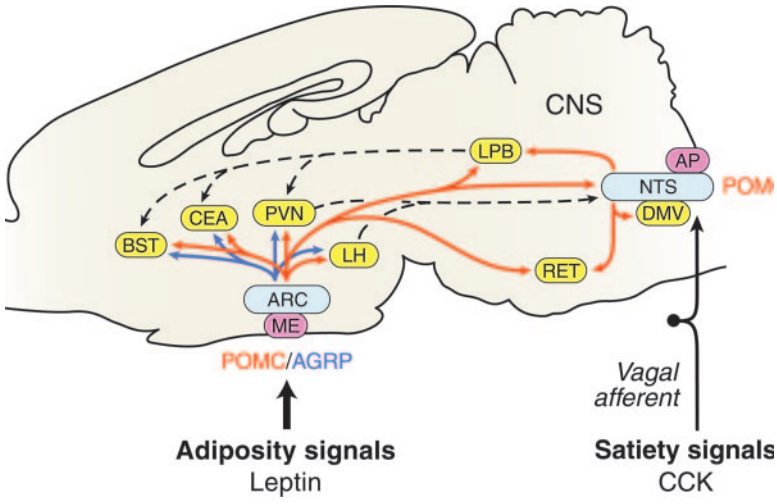


FIG. 5. Integration of adipostatic and satiety signaling by the melanocortin system. Receipt of long-term adipostatic signals and acute satiety signals by POMC neurons in arcuate nucleus and brainstem, respectively. *Blue*: Nuclei containing POMC neurons; *yellow*: nuclei containing MC4-R neurons that may serve to integrate adipostatic and satiety signals; *pink*: circumventricular organs adjacent to sites of POMC expression; *red arrows*: POMC projections; *blue arrows*: AgRP projections. BST, Bed nucleus of the stria terminalus; CEA, central nucleus of the amygdala; PVN, paraventricular nucleus of the hypothalamus; LH, lateral hypothalamic area; LPB, lateral parabrachial nucleus; AP, area postrema. [Adapted from W. Fan *et al.*: *Nature Neuroscience* 7:335–336, 2004 (85) with permission from Nature Publishing Group.]

iological responses relevant to energy homeostasis could be shown to be defective in these animals? Andrew Butler began a series of studies perturbing these animals from homeostasis using a variety of stimuli, then measuring the responses (68). Interestingly, the animals exhibited rather normal rebound hyperphagia to a fast and demonstrated normal cold-induced hyperphagia. Interestingly, the MC4-R null mice exhibited an extremely altered response to a change in diet. When normal C57BL/6J are switched from regular chow (4.5% fat) to a moderate-fat chow (11% fat), after an initial brief period of hyperphagia, they exhibit a decrease in food intake by mass, thus retaining an overall isocaloric intake. In contrast, the MC4-R null mice did not decrease the mass of food intake, but actually increased intake significantly. Although the rate of weight gain on the moderate-fat chow did not increase in the C57BL/6J mice, it increased dramatically in the MC4-R null animals. The defective responses in the MC4-R null were not limited to energy intake because a defect in regulation of energy expenditure was also found. Although C57BL/6J and even $Lep^{ob}$/ $Lep^{ob}$ mice exhibited a measurable diet-induced thermogenesis response to the increase in fat intake, the MC4-R null animals exhibited no measurable diet-induced thermogenesis response. These data suggest that the central melanocortin system is also important for the acute regulation of expenditure in response to calories ingested, an example of acute responses regulated by the melanocortin system under conditions in which changes in leptin levels are unlikely to be an operating factor. These defective responses in energy homeostasis have also been demonstrated in rats treated with SHU9119 (92), and this system has also been used to demonstrate that MC4-R blockade results in defective responses to dietary fats, but not dietary carbohydrates. Furthermore, pharmacological blockade of the MC4-R was not found to inhibit the satiating effects of protein in the diet (92).

