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SPONTANEOUS AND INDUCED GENETIC MUTATIONS OF THE POMC SYSTEM

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INTRODUCTION

Two decades ago a series of biochemical experiments conclusively demonstrated that adrenocorticotropin (ACTH) and β -lipotropin (β LPH) peptides were stoichiometrically generated from a common precursor (1). This result was heralded by one of the landmark studies in the nascent field of eukaryotic cloning, the sequencing of a pituitary cDNA whose deduced amino acids encoded both peptides separated by a pair of basic amino acids (Lys-Arg) (2). Subsequent work demonstrated that the proopiomelanocortin (POMC) prohormone is posttranslationally processed into several biologically active peptides in addition to ACTH and β LPH. Among these are the melanocortin melanocyte-stimulating hormone (α MSH) and the potent opioid β -endorphin (β END). The mechanism of POMC processing became a paradigm in cellular biology for the elucidation of vesicular trafficking and enzymatic cleavage of secreted peptides from prohormone precursors. A renaissance of scientific interest in POMC has occurred in more recent years with the cloning of a gene family of melanocortin receptors and the demonstration of central melanocortin effects on energy homeostasis. This chapter will review the ongoing role that spontaneous and induced (transgenic and "knockout") mutant mouse models have played in the analysis of POMC gene regulation and biological function.

Biosynthesis and Post-translational Processing of POMC

Several comprehensive reviews of POMC processing have been published (3, 4). This section will serve only to introduce major points relevant to the mouse models under consideration and is summarized in Table 1. The principal sites

of POMC expression are corticotrophs and melanotrophs of the pituitary gland and subsets of neurons in the arcuate nucleus (ARC) of the hypothalamus and in the caudal nucleus of the solitary tract (NTS) within the dorsal vagal complex of the medulla (5-7). Endoproteolytic cleavage of POMC by a prohormone convertase (PC1/PC3) in corticotrophs yields ACTH, β LPH, and an NH_2 -terminal peptide (Pro- γ MSH). The latter peptide and/or further proteolytic derivatives have been postulated to mediate paracrine growth and secretory effects within the anterior lobe and to stimulate mitogenesis in the adrenal cortex (8, 9). A small proportion of β LPH is cleaved to yield γ LPH and β END1-31. However, in melanotrophs of the intermediate lobe virtually all β LPH is cleaved by PC2 to γ LPH and β END1-31. The latter peptide is acetylated on its NH_2 -tyrosine and truncated by the action of a carboxypeptidase to yield Ac- β END1-27 and Ac- β END1-26. The acetylated opioid peptides have a greatly reduced affinity for μ and δ opioid receptors and their biological function has not been satisfactorily determined. α MSH is acetylated on its NH_2 -serine and amidated on its COOH-terminal to yield a fully bioactive peptide. β MSH is an additional endoproteolytic product of human γ LPH but is not generated from rodent γ LPH because it lacks the necessary consensus amino acid sequence for cleavage by a PC. Endoproteolytic processing of POMC in ARC neurons closely mirrors the processing in melanotrophs. The neuronal α MSH is fully amidated, but there is minimal NH_2 -acetylation of peptides or COOH-terminal shortening of β END1-31. γ MSH and/or γ_3 MSH are processed from the NH_2 -POMC domain in ARC.

Table 1. POMC Peptides and their Cognate G-Protein Coupled Receptors

Peptide	Cell of origin	Receptor	Target site	Function
ACTH	Corticotrophs	MC2-R	Adrenal cortex	Cort. secretion
Pro- γ MSH	Corticotrophs	MC3-R, ?	Pituitary cells	Paracrine action
Ac- α MSH- NH_2	Melanotrophs	MC1-R	Melanocytes	Pigmentation
Ac- β END1-27	Melanotrophs	$\mu, \delta \uparrow K_d$	Peripheral sites	Modulation ?
DesAc- α MSH	Pituitary	MC5R	Exocrine glands	Secretion
γ MSH, γ_3 MSH	ARC neurons	MC3-R	Limbic system	Homeostasis
DesAc- α MSH- NH_2 , ACTH	ARC neurons	MC4-R	Hypothalamus	Homeostasis
β END1-31	ARC neurons	$\mu = \delta > \kappa$	Diffuse CNS	Analgesia, reward

Five distinct members of a G-protein coupled, melanocortin receptor gene family have been cloned (reviewed in (10)). MC1-R is expressed on melanocytes within the skin and its activation by Ac- α MSH- NH_2 leads to increased membrane adenylyl cyclase activity by interaction with G_{sa} and subsequently greater production of the brown-black eumelanin pigment and less of the yellow-red pheomelanin. MC2-R is the classical ACTH receptor

expressed on zona glomerulosa and fasciculata cells of the adrenal cortex and it has very low affinity for the various melanocortin peptides other than ACTH and ACTH₁₋₂₄ (11). Two additional members of the gene family, MC3-R and MC4-R are expressed exclusively in the CNS in partially overlapping but distinct neuroanatomical areas with particularly high abundance in the hypothalamus and limbic system (12, 13). The MC3-R has the highest affinity for γ MSH, although it is also activated by other melanocortins, while the MC4-R is activated most selectively by α MSH and ACTH. Together these receptor subtypes mediate most of the described melanocortin effects on appetite, metabolism, thermoregulation, autonomic outflow, and behavior. The MC5-R is the most ubiquitously expressed of the melanocortin receptors and its best-documented function is the stimulation of exocrine function including the sebaceous, preputial, Harderian, and lacrimal glands (14).

Three opioid receptor genes have been cloned corresponding to the μ , δ , and κ receptors originally defined by their pharmacological profiles (15, 16). β END₁₋₃₁ has highest and nearly equivalent affinity to both μ and δ receptors (17). However, each of these subtypes has significant affinity for other opioid peptides derived from the proenkephalin and prodynorphin precursors.

POMC Peptides and Energy Homeostasis

Classical ablation or disconnection experiments utilizing electrolytic and chemically generated lesions or knife cuts of the mammalian brain revealed a number of discrete areas within the medial-basal hypothalamus to be nodal-points in the regulation of energy homeostasis (reviewed in (18)). One principal area characterized in this fashion is the ARC located at the base of the hypothalamus and adjacent to the third ventricle. The ARC extends rostrocaudally from the posterior border of the optic chiasm to the anterior border of the mammillary nuclei. A subset of hypothalamic neurons distributed throughout the ARC express the POMC gene. These POMC neurons densely innervate other brain areas implicated in energy homeostasis, motivated behavior, and autonomic control including the ventromedial nucleus of the hypothalamus (VMN), dorsomedial nucleus of the hypothalamus (DMH), paraventricular nucleus of the hypothalamus (PVN), lateral hypothalamic area (LH), ventral tegmental area (VTA), parabrachial nucleus, and NTS.

