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Targeted Disruption of the Melanocortin-4 Receptor Results in Obesity in Mice

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Summary

The melanocortin-4 receptor (MC4-R) is a G protein–coupled, seven-transmembrane receptor expressed in the brain. Inactivation of this receptor by gene targeting results in mice that develop a maturity onset obesity syndrome associated with hyperphagia, hyperinsulinemia, and hyperglycemia. This syndrome recapitulates several of the characteristic features of the *agouti* obesity syndrome, which results from ectopic expression of agouti protein, a pigmentation factor normally expressed in the skin. Our data identify a novel signaling pathway in the mouse for body weight regulation and support a model in which the primary mechanism by which agouti induces obesity is chronic antagonism of the MC4-R.

Introduction

Over the past few years, there has been considerable progress in identification and characterization of the mutations underlying five monogenic murine models of obesity: obese (*ob*), diabetes (*db*), fat (*fat*), tubby (*tub*), and obese yellow (e.g., A^y). In particular, cloning of the *ob* and *db* genes has led to identification of a signaling system that monitors the status of energy stores in the body. The *ob* gene product, leptin, is a circulating protein secreted by adipose tissue that communicates body fat content to the brain through the OB receptor (OB-R), the product of the *db* gene. The OB-R is a member of the class 1 cytokine receptor family expressed in the hypothalamus and other tissues (Zhang et al., 1994; Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Tartaglia et al., 1995; Baumann et al., 1996; Chen et al., 1996; Chua et al., 1996; Ghilardi et al., 1996; Lee et al., 1996).

The identification of the leptin signaling pathway represents a significant advance in the elucidation of the mechanisms by which body weight and energy expenditure are regulated. However, it is clear that the control of body weight is a complex process that will likely involve the interplay of additional, as yet undefined, neural circuits and molecular mechanisms (Friedman and Leibel, 1992; Campfield et al., 1996; Spiegelman and Flier, 1996). The existence of one such pathway, involving signaling via neural melanocortin receptors, has been hypothesized on the basis of studies of the action of the agouti pigmentation factor (Lu et al., 1994).

Agouti, a 131 amino acid secreted protein expressed exclusively within the hair follicle, regulates the synthesis of brown-black (eumelanin) and yellow-red (phaeomelanin) pigment to produce the wild-type (agouti) pattern of coat coloration: black hairs with a subapical yellow band (Bultman et al., 1992; Miller et al., 1993; reviewed in Silvers, 1979). Agouti acts in a paracrine fashion on melanocytes (Silvers and Russell, 1955) to induce a switch from eumelanin to phaeomelanin synthesis by antagonism of the melanocortin-1 receptor (MC1-R) (Lu et al., 1994), also known as the melanocyte-stimulating hormone receptor (MSH-R), a G protein–coupled, seven-transmembrane receptor (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992; Robbins et al., 1993).

Dominant alleles of the *agouti* locus resulting in widespread ectopic expression of agouti give rise to a pleiotropic obesity syndrome referred to as the obese yellow, or *agouti*, syndrome (Bultman et al., 1992; Michaud et al., 1993; Miller et al., 1993; Duhl et al., 1994; Michaud et al., 1994a; 1994b). The best characterized and most dominant of the allele series are the lethal yellow (A^y) and viable yellow (A^w) mutants. Obese yellow mice are characterized by maturity-onset obesity (in contrast to the early-onset obesity of *ob* and *db* mice), hyperinsulinemia, hyperglycemia in males, and yellow coat color (reviewed by Yen et al., 1994). The mice are hyperphagic (Frigeri et al., 1988; Shimizu et al., 1989) and display increased rates of hepatic lipogenesis (Yen et al., 1976b) and decreased rates of lipolysis in adipocytes (Yen et al., 1970). The increased adiposity of dominant agouti mutants is primarily due to fat cell hypertrophy, as is true of most human forms of obesity, rather than the more prevalent hyperplasia characteristic of *ob* and *db* mice (Johnson and Hirsch, 1972). In addition, obese yellow mice are unique among rodent obesity models in exhibiting a slight increase in linear growth, in contrast to *ob* and *db* mice, which are somewhat shorter than their wild-type littermates (Heston and Vlahakis, 1962; Wolff, 1963).