*2. Hunger.* Additional evidence of a role for the hypothalamic melanocortin system in responding directly to acute satiety/hunger signals comes from data on ghrelin. Identified as an endogenous ligand for the GH secretagogue receptor (GHS-R) (93, 94), ghrelin is an acylated 28-amino-acid peptide predominantly secreted by the stomach, regulated by ingestion of nutrients (95–98) with potent effects on appetite (97, 98). GHS-Rs have been demonstrated on arcuate NPY containing neurons (99), and pharmacological doses of ghrelin injected peripherally or into the hypothalamus activate c-fos solely in arcuate NPY neurons in rats (100) and stimulate food intake and obesity. Evidence indicates the melanocortin system is central to ghrelin's effects on food intake. Stimulation of food intake by ghrelin administration is blocked by administration of NPY/Y1 and Y5 antagonists (101), and reduced in the NPY −/− mouse. Administration of the melanocortin agonist MTII blocks further stimulation of weight gain by GH-releasing peptide-2, a synthetic GHS-R agonist, in the NPY −/− mouse (102). Finally, peripheral administration of ghrelin activates c-fos expression only in arcuate NPY/AGRP neurons, not in other hypothalamic or brainstem sites (103), and ablation of the arcuate nucleus blocks the actions of ghrelin administration on feeding but not elevation of GH (104). Electrophysiological analyses suggest that ghrelin acts on the arcuate NPY/AGRP neurons to activate these orexigenic cells coordinately and inhibit the anorexigenic POMC cells by increasing γ-aminobutyric acid (GABA) release onto them (105).

*3. Satiety.* The brainstem is classically understood as the center for detection and response to hunger/satiety signals (Fig. 5). An alternate route for satiety factors and nutrients to activate the hypothalamic POMC neurons would involve the known action of these factors at the brainstem because sites of vagal afferent and gut peptide action involve brainstem cell groups like the NTS that send dense projections to mediobasal hypothalamic cell groups like the arcuate nucleus (106).

Several lines of evidence suggest an important role for melanocortin signaling within the brainstem in satiety and vago-vagal reflexes. First, a discrete site of POMC expression in the brainstem has been identified in the commissural NTS (75, 107, 108). ACTH, α-MSH, and β-endorphin immunoreactivity have been identified in these cells (108). POMC NTS fibers are found in many sites in the caudal mesencephalon and spinal cord, with sites of dual innervation by hypothalamic and brainstem POMC fibers seen in the locus coeruleus, parabrachial nucleus, rostral NTS, DMV, and lateral reticular nucleus. MC4-R mRNA is also expressed in multiple sites within the brainstem, including quite high levels within the DMV, NTS, and parabrachial nucleus (10, 109). Fourth ventricular (110) and parenchymal DVC injections (111) of melanocortin agonists and antagonists produced effects on both food intake and weight gain comparable to those seen with lateral ventricle injections. These data infer a remarkable degree of coordination between the brainstem and hypothalamic melanocortin systems.

In addition to signals from gut distension, gut peptides stimulated by meal intake mediate satiety through centers in the brainstem. These signals then are thought to interact primarily with long-term weight regulation centers via neural connections to the hypothalamus to regulate total daily intake by adjusting meal size, number, or both.

Cholecystokinin (CCK) is a good example of such a gut peptide. Produced by the gastrointestinal tract in response to meal ingestion, CCK's diverse actions include stimulation of pancreatic enzyme secretion and intestinal motility, inhibition of gastric motility, and acute inhibition of feeding. Early experiments administering CCK peripherally supported a role for increased CCK levels in the early termination of a meal (112, 113). Central administration of insulin and leptin potentates the satiety-inducing effects of peripherally administered CCK (114, 115).

We sought to test if brainstem POMC and/or MC4-R neurons may be involved in transmitting CCK's satiety signal. Approximately 30% of the POMC NTS neurons are activated (Fig. 6), as determined by induction of c-fos immunoreactivity, after ip administration of a dose of CCK that initiates satiety (85). Furthermore, the central melanocortin system as a whole is clearly important for the satiety effect mediated by CCK because MC4-R −/− mice are largely resistant to CCK-induced satiety, and 4th ventricular administration of the melanocortin antagonist SHU9119 appears more potent than the third ventricular in blocking the actions of CCK (85). It