The lesion studies showed that disruption of the mammalian ARC correlated with an obese phenotype and led to the concept that this area of the hypothalamus contained a satiety center. Later efforts focused on determining the specific neuronal phenotype in the ARC that was involved in

the regulation of energy homeostasis. An early indication of the importance of POMC peptides was the detection of MSH binding by autoradiography in the VMN, another hypothalamic nucleus involved in regulating appetite and feeding (19). In addition, central or peripheral injections of melanocortin peptides were shown to affect food intake. Melanocortins appear to have dual roles in energy balance, depending on the site of action. Administration of ACTH and Ac- α MSH directly into adipose tissue increases lipolysis whereas intramuscular injection of DesAc- α MSH increases food intake. Furthermore, peripheral DesAc- α MSH inhibits pituitary production of ACTH and Ac- α MSH, which may lead to increased lipogenesis (20). MSH analogues administered i.c.v. or injected stereotactically directly into the PVN of rodents have potent anorexigenic effects and stimulate metabolic rate (21). Exogenous opiates and endogenous opioid peptides have also been shown to affect food consumption and food preference, suggesting a role for POMC-derived β -endorphin, in addition to the melanocortin peptides, as a modulator of energy homeostasis (22-24). The physiology of this complex neuroendocrine system is virtually impossible to replicate in *in vitro* models. Thus, transgenic mice and mice with naturally occurring mutants or targeted genetic alterations have been extremely informative tools for the analysis of POMC peptides and their receptors in the control of food intake, energy partitioning, and body weight.

One of the first mutant mouse models of obesity to be studied was the lethal yellow (A^Y/a) mouse (25). The mutation arose spontaneously in the C57BL/6 inbred strain and is associated with a striking yellow coat color and the development of obesity and hyperphagia. Genetically, the mutation was shown to be dominant to the extension (E) locus, later identified as the gene encoding the MC-1 receptor (26). Cloning of the agouti gene revealed that it encodes a secreted protein characterized by multiple pairs of intrachain disulfide bonds in its C-terminal domain. Agouti has high affinity binding for the MC1-R, antagonizes receptor activation by MSH (27), and is normally transiently expressed only in the hair follicles accounting for the yellow banding of individual black hairs in the fur of many mammals. The mutant A^Y allele results from a 120 kb genomic deletion and juxtaposition of the agouti coding sequences to the Raly gene locus and subsequent ubiquitous expression of agouti protein from the Raly promoter (25). These observations led to the agouti-obesity hypothesis, which states that agouti protein expressed within the brain antagonizes the action of central MSH to a neural MC receptor involved in regulation of energy balance. Some of the experimental models reviewed in this chapter provide essential data in support of this hypothesis, which has been further validated by the discovery and cloning of an agouti homologue, agouti gene-related protein (AGRP).

AGRP is expressed in the nervous system and functions as an antagonist of central MC receptors (28).

Genetic linkage studies and quantitative trait loci analyses have strongly implicated the POMC gene locus as an important determinant of weight homeostasis in humans of many different ethnic populations, although specific alleles associated with obesity have not yet been demonstrated (29, 30). Because no mutations within the coding region of the POMC gene that alter peptide activity have been identified in these populations, a current hypothesis is that mutations in regulatory regions of the gene decrease the level of POMC expression in the brain. However, a small number of children from consanguineous parents have been found to have null mutations in the POMC gene resulting in the absence of detectable circulating ACTH (31). These children presented with a syndrome of red hair, adrenal insufficiency and severe, early-onset obesity. In addition, both dominant and recessive mutations in the MC4-R gene have been found in the human population, and MC4-R mutations have been proposed to play a role in as many as 5% of pediatric obesity cases (32, 33).

POMC GENE REGULATION

A standard method to characterize the *cis* DNA elements and transcription factors that control POMC gene transcription has been the expression of fusion genes, consisting of POMC promoter sequences and a reporter sequence, in transfected cell lines. The most commonly used cell line is AtT20, originally derived from a radiation-induced pituitary tumor (34). These cells have many characteristics of primary corticotrophs. Small cell lung cancers often ectopically express POMC and cell lines derived from these tumors have also been used for the analysis of POMC gene expression (35). It is clear, however, that neither of these cell lines are adequate models for elucidation of the mechanisms governing neural-specific expression of the POMC gene. For this reason, our laboratory has focused on a transgenic mouse approach for the identification of cell-type specific regulatory elements in the POMC gene.

Promoter Analysis in Transgenic Mice

The Drouin laboratory was the first to report the generation of transgenic mice expressing a neomycin-resistance coding sequence (*neo*) from 770 bases of the rat POMC promoter (36). Although the expression pattern in pituitary and regulation by adrenalectomy and dexamethasone were consistent with accurate cellular expression of the transgene, the low levels of transcript from the *neo* reporter prevented the direct demonstration of corticotroph and melanotroph specific transcription. Our laboratory initially

approached this problem using an *E. coli lacZ* reporter gene encoding β -galactosidase, which was useful for both the precise cellular identification of transgene expression and quantitation of transcriptional activity by an enzymatic assay utilizing a fluorogenic substrate. These studies demonstrated that 770 bases of the rat POMC promoter from nucleotide -706 in the 5' flanking sequences to +64 in the 5' untranslated region of exon 1 were sufficient to direct *lacZ* expression selectively to pituitary corticotrophs and melanotrophs (Figure 1, POMC-LacZ) (37). The ontogeny of transgene expression also closely approximated the time course of endogenous POMC gene expression in the two lobes of the pituitary gland. However, there was no detectable expression of the -706POMC-*lacZ* transgene in the brain suggesting that additional genomic sequences were necessary for neuronal transcription.