The dominant *agouti* alleles that give rise to obesity result in constitutive deregulated synthesis of wild-type agouti protein within the hair follicle, accounting for the yellow coat color, and ectopic expression of agouti throughout the animal, presumably accounting for the other characteristics of the pleiotropic obesity syndrome (Bultman et al., 1992; Michaud et al., 1993; Miller et al., 1993; Duhl et al., 1994). That this phenotype is directly related to ectopic expression of agouti, rather than to other potential effects of the various agouti promoter mutations, is supported by the demonstration

that transgenic mice ectopically expressing agouti from ubiquitous promoters recapitulate the obese hyperinsulinemic phenotype (Klebig et al., 1995; Perry et al., 1995). Furthermore, the obesity phenotype is not dependent on synthesis of yellow pigment, or disruption of MC1-R function, since A^{vy} mice carrying the gain of function MC1-R mutation somber (E^{so}) are black yet still obese (Wolff et al., 1978) and yellow *e/e* mice, which lack MC1-R function (Robbins et al., 1993), are not obese (Searle, 1968).

One hypothesis for the mechanism by which ectopic agouti expression induces obesity is aberrant antagonism of melanocortin receptors expressed in regions of the brain known to be involved in regulating feeding (Lu et al., 1994; Mountjoy et al., 1994). In addition to the MC1-R, four other members of the melanocortin receptor family have been identified: the MC2-R (Mountjoy et al., 1992), MC3-R (Gantz et al., 1993a; Roselli-Rehfuss et al., 1993), MC4-R (Gantz et al., 1993b; Mountjoy et al., 1994), and MC5-R (Chhajlani et al., 1993; Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994). All are G protein–coupled receptors that activate adenylyl cyclase and bind ligands collectively referred to as the melanocortins (e.g., adrenocorticotrophin [ACTH] and the α-, β-, and γ-melanocyte–stimulating hormones) derived from the precursor proopiomelanocortin (POMC). The MC2-R is the ACTH receptor, expressed primarily in the adrenal cortex (Mountjoy et al., 1992). The MC3-R and MC4-R are neural melanocortin receptors. The MC3-R is expressed in the brain in regions of the hypothalamus and limbic system, as well as in the placenta and gut (Gantz et al., 1993a; Roselli-Rehfuss et al., 1993), whereas expression of the MC4-R is restricted primarily to the brain, where it is widely expressed (Gantz et al., 1993b; Mountjoy et al., 1994; reviewed in Low et al., 1994). The MC5-R, on the other hand, is widely expressed at low levels throughout the body (Gantz et al., 1994; Griffon et al., 1994; Labbe et al., 1994). To test for agouti antagonism, Lu et al. (1994) expressed melanocortin receptors in 293 cells and assayed the ability of agouti to inhibit α-MSH–induced stimulation of adenylyl cyclase activity. The results demonstrated that agouti was a high affinity antagonist of both the MC1 and MC4 receptors in vitro, but not of the MC3 or MC5 receptors, suggesting that agouti antagonism of the MC4-R could be the cause of the *agouti* obesity syndrome.

To evaluate whether signaling via the MC4-R plays a role in the regulation of body weight, mice lacking the receptor were generated by gene targeting in embryonic stem (ES) cells. Our results show that absence of the MC4-R produces an obesity syndrome strikingly reminiscent of the *agouti* syndrome, defining a novel function for the MC4-R in the regulation of energy balance and supporting aberrant antagonism of the MC4-R as the primary cause of the *agouti* obesity syndrome.

Results

Generation of MC4-R–Deficient Mice

The murine MC4-R gene consists of approximately 1 kb of coding sequence contained within a single exon