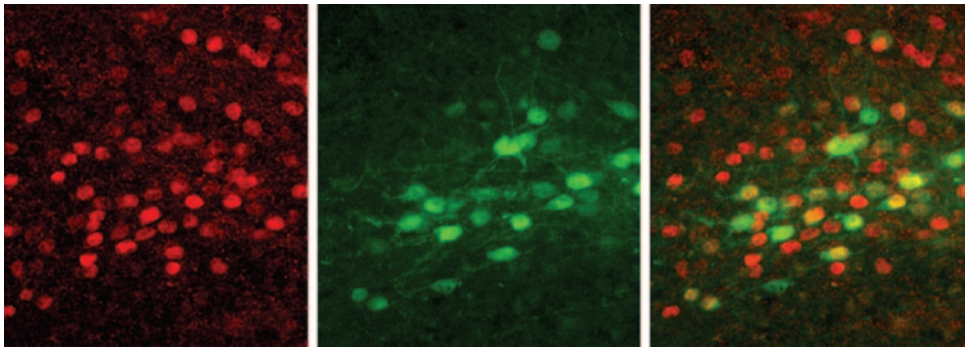

FIG. 6. Regulation of the NTS POMC neurons by the satiety factor CCK. *Left panel*: ip administration of CCK-8s (10 μg/kg) activates c-fos (*red*) in NTS neurons. *Middle panel*: Expression of POMC in the NTS. *Right panel*: ip administration of CCK-8s (10 μg/kg) activates c-fos in POMC NTS neurons (*red*, c-fos; *green*, GFP; *yellow-orange*; c-fos plus GFP. [Adapted from W. Fan *et al.*: *Nature Neuroscience* 7:335–336, 2004 (85) with permission from Nature Publishing Group.]

is important to note that only a very small fraction of cells activated in the brainstem by CCK-induced satiety are POMC NTS cells. Thus, although CCK-mediated satiety appears dependent on MC4-R signaling, its dependence on the POMC NTS cells is unlikely. C-fos up-regulation of 10–15% of these cells was also achieved by feeding-induced satiety (85). Finally, the POMC NTS cells are also activated by leptin (116). Approximately 50% of the cells showed induction of c-fos immunoreactivity after peripheral leptin treatment, and approximately 30% of all c-fos immunoreactive cells in the NTS after leptin treatment were POMC-green fluorescent protein (GFP) positive. Thus, they represent a novel class of NTS neurons regulated by both leptin as well as acute satiety signals. Interestingly, in a different mouse strain, the POMC NTS cells have been reported to be nonresponsive to leptin (117).

Peptide YY (PYY), a peptide related to NPY and pancreatic peptide, is postprandially released by endocrine cells in the ileum and colon (118). PYY is found *in vivo* in both a full-length 36-amino-acid and 34-amino-acid form ($PYY_{3–36}$) in approximately a 2:1–1:1 molar ratio (119). PYY is a potent agonist of both Y1 and Y2 receptors, whereas $PYY_{3–36}$ is a Y2-specific agonist, with approximately 1000 times greater affinity for the Y2 *vs.* the Y1 receptor (120). $PYY_{3–36}$ was demonstrated to inhibit food intake acutely in rats in a Y2 dependent manner and to activate c-fos in a small number (10–15%) of arcuate POMC neurons (121, 122). There was initially considerable controversy surrounding $PYY_{3–36,}$ first, concerning whether or not $PYY_{3–36}$ even inhibits food intake (123). Ilia Halatchev demonstrated that stress-induced anorexia in mice masks the inhibition of food intake by $PYY_{3–36,}$ and this probably explains much of the difficulty in reproducing the finding (121). The result has now been repeated in multiple species, and the fact that PYY null mice exhibit obesity supports a physiological role for the peptide in restraining food intake and weight gain (124). More recently, there has been additional controversy regarding the mechanism of action of the peptide. Initially, we proposed that $PYY_{3–36}$ might access arcuate Y2 receptor sites, altering food intake by activating arcuate POMC neurons (122). However, more recent data demonstrates that $PYY_{3–36}$-induced inhibition of food intake can be seen in both MC4-R (121) and POMC (125) knockout mice, making this model less likely. Furthermore, careful electrophysiological studies show clearly that $PYY_{3–36}$ potently inhibits POMC neurons, thus making hormonal activation of these neurons an unlikely scenario for the anorexigenic actions of administered peptide (126, 127). Finally, peripheral administration of $PYY_{3–36}$ also activates c-fos in the brainstem and induces conditioned taste inversion, suggesting that the brainstem may be the primary site of action of the peptide (128).