Additional studies from our laboratory analyzed the rat POMC promoter in more detail to define the minimal elements essential for pituitary expression (38, 39). Accurate cell-specific colocalization was verified in all instances by double-label immunofluorescence histochemistry. Truncations of the rat POMC promoter to nucleotide -323 or -234 had minimal effects on both the ratio of expression-positive to total transgenic pedigrees and qualitative levels of reporter expression in the positively expressing lines. Further deletion of the 5' flanking sequences to nucleotide -160 abolished expression in the pituitary gland. Transfection studies in AtT20 cells had identified important regulatory elements in the core promoter region of the POMC gene. However, we demonstrated that the enhancerless promoter and TATA box from the HSV1 thymidine kinase gene were equivalent to the native POMC promoter sequences between nucleotides -34 and +64, in the context of upstream flanking sequences of POMC, to support pituitary cell-specific expression and normal upregulation by adrenalectomy. Based on these promoter truncation studies in transgenics and our data from DNase I protection assays and gel-shift assays utilizing POMC oligonucleotide probes and fractionated nuclear proteins from AtT20 cells, we designed a series of more discrete mutations in the rat POMC promoter. This second generation of transgenic experiments identified two functionally important protein binding sites at nucleotide positions -262/-253 and -202/-193 of the POMC gene (39). At least one of these sites had to be intact to support detectable pituitary expression of the transgenes. In addition, the ubiquitous transcription factor SP1 appeared to play a supportive role in POMC transgene expression through its interaction with DNA sites at positions -201/-192 and -146/-136.

Fig 1. Restriction maps of the mouse POMC gene locus, transgene constructs utilized to map the location of tissue-specific regulatory elements, and targeted POMC alleles that either selectively prevent β -endorphin production or result in the absence of all POMC peptides. See text for details of the individual alleles.

None of the transgene constructs that we studied selectively directed expression to only pituitary melanotrophs or corticotrophs, suggesting that both cell types share one or more essential components for POMC gene transcription. In common with most published transgenic promoter analyses, we observed a large quantitative and qualitative range of expression levels among independent pedigrees for each construct. It has been suggested that these mosaic patterns of gene expression are influenced by repressive effects of multiple-copy transgene integration in some cases (40). In general, basal expression of the transgenes appeared to be greater in individual melanotrophs than corticotrophs, regardless of the type of reporter molecule.

The data from our transgenic promoter analyses revealed a number of inconsistencies with the mapping of functional POMC promoter elements by transfection studies in AtT20 cells. These differences may be due in part to

the more stringent requirement in transgenic mice for the definition of cell-specific expression and the importance of chromatin remodeling during *in vivo* development. However, there is good agreement between both kinds of studies for the key importance of DNA elements between nucleotides -160 and -323 in the 5' flanking domain of the POMC gene. This region contains a site between -173/-160 that appears to mediate the transcriptional activating effects of both corticotroph releasing hormone (CRH) and the cytokine leukemia inhibitory factor (LIF), although it is neither a binding site for CREB, AP-1 or STAT proteins (41). In contrast, the POMC TATA box, a putative AP-1 binding site in exon 1, and more distal sites in the POMC 5' flanking sequences that bind HLH proteins and confer a synergistic activation of POMC expression in AtT20 cells do not appear to be essential for pituitary cell-specific expression in the *in vivo* paradigm (42).

Generation of Novel POMC Expressing Cell Lines

Targeted cellular expression of oncogenes with tissue-specific promoters has led to new insights into the molecular basis of tumorigenesis. Several laboratories have used this technique to isolate novel differentiated cell lines (43-46). We used the rat POMC promoter elements to express two isoforms of the simian virus SV40-Large T antigen in pituitary corticotrophs and melanotrophs. Wild-type SV40Tag consistently induced tumors in the pituitary gland, as predicted, but also in the thymus gland with incomplete penetrance of the phenotype (47). However, the incidence of thymic tumors decreased and eventually disappeared after 15 generations of backcrossing to an outbred CD-1 genetic background, suggesting that epistatic interactions with gene alleles from the original B6D2 genetic background were necessary for expression of the transgene in thymus. In contrast, the pituitary tumor phenotype remained stable after 27 generations of breeding (48). The variable latency to tumor development in these mice is consistent with the idea that stochastic secondary events likely occur in individual T-antigen expressing cells before they are transformed and lose their inhibitory growth controls. Other transgenic mice expressing the temperature-sensitive tsA58 mutant form of SV40Tag from the same POMC promoter sequences only developed pituitary tumors after 1 year of age (our unpublished data). The longer latency was presumably due to the expression of minimal amounts of biologically stable tsA58-Tag at the partially permissive core body temperature of the mice.

Although the POMC-Tag transgene is expressed in both pituitary corticotrophs and melanotrophs, every pituitary tumor that has developed in the mice has been of melanotroph origin based on visual inspection and biochemical and molecular studies. The melanotroph tumor cells are fully transformed because they will produce secondary tumors after subcutaneous

passage in nude mice and will freely grow in suspension culture (47, 49). Unlike the strict dependence for IGF-II in the induction and progression of pancreatic islet β -cell tumors from an insulin promoter-Tag transgene (50), we found no evidence for a critical role of the IGF system in the POMC-Tag induced pituitary tumors (51). Posttranslational processing of POMC in the primary tumors was indistinguishable from normal melanotrophs and was characterized by high proportions of NH_2 -acetylated and COOH -truncated forms of β -endorphin and $\text{Ac-}\alpha\text{MSH}$ with virtually no ACTH (47). Even though the tumors expressed high levels of PC2, which is characteristic of melanotrophs, there was frequently a sufficient quantity of an ACTH-like peptide secreted from the neoplastic glands to induce adrenal cortical hyperplasia and elevated serum corticosterone. A syndrome of fatal melanotroph-dependent Cushing's disease was recently reported in mice with a null mutation in the gene encoding the neuroendocrine protein 7B2 (52) (see Chapter 8). 7B2 has been implicated in the activation of PC2, accounting for the lack of functional PC2 production and generation of ACTH instead of $\text{Ac-}\alpha\text{MSH}$ and CLIP in melanotrophs of the 7B2 null mice. However, it remains unexplained why ACTH is constitutively secreted from the intermediate lobe of these mice because a knockout of the PC2 gene results in similarly high ACTH production in melanotrophs without the unregulated secretion of the hormone (53). Therefore, 7B2 may have an additional function in vesicular trafficking or secretion.