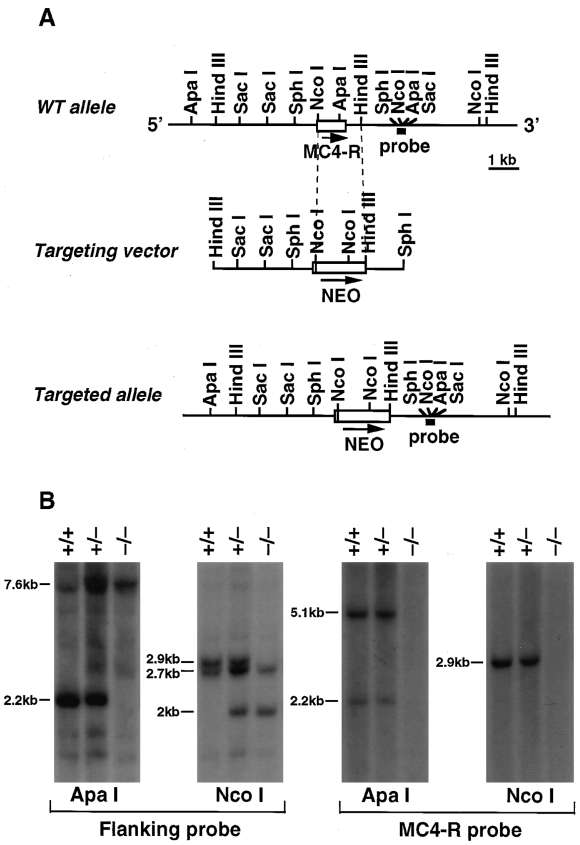

Figure 1. Deletion of the Mouse MC4-R

(A) Schematic diagrams and partial restriction maps of the MC4-R locus, the MC4-R targeting vector, and the predicted structure of the MC4-R locus following homologous recombination with the targeting vector. The open box represents MC4-R coding sequences, the closed box is the 200 bp SphI–SacI flanking probe used to identify the targeted MC4-R locus, and the shaded box indicates the PGK–*neo* expression cassette. The arrows indicate the direction of transcription.

(B) Autoradiogram of a Southern blot analysis of tail DNA from F2 progeny. Genomic DNA was digested with ApaI or NcoI, as indicated, and hybridized with the radiolabeled probe shown in (A), then stripped and rehybridized with a radiolabeled probe consisting of the human MC4-R coding sequence. +/+, +/−, and −/− denote DNA from wild-type, heterozygous, and homozygous F2 littermates, respectively.

(Figure 1A). A targeting vector was designed to delete virtually all MC4-R coding sequence following homologous recombination with the locus in embryonic stem cells. As shown in Figure 1A, the vector consists of a total of approximately 4.5 kb of strain 129/Sv mouse genomic DNA flanking a deletion of 1.5 kb. This deletion extends from the NcoI site located approximately 20 nucleotides downstream of the MC4-R initiation codon to the HindIII site situated approximately 0.5 kb 3′ of the gene. The deleted MC4-R sequences have been replaced by the *neo* gene under the control of the phosphoglycerate kinase-1 (PGK-1) promoter.

A total of 809 G418-resistant colonies were screened for homologous recombination by Southern blot hybridization of ApaI-digested genomic DNA with the flanking probe shown in Figure 1A. One clone showed the predicted 7.6 kb targeted ApaI DNA fragment in addition

to the expected 2.2 kb wild-type fragment. Injection of this clone into C57BL/6J blastocysts produced several male chimeras that, when bred to C57BL/6J females, transmitted the targeted MC4-R allele to their F1 129/B6 offspring. F1 heterozygotes were interbred and their offspring genotyped by Southern blot hybridization of ApaI- or NcoI-digested tail DNA with the flanking probe. As described above, ApaI digestion generates a wild-type fragment of 2.2 kb and a targeted fragment of 7.6 kb (note that this 7.6 kb is distinct from a background band of slightly lower molecular weight that is present in all samples; see Figure 1B). NcoI digestion generates two wild-type fragments of 2.7 and 2.9 kb, since the NcoI site is situated within the sequences recognized by the flanking probe (Figures 1A and 1B). The 2.7 kb NcoI fragment represents genomic sequences extending 3′ of the probe that are unaffected by MC4-R targeting, whereas the 2.9 kb band includes the MC4-R gene sequences. Following targeting, this latter fragment is reduced to a 2 kb band diagnostic of the mutated MC4-R allele. As shown in Figure 1B, heterozygous intercrosses produced homozygous mutant, heterozygous, and wild-type F2 progeny. To verify deletion of the MC4-R gene in homozygous mutants, the filters were stripped and rehybridized with an MC4-R probe. No MC4-R hybridization was detected in homozygous mutant mice, whereas the predicted 2.2 kb and 5.1 kb ApaI bands (ApaI cuts within the MC4-R gene generating two MC4-R–containing fragments; see Figure 1A) and 2.9 kb NcoI fragment were observed in both heterozygous and wild-type littermates (Figure 1B).

Body Weight and Size of MC4-R–Deficient Mice

F2 animals were maintained on a chow diet ad libitum and their weights monitored regularly. The weights of MC4-R–deficient mice and their wild-type littermates were largely indistinguishable for the first 4 weeks of life. However, by approximately 5 weeks of age, most of the homozygous mutants, both males and females, were heavier than their wild-type siblings of the same sex, and by 7 weeks of age all of the null mutants were heavier than the controls (Figures 2A and 2C). By 15 weeks of age, homozygous mutant females were on average twice as heavy as their wild-type siblings, while homozygous mutant males were approximately 50% heavier than wild-type controls. By about 24 weeks of age the weight of female null mice averaged approximately 63 grams ($n = 3$), and males averaged approximately 65 grams ($n = 8$). Mice heterozygous for MC4-R deletion showed a weight gain intermediate to that seen in wild-type and homozygous mutant sibs (Figures 2B and 2D), demonstrating a gene dosage effect of MC4-R ablation on body weight regulation.