## IX. Cachexia

The ability of MC4-R blockade to increase food intake, decrease expenditure, and increase lean mass suggested that chronic melanocortin stimulation could produce a response known as cachexia, a condition found associated with diseases such as some cancers, infectious diseases, and cardiovascular disease. Under normal conditions, decreased energy intake is associated with decreased metabolic rate, preferential loss of adipose mass, and a number of neuroendocrine changes (*e.g.*, hypothyroidism, hypothalamic hypogonadotropism) that act to conserve energy. In contrast, in cachexia or disease wasting, decreased food intake due to loss of appetite is associated with increased metabolic rate and loss of lean as well as adipose mass. Daniel Marks tested the hypothesis that central melanocortin signaling could be important in cachexia by examining the effects of both pharmacological and genetic blockade of the MC4-R in mouse models of cachexia (72, 129, 130). Several aspects of cachexia modeled by administration of the cytokine IL-1β, including the febrile response and the activation of the hypothalamic-pituitary-adrenal axis were unaffected by MC4-R blockade. In contrast, MC4-R blockade inhibits the anorexia, weight loss, and loss of lean mass in IL-1β-induced cachexia, as well as in mouse models of cachexia induced by tumor implantation or diminution of kidney function. As mentioned above, MC3-R blockade actually enhances the cachexigenic response to IL-1β, thus supporting the concept that the MC3-R is an inhibitory autoreceptor in the central melanocortin system and suggesting that specific MC4-R antagonists may have clinical utility in the treatment of cachexia.

## X. Molecular and Cellular Approaches to Understanding Melanocortin Signaling

To begin to characterize the arcuate melanocortin system on the cellular level, we sought to develop an electrophysiological model system for characterizing the responsiveness of the POMC neurons to a variety of hormones and nutrients. Because both POMC and NPY/AGRP neurons comprise only a fraction of total arcuate neurons, a transgenic line

(POMC-enhanced GFP) was created by Dr. Malcolm Low using the hypothalamic POMC promoter driving expression of enhanced GFP. Colocalization studies using an antibody against the POMC peptide β-endorphin demonstrated that greater than 99% of arcuate POMC neurons expressed detectable GFP. Whole cell patch clamp recordings and a loose cell attached patch method using this system were then developed to demonstrate the responsiveness of these neurons to leptin (70), ghrelin (105), serotonin (131), and PYY (126, 127). Leptin was found to activate these neurons primarily by inhibiting the release of GABA from NPY terminals synapsing onto POMC neurons, and immunoelectronmicroscopy demonstrated that many POMC cell bodies are contacted by terminals containing both GABA and NPY. Transgenic lines in which GFP variants are expressed under the control of the NPY (132) and MC4-R (133) promoters also allow investigators to record directly from these neurons as well, and together these data have led to the development of a model in which hormones and peptides appear to control the signal coming from POMC and NPY/AgRP neurons coordinately in the arcuate nucleus (Fig. 7). Remarkably, although levels of NPY, POMC, and particularly AgRP gene expression in the central melanocortin system reflect metabolic state (134–137), the electrical activity of the NPY/AgRP neurons do as well, and this property is maintained in hypothalamic slices used for electrophysiological studies (138). The spontaneous firing rate of the NPY/AgRP neuron is generally quite low (0.5 Hz) and is activated 3-fold by fasting. The activation can be prevented by administration of leptin to the fasted animal (138).