Two cell lines with distinct phenotypes were isolated from cultures of the mouse melanotroph tumors (54). mIL39 cells are melanotrophs based on their expression of both POMC and the dopamine D2 receptor. They are small, bipolar cells and rapidly produce secondary tumors after transplantation into nude mice. In contrast, mIL5 cells fail to express either POMC or detectable levels of SV40Tag. The exact origin of these latter cells is unknown but they are of interest because they secrete a prolactin-regulating factor. The lack of concordance between this prolactin-regulating activity and POMC gene expression suggests that the activity is not due to a peptide product of POMC. More recently our laboratory has isolated another melanotroph-like cell line from a pituitary tumor induced by the tsA58SV40Tag (Chronwall et al, submitted). These cells express high levels of POMC mRNA and POMC prohormone but they have few secretory granules and limited posttranslational processing of POMC. Preliminary studies have demonstrated the expression of several G-protein coupled receptors including dopamine D2, CRH R1, and GABA_B that are normally expressed on melanotrophs. These receptors are functional and therefore this cell line may be useful for analyzing the biochemical cross-talk in signaling pathways that are unique to pituitary melanotrophs.

Targeting POMC Neurons *In Vivo*

Because neither 770 or 4000 bp of the 5' flanking sequence from the rat POMC gene were sufficient to produce appropriate neuronal expression in transgenic mice (55), longer mouse genomic POMC clones were isolated from a cosmid library. Exon 3 was marked with an oligonucleotide sequence to distinguish transgenic mRNA from endogenous POMC mRNA and to enable the simultaneous quantitation of both mRNA species by an RNase protection assay. A new series of transgenic mice was generated and analyses of mRNA expression in the mice clearly established the location of a putative neuron-specific enhancer within an 11 kb stretch of 5' flanking DNA sequences between -13 kb and -2 kb relative to the transcriptional start site (Figure 1, $\Delta 3'P27^*$ and HAL*) (56). Transgene constructs containing these sequences have had a 100% penetrance of correctly targeted expression in neurons of both the ARC and NTS, consistent with the existence of a locus-control type regulatory element within the POMC gene. There is also total concordance of pituitary expression with neural expression from the longer genomic constructs indicating that an overlapping subset of POMC regulatory elements and cognate transcription factors may be required for transcription in both cell types. The recent identification of a novel neuropeptide precursor gene, cocaine and amphetamine responsive-transcript (CART), as a cotransmitter in POMC arcuate neurons suggests that these two distinct genes may also share a subset of regulatory factors (57, 58). Ongoing functional analyses of the distal POMC genomic sequences combined with recent advances in the human and mouse genome projects should rapidly lead to the identification of the minimal DNA elements that are responsible for neural-specific expression. It will then be possible to compare these sequences with the corresponding regions of DNA amplified from obese subjects to test the hypothesis that mutations in POMC regulatory sites are the molecular basis for the genetic linkage established by QTL analyses (29). Ultimately, the functional significance of nucleotide differences identified by this type of horizontal genetic comparison can be tested in mice by targeted mutagenesis and analyses of POMC expression in hypothalamus, feeding behavior, and energy partitioning.

Our characterization of neural-specific regulatory regions in the POMC gene provided a means to identify viable POMC neurons using a nondestructive, green fluorescent protein (enhanced-GFP) reporter (Cowley et al., submitted). Previously, the analysis of cellular events in POMC neurons has been limited because of their small number and sparse distribution. Bright green fluorescence was readily identified in the basal hypothalamus and in the NTS of transgenic mice expressing a POMC-EGFP construct (Figure 1). Double-labeling immunofluorescence with a β -endorphin antisera revealed >99% cellular colocalization of EGFP and endogenous POMC peptide

expression within the ARC. The developmental onset of EGFP expression in both hypothalamic and pituitary cells also matched the ontogeny of native POMC.

POMC neurons are an important site of leptin receptor expression in the hypothalamus (59). The adipocyte derived hormone leptin regulates long-term aspects of energy homeostasis by signaling the levels of fat mass to the brain. Leptin also has acute actions to stimulate *c-fos* and *Socs3* expression in POMC neurons and can cause the release of β -endorphin from hypothalamic fragments *in vitro* (60). Therefore, the POMC-EGFP transgenic mice will be a useful tool to test the hypothesis that leptin directly activates POMC neurons. Preliminary electrophysiological experiments in hypothalamic slices suggest that leptin has both pre- and postsynaptic actions on POMC neurons to increase the frequency of spontaneous action potentials. Additional studies using this transgenic mouse line will decipher the role of other peptidergic modulators, cytokines, and amino acid neurotransmitters on POMC neuronal activity. EGFP-labeled POMC neurons can also be used for investigation of neuronal migration during development and as a unique source of mRNA for differential gene expression analyses. Similar techniques to identify rare neuronal populations *in vivo* are increasingly being used by other laboratories. A prime example is the identification of GnRH neurons as described in Chapter 3 of this volume and by other investigators (61).

In principle, the neural-specific regulatory sequences of the POMC gene can be used to target expression of foreign or mutated proteins to POMC neurons to investigate intracellular signaling pathways *in situ*. For example, leptin resistance may be associated with alterations in the capacity for leptin signaling through either JAK/Stat or MAPK cascades. Individual molecular steps in these pathways could be perturbed by the use of dominant-negative factors or expression of natural inhibitor proteins. Finally, targeted oncogene expression to POMC neurons may lead to the isolation of a differentiated POMC neuronal cell line, which would greatly facilitate molecular studies of POMC gene regulation and the membrane properties of the neurons themselves.

MUTATIONAL ANALYSIS OF POMC FUNCTION

TARGETED MUTAGENESIS OF THE POMC GENE

β -endorphin-Deficient Mice

β -endorphin-deficient mice were generated by the introduction of a point mutation into the POMC gene by homologous recombination in embryonic

stem cells (62). The nucleotide insertion at the amino-terminal tyrosine of β END introduced a premature translational stop-codon into exon 3 (Figure 1, construct POMCX*4). Homozygous mice carrying this mutated allele express normal levels of POMC mRNA in pituitary and ARC. The mRNA encodes a COOH-truncated POMC prohormone that is processed normally to ACTH and other melanocortin peptides (63). The mice have no detectable immunoreactive β END and have been used to infer the normal physiological function of this specific endogenous opioid peptide. Basal activity of the hypothalamic-pituitary-adrenal axis is normal and the β END-deficient mice have normal corticosterone responses to a variety of stressors. β END-deficient mice exhibit unaltered nociceptive thresholds and unaltered antinociceptive responses to i.p. morphine administration. However, β END-deficient mice fail to exhibit endogenous opioid-mediated stress-induced antinociception and have increased levels of nonopioid (naloxone insensitive) stress-induced antinociception (63). Complimentary studies in enkephalin-deficient mice have demonstrated that they retain endogenous opioid stress-induced antinociception (64). Taken together, these data provide convincing genetic evidence that β END derived from the POMC prohormone, and not the more abundant enkephalin peptides, is the natural opioid mediating stress-induced antinociception. The most likely relevant neuroanatomical substrate is the projection of ARC POMC neurons to the central periaqueductal gray known to modulate descending antinociceptive pathways to the brainstem and spinal cord.