To determine whether mice lacking the MC4-R exhibited alterations in linear growth, body length (naso–anal) measurements of F2 progeny were taken at approximately 19 weeks of age (between 132 and 138 days). As shown in Figure 3, MC4-R–deficient mice are significantly longer than wild-type controls. The mean length of homozygous mutant females is increased approximately 11% relative to wild-type F2 mice, and heterozygous females are approximately 7% longer than controls. Male homozygotes and heterozygotes are approximately 8% and 2.5% longer than controls, respectively.

Food Consumption

To determine whether food consumption was increased in mice lacking the MC4-R, homozygous mutant females and wild-type F2 controls were monitored for food intake over a two-week period. A^y mutants, on a C57BL/6J background, and C57BL/6J controls were also monitored. As previously documented (Frigeri et al., 1988; Shimizu et al., 1989), A^y mice were hyperphagic, eating 36% more than C57BL/6J controls. Absence of the MC4-R also resulted in a significant increase (46%) in food consumption over wild-type F2 controls (Figure 4).

Serum Glucose, Insulin, and Leptin Concentrations

Blood was collected from MC4-R–deficient mice and wild-type controls over three time intervals (4–8 weeks, 10–14 weeks, and 17–23 weeks) and serum assayed for glucose and insulin levels. Serum glucose levels were essentially unchanged in females heterozygous or homozygous for MC4-R deletion, but both heterozygous and homozygous males were hyperglycemic (Figures 5A and 5B). This was first evident for homozygous males at the 10–14 week interval, at which time glucose levels were elevated over 2-fold above controls, to 390 mg/dl, but heterozygous mutants showed only a slight elevation of serum glucose at this age. By 17–23 weeks of age, both heterozygous and homozygous male mutant mice showed a doubling of normal serum glucose levels (334 and 361 mg/dl, respectively) relative to controls (156 mg/dl).

Both male and female mutant mice were hyperinsulinemic (Figures 5C and 5D). Inceases in insulin levels by 9-fold and 5-fold were evident in the sera of homozygous mutant females and males, respectively, at 4–8 weeks of age. These levels increased dramatically over time, such that by 17–23 weeks of age the mean concentration of insulin in the serum of homozygous mutant females was approximately 65 ng/ml, and for males approximately 130 ng/ml, representing appoximately 60- and 14-fold increases, respectively, over insulin levels in F2 wild-type controls. Heterozygous mutants were also hyperinsulinemic, although less so than homozygous mutants. For both male and female heterozygotes, a significant difference in insulin levels relative to controls was first observed at the 10–14 week interval. By 17–23 weeks, mean insulin levels of heterozygotes were elevated to approximately 10 ng/ml (females) and 85 ng/ml (males).

In addition to glucose and insulin, serum leptin and corticosterone levels were also determined. Leptin levels are elevated in A^y mice (Maffei et al., 1995; Mizuno et al., 1996), indicating that the syndrome does not result from defects in leptin production. Consistent with the postulated role of leptin in communicating body fat depot contents to the brain (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995; Campfield et al., 1996), leptin is also elevated in MC4-R–deficient mice (Figures 5E and 5F). At 4–8 weeks of age, leptin was elevated 4.5-fold and 1.5-fold in the serum of female