Much remains to be learned on the cellular or molecular level regarding melanocortin signaling downstream of the ligand-receptor interaction. The most intriguing insights thus far derive from two naturally occurring suppressors, the dominant agouti alleles, known originally as mahogany and mahoganoid. Both of these genes, when homozygous recessive, block agouti-induced obesity and pigmentary changes; thus, the function of these genes is required for agouti action at the MC1-R and MC4-R. Mahogany, now known as attractin, is a widely expressed single spanning transmembrane protein that is proposed to be a low-affinity agouti binding protein (139, 140). Mahoganoid, now known as mahogunin, is an E2 ubiquitin ligase (141). Although attractin does not appear to be required for AgRP action (142), the MC4-R has been shown to bind attractin-like protein, an orthologue of attractin that is coexpressed with MC4-R in some brain sites (143). It is possible that ubiquitination plays an important role in the cell sorting or regulation of the MC4-R and MC1-R.

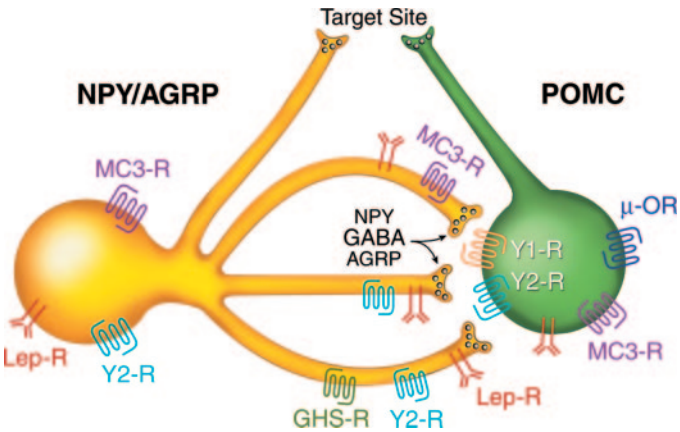


FIG. 7. Coordinate control of energy homeostasis by POMC and NPY/AgRP neurons. Electrophysiological and neuroanatomical studies demonstrate that arcuate POMC and NPY/AgRP coordinately regulate key target neurons involved in energy homeostasis. Some of the neurotransmitters, hormone receptors, and neuropeptide receptors known to regulate these neurons are indicated. [Adapted from M. A. Cowley *et al.*: *Nature* 411:480–484, 2001 (70) with permission from Nature Publishing Group.]

## XI. Future Directions: Simple Genetic Models for Understanding Melanocortin Signaling

Characterization of monogenic obesity mutants in the mouse and candidate gene approaches have led to identification of a few dozen genes that play important roles in energy homeostasis. Given the complexity of the process, there are likely to be hundreds. The recent discovery of leptin (1994), and ghrelin (1999), for example, suggest that we are still at an early stage of the discovery process in this field. Clearly, the development of model systems for the analysis of energy homeostasis would be a highly valuable approach because entire collections of genes physiologically involved in the process could be identified in an unbiased fashion, perhaps even identifying entirely new regulatory pathways. Recently, we and others have demonstrated that some of the key central regulators of energy homeostasis, including POMC, AgRP, MC4-R, and ghrelin, are conserved in teleosts and play a functional role in the regulation of food intake. In particular, AgRP and POMC are both expressed exclusively in the equivalent structure to the arcuate nucleus in the fish CNS. Furthermore, AgRp is up-regulated 3- to 8-fold by fasting in the zebrafish (144). Importantly, intracerebroventricular administration of the melanocortin agonist, MTII, in the goldfish inhibited feeding (145), whereas the MC4-R antagonist HS024 stimulated food intake; these data strongly argue that the central melanocortin system also regulates food intake in fish. Many fascinating problems remain in understanding the function of the central melanocortin system, and of course many aspects of energy homeostasis remain to be elucidated. Based on the knowledge that aspects of these processes are conserved across vertebrates and the advantages of zebrafish for large-scale forward genetic analyses, it should ultimately be possible to conduct screens for mutants in energy homeostasis using this system.

## Acknowledgments

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Disclosure Statement: OHSU and Dr. Cone hold stock in Orexigen, and Dr. Cone also has equity interests in Neurocrine Biosciences and Znomics. Orexigen and Neurocrine have licensed technology from OHSU of which Dr. Cone is an inventor. The technology is used in this research. Dr. Cone also receives research support from the Bristol-Myers Squibb Foundation. This potential conflict was reviewed and a man-

agement plan approved by the OHSU Conflict of Interest in Research Committee and the Integrity Program Oversight Council was implemented.

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