More recent studies have demonstrated that the β END-deficient mice have altered antinociceptive responses to morphine administered i.c.v. or i.t. The mutant mice are more sensitive to the antinociceptive action of morphine injected centrally into the lateral ventricle but less sensitive to morphine injected into the spinal compartment, compared to wild-type control mice (65). The μ -specific agonist DAMGO produced qualitatively identical results indicating the importance of the μ opioid receptor in the phenotype. Total binding sites for the μ , δ , and κ receptors, assessed by quantitative autoradiography, were normal in the β END-deficient mice suggesting that subtle alterations in μ receptor signaling may be the mechanism for the opposing alterations in central and spinal sensitivity to morphine. An electrophysiological study demonstrated a normal dose-response to DAMGO for the induction of an inwardly rectifying K^+ current (GIRK) in mediobasal hypothalamic neurons of the mutant mice (66) consistent with the suggestion that μ receptor number, affinity, and coupling to G proteins are not altered by the complete absence of β END.

Opioids, including β END, have generally been shown to increase food intake in pharmacological studies (67). Paradoxically, β -endorphin deficient mice exhibit a sexually dimorphic obesity phenotype due to increased white fat mass in males (Appleyard et al, submitted). The male mice are slightly hyperphagic and have a normal basal metabolic rate. It is not yet clear if POMC derived melanocortins and β END are involved in a parallel, redundant anorexigenic pathway within the hypothalamus or if β END additionally plays a role in an opioid reward pathway that is activated by feeding.

Null Mutation of the POMC Gene

Mice that are deficient in all POMC peptides (POMC Null) were generated by the Hochgeschwender laboratory by homologous recombination of a POMC allele containing a deletion of exon 3 (Figure 1, POMC Null). The mice develop hyperphagia and obesity similar to that seen in the lethal yellow (A^Y/a) mice and MC4-R deficient mice (68). In contrast to the other obesity models, POMC Null mice have no detectable levels of circulating corticosterone or epinephrine and their adrenal glands are one-third the size of wildtype littermates. The adrenal deficiencies in these mice strengthen the hypothesis that normal POMC expression is needed for complete adrenal maturation, although the role of NH_2 -terminal peptides in addition to ACTH remains an open question that can now be approached experimentally *in vivo*. A partial perinatal lethality occurs in litters produced from heterozygous breeder pairs leading to significantly decreased numbers of homozygous POMC Null offspring compared to Mendelian predictions. Homozygous POMC Null adults are fertile and females carry their litters to term but all pups die in the immediate perinatal period (our unpublished data). The mechanism for this pattern of perinatal lethality from both heterozygous and homozygous POMC Null female mice has not yet been explained, but may result from the combined lack of glucocorticoids and epinephrine in the POMC Null pups.

In principle, the absence of all POMC peptides should recapitulate the sum of phenotypes observed in mice deficient in the various receptor subtypes for the peptides. Although not all the characteristics of the various receptor-deficient mice have been investigated, some of the predicted phenotypes are absent in the POMC Null mice. In particular, POMC Null mice do not exhibit the hair pigmentation that would be expected based on the phenotypes of A^Y/a yellow and null MC1-R mutants (see discussion in the following section). The original POMC Null mice bred on an agouti (A/A) 129 genetic background had patchy alterations in their coat color, but were clearly not a uniform yellow (68). We have crossed the POMC Null allele onto the C57BL/6 (a/a) genetic background and observed an unaltered black

coat color in the mutant mice despite the absence of MSH, the only known natural agonist for the MC1-R (unpublished data). In sum, POMC deficient mice will be invaluable in deciphering the physiological roles of individual POMC peptides in the neuroendocrine and immune systems by transgenic strategies designed to rescue the expression of subsets of POMC products to either the pituitary and/or neural compartments.

MELANOCORTIN AND OPIOID RECEPTOR GENE MUTATIONS

Hypomorphic and Constitutively Active MC1-R Gene Alleles

Classical genetic studies indicated that two independent loci, extension (*E*) and agouti (*A*), primarily controlled the ratio of eumelanin and pheomelanin in melanocytes, and hence the color of individual hairs. The extension locus is cell-autonomous and approximately a decade ago was demonstrated to encode the MCR-1 or MSH receptor (26). Numerous naturally occurring mutations at the extension locus have been characterized genetically because of the relative ease in tracing the mutant alleles during backcrosses and intercrosses by observation of coat color. Recessive mutations of the mouse extension locus (*e*) result in a yellow coat color due to low levels of tyrosinase activity in melanocytes and a high pheomelanin/eumelanin pigment ratio. The *e* allele encodes a nonfunctional MCR-1 resulting from a frameshift mutation and premature translational stop codon between the fourth and fifth transmembrane domains. Dominant mutations of the mouse *E* locus result in black coat color and are epistatic to agouti. The sombre (*E^{so}*) allele encodes a MC1-R containing a single activating point mutation. This MC1-R is constitutively active in the absence of MSH and therefore not antagonized by agouti protein, accounting for the genetic epistasis. These original data and the analyses of other mutant *E* alleles have been the basis of structure-function models to explain receptor activation by agonist ligand binding. Interestingly, identical mutations introduced into the other highly homologous MCR subtypes have generally not resulted in receptor activation emphasizing the important functional differences among these related G-protein coupled receptors and the danger of over extrapolating data concerning ligand interactions from one receptor subtype to another.

Null Mutation of the MC4-R Gene

Targeted deletion of the MC4-R has shown it to play an important role in energy homeostasis. MC4-R is the predominant melanocortin receptor in the brain and genetically engineered mice homozygous for a null MC4-R allele are obese, hyperphagic, and exhibit increased longitudinal growth (69), all features shared with the *A^y/a* yellow mouse. These data and the inability of the potent MSH analogue MTII to decrease food intake in MC4-R knockout

mice (70) indicate that the MC4-R is essential for the anorectic effects of central melanocortins.

Null Mutation of the MC5-R Gene

Transgenic mice deficient in the MC5-R do not exhibit any phenotypes that are directly associated with weight regulation or feeding but they do exhibit thermoregulatory defects and fail to repel water from their coat. These phenotypes were shown to be a result of reduced production and secretion of lipids from sebaceous glands in MC5-R deficient mice (14). Additionally, the mice exhibited decreased production and attenuated stress-induced synthesis of porphyrins by the Harderian glands and decreased protein secretion from lacrimal glands. All of these exocrine glands, including the preputial gland, are normally sites of high MC5-R expression. The results suggest the existence of a hypothalamic-pituitary-exocrine axis that potentially regulates pheromone production from exocrine glands in coordination with the behavioral arousal state of the mouse.

Null Mutations of the MC3-R Gene

Null mutations of the POMC (68) and MC4-R (69) genes, and transgenic overexpression or misexpression of the MC4-R antagonists agouti and AGRP (28, 71) all produce phenotypes that can be attributed to diminished MC4-R signaling. Although these genetic models have provided great insight into the role of POMC and MC4-R in energy homeostasis, little specific information can be extracted regarding a contributory role of MC3-R signaling. The MC3-R, like the MC4-R, is expressed in the CNS but in a more restricted distribution including ARC and VMN. Recently, two groups independently published reports of knockout mice lacking the MC3-R (72, 73). MC3-R deficient mice exhibit nearly normal growth curves and body weight, but carcass analyses reveal a significant increase in white adipose mass. Additionally, MC3-R deficient mice are more susceptible than controls to developing obesity on a high fat diet. In contrast to the obesity phenotype exhibited in MC4-R deficient mice, which can mostly be explained by alterations in feeding behavior, the MC3-R deficient mice apparently have a predominant metabolic defect involving energy partitioning. They have higher feed efficiency and decreased lean mass. Mice lacking both the MC3-R and MC4-R gain more weight than single MC4-R knockouts (73). Additional studies are needed to define the specific neural circuits responsible for the divergent actions of melanocortin peptides, MC3-R, and MC4-R on the regulation of food intake versus metabolic rate and energy expenditure.

Null Mutations of the Opioid Receptor Genes

Each of the three genes encoding the classical opioid receptor subtypes has been deleted by homologous recombination in mice (16). Surprisingly, there have been no published reports regarding the effects of these mutations on food intake and energy homeostasis. Based on our findings in the β END-deficient mice, we would predict that μ receptor knockout mice are the most likely to have a phenotype relevant to the physiologic regulation of body weight and fat mass.

SUMMARY AND FUTURE PROSPECTS

Complex interactions between the nervous system and the endocrine system are essential for the regulation of energy homeostasis. Transgenic and targeted gene mutational studies have demonstrated that neuropeptides derived from the POMC prohormone and their cognate G-protein coupled receptors are key elements of communication between and within these two important biological systems. Transgenic models have identified POMC neurons in the ARC as a prominent component of the hypothalamic neural circuitry that modulates energy homeostasis. Moreover, melanocortin and opioid peptides derived from POMC may have both additive and opposing actions in the balance of caloric intake and expenditure. Many of the mutations described in this chapter give rise to multisystem physiological disturbances and could also involve undetected developmental alterations, therefore one must be circumspect in the assignment of a specific peptide or receptor subtype as the major determinant in a disease. As a result, newer and more sophisticated molecular and genetic approaches to *in vivo* gene mutations, such as conditional gene deletion and regulation, are currently being explored. These newer techniques will permit a more detailed characterization of specific endocrine-disease pathophysiology, and in addition, help to reveal the function of each biologically active POMC peptide *in vivo*.

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REFERENCES

1. Eipper BA, Mains R.E. Structure and biosynthesis of pro-adrenocorticotropin/endorphin and related peptides. *Endocr Rev* 1980;1:1-27.
2. Nakanishi S, Inoue A, Kita T, Nakamura M, Chang AC, Cohen SN, Numa S. Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. *Nature* 1979;278:423-427.

3. Castro MG, Morrison E. Post-translational processing of proopiomelanocortin in the pituitary and in the brain. *Crit Rev Neurobiol* 1997;11:35-57.
4. Smith AI, Funder JW. Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues. *Endocr Rev* 1998;9:159-179.
5. Finley JCW, Lindström P, Petrusz P. Immunocytochemical localization of β -endorphin-containing neurons in the rat brain. *Neuroendocrinol* 1981;33:28-42.
6. Elkabes S, Loh YP, Nieburgs A, Wray S. Prenatal ontogenesis of pro-opiomelanocortin in the mouse central nervous system and pituitary gland: an in situ hybridization and immunocytochemical study. *Brain Res Dev Brain Res* 1989;46:85-95.
7. Palkovits M, Mezey É, Eskay RL. Pro-opiomelanocortin-derived peptides (ACTH/ β -endorphin/ α -MSH) in brainstem baroreceptor areas of the rat. *Brain Res* 1987;436:323-328.
8. Seger MA, Bennett HP. Structure and bioactivity of the amino-terminal fragment of pro-opiomelanocortin. *J Steroid Biochem* 1986;25:703-710.
9. Deneff C, Van Bael A. A new family of growth and differentiation factors derived from the N-terminal domain of proopiomelanocortin (N-POMC). *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 1998;119:317-324.
10. Cone RD, Lu D, Koppula S, Vage DI, Klungland H, Boston B, Chen W, Orth DN, Pouton C, Kesterson RA. The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. *Recent Prog Horm Res* 1996;51:287-317.
11. Mountjoy KG, Robbins LS, Mortrud MT, Cone RD. The cloning of a family of genes that encode the melanocortin receptors. *Science* 1992;257:1248-1251.
12. Roselli-Rehfuß L, Mountjoy KG, Robbins LS, Mortrud M, Low MJ, Tatro J, Entwistle M, Simerly R, Cone RD. Identification of a receptor for γ -MSH and other proopiomelanocortin peptides in the hypothalamus and limbic system. *Proc Natl Acad Sci USA* 1993;90:8856-8850.
13. Mountjoy KG, Mortrud M, Low MJ, Simerly R, Cone RD. Characterization of a melanocortin receptor (MC4-R) localized in neuroendocrine and autonomic control circuits in the brain. *Mol Endocrinol* 1994;8:1298-1308.
14. Chen W, Kelly MA, Optiz-Araya X, Low MJ, Cone RD. Exocrine gland dysfunction in MC5-R deficient mice. *Cell* 1997;91:789-798.
15. Mansour A, Fox CA, Akil H, Watson SJ. Opioid-receptor mRNA expression in the rat CNS: anatomical and functional implications. *Trends Neurosci* 1995;18:22-29.
16. Hayward MD, Low MJ. Targeted mutagenesis of the murine opioid system. *Results Probl Cell Differ* 1999;26:169-191.
17. Pasternak GW. Pharmacological mechanisms of opioid analgesics. *Clin Neuropharmacol* 1993;16:1-18.
18. Elmquist JK, Elias CF, Saper CB. From lesions to leptin: hypothalamic control of food intake and body weight. *Neuron* 1999;22:221-232.
19. Tatro JB. Melanotropin receptors in the brain are differentially distributed and recognize both corticotropin and alpha-melanocyte stimulating hormone. *Brain Res* 1990;536:124-132.
20. Mountjoy KG, Wong J. Obesity, diabetes and functions for proopiomelanocortin-derived peptides. *Mol Cell Endocrinol* 1997;128:171-177.
21. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of melanocortinergic neurons in feeding and the agouti obesity syndrome. *Nature* 1997;385:165-168.
22. Reid LD. Endogenous opioid peptides and regulation of drinking and feeding. *Am J Clin Nutr* 1985;42:1099-1132.
23. Hope PJ, Chapman I, Morley JE, Horowitz M, Wittert GA. Food intake and food choice: the role of the endogenous opioid peptides in the marsupial *Sminthopsis crassicaudata*. *Brain Res* 1997;764:39-45.

24. Levine AS, Grace M, Portoghesi PS, Billington CJ. The effect of selective opioid antagonists on butorphanol-induced feeding. *Brain Res* 1994;637:242-248.
25. Duhl DM, Stevens ME, Vrieling H, Saxon PJ, Miller MW, Epstein CJ, Barsh GS. Pleiotropic effects of the mouse lethal yellow (Ay) mutation explained by deletion of a maternally expressed gene and the simultaneous production of agouti fusion RNAs. *Development* 1994;120:1695-1708.
26. Robbins LS, Nadeau JH, Johnson KR, Kelly MA, Roselli-Rehffuss L, Baack E, Mountjoy KG, Cone RD. Pigmentation phenotypes of variant extension locus alleles result from point mutations that alter MSH receptor function. *Cell* 1993; 72:827-834.
27. Lu D, Willard D, Patel IR, Kadwell S, Overton L, Kost T, Luther M, Chen W, Woychik RP, Wilkison WO. Agouti protein is an antagonist of the melanocyte-stimulating-hormone receptor. *Nature* 1994;371:799-802.
28. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 1997;278:135-138.
29. Comuzzie AG, Hixson JE, Almasy L, Mitchell BD, Mahaney MC, Dyer TD, Stern MP, MacCluer JW, Blangero J. A major quantitative trait locus determining serum leptin levels and fat mass is located on human chromosome 2. *Nat Genet* 1997;15:273-276.
30. Hixson JE, Almasy L, Cole S, Birnbaum S, Mitchell BD, Mahaney MC, Stern MP, MacCluer JW, Blangero J, Comuzzie AG. Normal variation in leptin levels is associated with polymorphisms in the proopiomelanocortin gene, POMC. *J Clin Endocrinol Metab* 1999;84:3187-3191.
31. Krude H, Biebermann H, Luck W, Horn R, Brabant G, Gruters A. Severe early-onset obesity, adrenal insufficiency and red hair pigmentation caused by POMC mutations in humans. *Nat Genet* 1998;19:155-157.
32. Farooqi IS, Yeo GS, Keogh JM, Aminian S, Jebb SA, Butler G, Cheetham T, O'Rahilly S. Dominant and recessive inheritance of morbid obesity associated with melanocortin 4 receptor deficiency. *J Clin Invest* 2000;106:271-279.
33. Yeo GS, Farooqi IS, Challis BG, Jackson RS, O'Rahilly S. The role of melanocortin signalling in the control of body weight: evidence from human and murine genetic models. *QJM* 2000;93:7-14.
34. Furth J, Gadsden EL, Upton AC. ACTH-secreting transplantable pituitary tumors. *Proc Soc Exp Biol Med* 1953;84:253-254.
35. Picon A, Bertagna X, de Keyser Y. Analysis of proopiomelanocortin gene transcription mechanisms in bronchial tumour cells. *Mol Cell Endocrinol* 1999;147:93-102.
36. Tremblay Y, Tretjakoff I, Peterson A, Antakly T, Zhang CX, Drouin J. Pituitary-specific expression and glucocorticoid regulation of a proopiomelanocortin fusion gene in transgenic mice. *Proc Natl Acad Sci USA* 1988;85:8890-8894.
37. Hammer G, Fairchild-Huntress V, Low MJ. Pituitary-specific and hormonally regulated gene expression directed by the rat proopiomelanocortin promoter in transgenic mice. *Mol Endo* 1990;4:1689-1697.
38. Liu B, Hammer GD, Rubinstein M, Mortrud M, Low MJ. Identification of DNA elements cooperatively activating proopiomelanocortin gene expression in the pituitary gland of transgenic mice. *Mol Cell Biol* 1992;12:3978-3990.
39. Liu B, Mortrud M, Low MJ. DNA elements with A T-rich core sequences direct pituitary cell-specific expression of the proopiomelanocortin gene in transgenic mice. *Biochemical J* 1995;312:827-832.
40. Garrick D, Fiering S, Martin DI, Whitelaw E. Repeat-induced gene silencing in mammals. *Nat Genet* 1998;18:56-59.
41. Bousquet C, Ray DW, Melmed S. A common pro-opiomelanocortin-binding element mediates leukemia inhibitory factor and corticotropin-releasing hormone transcriptional synergy. *J Biol Chem* 1997;272:10551-10557.

42. Therrien M, Drouin J. Cell-specific helix-loop-helix factor required for pituitary expression of the pro-opiomelanocortin gene. *Mol Cell Biol* 1993;13:2342-2353.
43. Windle JJ, Weiner RI, Mellon PL. Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. *Mol Endocrinol* 1990;4:597-603.
44. Efrat S, Linde S, Kofod H, Spector D, Delannoy M, Grant S, Hanahan D, Baekkeskov S. β -cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene. *Proc Natl Acad Sci USA* 1988;85:9037-9041.
45. Adams JM, Cory S. Transgenic models of tumor development. *Science* 1991;254:1161-1167.
46. Alarid E, Windle J, Whyte D, Mellon P. immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. *Development* 1996;122:3319-3329.
47. Low MJ, Liu B, Hammer GD, Rubinstein M, Allen RG. Post-translational processing of proopioidmelanocortin (POMC) in mouse pituitary melanotroph tumors induced by a POMC-simian virus 40 large T antigen transgene. *J Biol Chem* 1993;268:24967-24975.
48. Low MJ, Kelly MA, Graham KE, Asa SL. Transgenic and induced-mutant mouse models of pituitary adenomas. In: *The Management of Pituitary Tumors*. (Ed S. Webb) pp 55-69. Bristol: BioScientifica Ltd, 1998.
49. Allen DL, Low MJ, Allen RG, Ben-Jonathan N. Identification of two classes of prolactin releasing factors in intermediate lobe tumors from transgenic mice. *Endocrinology* 1995;136:3093-3099.
50. Christofori G, Naik P, Hanahan D. Deregulation of both imprinted and expressed alleles of the insulin-like growth factor 2 gene during beta-cell tumorigenesis. *Nat Genet* 1995;10:196-201.
51. Grewal A, Bradshaw SL, Schuller AGP, Low MJ, Pintar JE. Expression of IGF system genes during T-antigen driven pituitary tumorigenesis. *Horm Metab Res* 1999;31:155-160.
52. Westphal CH, Muller L, Zhou A, Zhu X, Bonner-Weir S, Schambelan M, Steiner DF, Lindberg I, Leder P. The neuroendocrine protein 7B2 is required for peptide hormone processing in vivo and provides a novel mechanism for pituitary Cushing's disease. *Cell* 1999;96:689-700.
53. Furuta M, Yano H, Zhou A, Rouille Y, Holst JJ, Carroll R, Ravazzola M, Orci L, Furuta H, Steiner DF. Defective prohormone processing and altered pancreatic islet morphology in mice lacking active PC2. *Proc Natl Acad Sci USA* 1997;94:6646-6651.
54. Hnasko R, Khurana S, Shackleford N, Steinmetz R, Low MJ, Ben-Jonathan N. Two distinct pituitary cell lines from mouse intermediate lobe tumors: a cell that produces prolactin-regulating factor and a melanotroph. *Endocrinology* 1997;138:5589-5596.
55. Rubinstein M, Mortrud M, Liu B, Low MJ. Rat and mouse proopioidmelanocortin gene sequences target tissue-specific expression to the pituitary gland but not to the hypothalamus of transgenic mice. *Neuroendocrinology* 1993;58:373-380.
56. Young JJ, Otero V, Cerdán MG, Falzone TL, Chan EC, Low MJ, Rubinstein M. Authentic cell-specific and developmentally regulated expression of proopioidmelanocortin genomic fragments in hypothalamic and hindbrain neurons of transgenic mice. *J Neurosci* 1998;18:6631-6640.
57. Elias CF, Lee C, Kelly J, Aschkenasi C, Ahima RS, Couceyro PR, Kuhar MJ, Saper CB, Elmquist JK. Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* 1998;21:1375-1385.
58. Vrang N, Larsen PJ, Clausen JT, Kristensen P. Neurochemical characterization of hypothalamic cocaine-amphetamine-regulated transcript neurons. *J Neurosci* 1999;19:RC5.
59. Cheung CC, Clifton DK, Steiner RA. Proopioidmelanocortin neurons are direct targets for leptin in the hypothalamus. *Endocrinology* 1997;138:4489-4492.

60. Elias CF, Aschkenasi C, Lee C, Kelly J, Ahima RS, Bjorbaek C, Flier JS, Saper CB, Elmquist JK. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* 1999;23:775-786.
61. Spergel DJ, Kruth U, Hanley DF, Sprengel R, Seeburg PH. GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. *J Neurosci* 1999;19:2037-2050.
62. Rubinstein M, Japon JA, Low MJ. Introduction of a point mutation into the mouse genome by homologous recombination in embryonic stem cells using a replacement type vector with a selectable marker. *Nuc Acids Res* 1993;21:2613-2617.
63. Rubinstein M, Mogil JS, Japon M, Chan EC, Allen RG, Low MJ. Absence of opioid stress-induced analgesia in mice lacking β -endorphin by site-directed mutagenesis. *Proc Natl Acad Sci USA* 1996;93:3995-4000.
64. König M, Zimmer AM, Steiner H, Holmes PV, Crawley JN, Brownstein MJ, Zimmer A. Pain responses, anxiety and aggression in mice deficient in pre-proenkephalin. *Nature* 1996;383:535-538.
65. Mogil JS, Grisel JE, Hayward MD, Bales JR, Rubinstein M, Belknap JK, Low MJ. Disparate spinal and supraspinal antinociceptive responses in β -endorphin-deficient mutant mice. *Neuroscience* 2000;101:709-717.
66. Slugg RM, Hayward MD, Ronnekleiv OK, Low MJ, Kelly MJ. Effect of the μ -opioid agonist DAMGO on medial basal hypothalamic neurons in beta-endorphin knockout mice. *Neuroendocrinology* 2000;72:208-217.
67. Morley JE. Neuropeptide regulation of appetite and weight. *Endocrine Rev* 1987;8:256-287.
68. Yaswen L, Diehl N, Brennan MB, Hochgeschwender U. Obesity in the mouse model of pro-opiomelanocortin deficiency responds to peripheral melanocortin. *Nat Med* 1999;5:1066-1070.
69. Huszar D, Lynch CA, Fairchild-Huntress V, Dunmore JH, Fang Q, Berkemeier LR, Gu W, Kesterson RA, Noston BA, Cone RD. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. *Cell* 1997;88:131-141.
70. Marsh DJ, Hollopeter G, Huszar D, Laufer R, Yagaloff KA, Fisher SL, Burn P, Palmiter RD. Response of melanocortin-4 receptor-deficient mice to anorectic and orexigenic peptides. *Nat Genet* 1999;21:119-122.
71. Klebig ML, Wilkinson JE, Geisler JG, Woychik RP. Ectopic expression of the agouti gene in transgenic mice causes obesity, features of type II diabetes, and yellow fur. *Proc Natl Acad Sci USA* 1995;92:4728-4732.
72. Butler AA, Kesterson RA, Khong K, Cullen MJ, Pelleymounter MA, Dekoning J, Baetscher M, Cone RD. A unique metabolic syndrome causes obesity in the melanocortin-3 receptor-deficient mouse. *Endocrinology* 2000;141:3518-3521.
73. Chen AS, Marsh DJ, Trumbauer ME, Frazier EG, Guan XM, Yu H, Rosenblum CI, Vongs A, Feng Y, Cao L. Inactivation of the mouse melanocortin-3 receptor results in increased fat mass and reduced lean body mass. *Nat Genet* 2000;26:97-102.