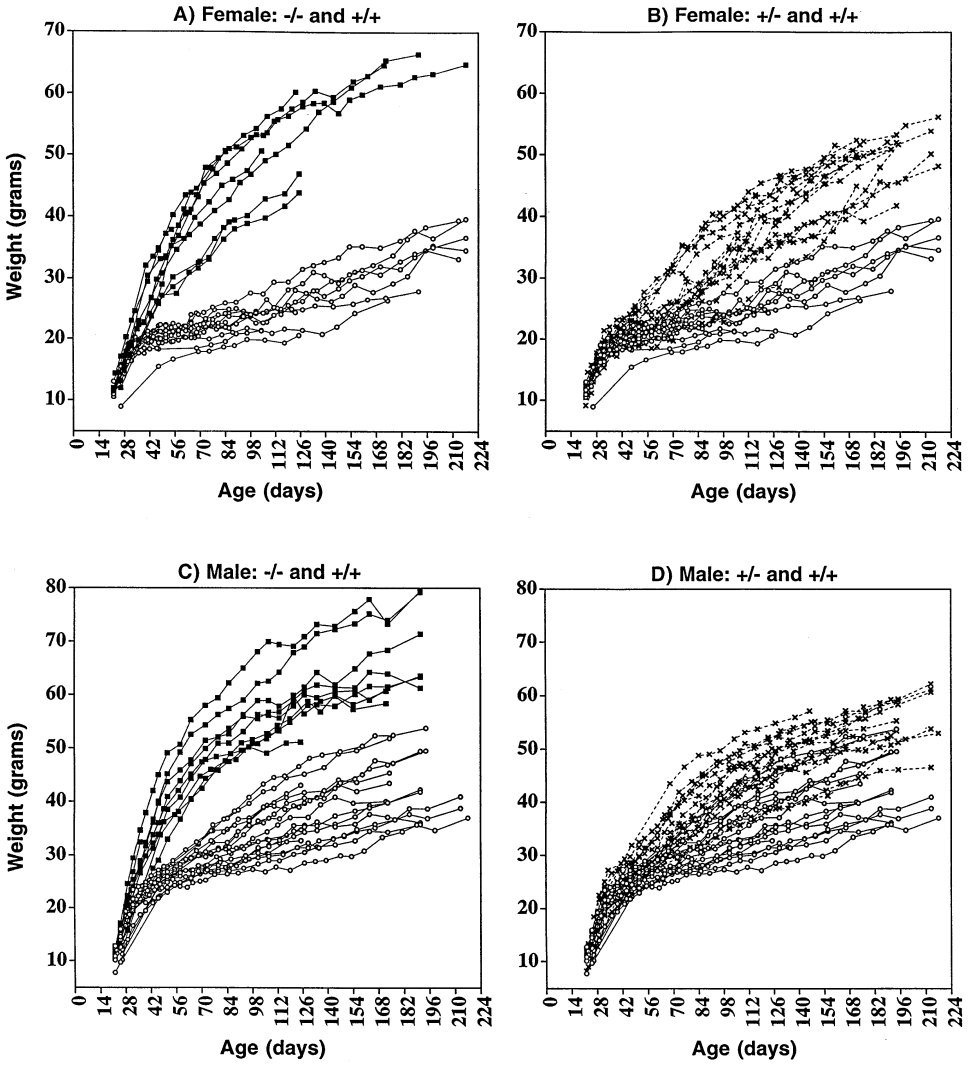

Figure 2. Weight Gain of MC4-R–Deficient Mice and Control Littermates

Each line represents the weight gain of an individual mouse.

(A) Weight gain of female homozygous (−/−) mutant mice (closed squares) and wild-type (+/+) F2 controls (open circles). The weights of 9 homozygous and 12 control mice were taken at the times indicated.

(B) Weight gain of female heterozygous (+/−) mutant mice (x) and wild-type (+/+) F2 controls (open circles). The weights of 18 heterozygous and 12 control mice were taken at the times indicated.

(C) Weight gain of male homozygous (−/−) mutant mice (closed squares) and wild-type (+/+) F2 controls (open circles). The weights of 9 homozygous and 17 control mice were taken at the times indicated.

(D) Weight gain of male heterozygous (+/−) mutant mice (x) and wild-type (+/+) F2 controls (open circles). The weights of 18 heterozygous and 17 control mice were taken at the times indicated.

and male homozygous mutants, respectively, relative to wild-type controls. By 17–23 weeks of age serum leptin levels in females had reached approximately 97 ng/ml, in males approximately 58 ng/ml, representing increases of 6.5- and 2.5-fold, respectively. Heterozygous mice, for the most part, showed leptin levels intermediate between that observed for wild-type mice and homozygous mutants.

Since glucocorticoids can profoundly affect weight homeostasis and somatic growth, basal serum corticosterone was measured in three sets of sex-matched littermates, each containing a wild-type, heterozygous, and homozygous mutant animal (Figure 6). No effect of MC4-R gene knockout on basal corticosterone levels was detected.

POMC Gene Expression

To assess whether the observed effects of MC4-R deletion on weight homeostasis could be attributed to the induction of compensatory changes in expression of the sole known source of ligand for the MC4-R, the POMC gene, central POMC gene expression was examined in wild-type mice, mice heterozygous for MC4-R deletion, and homozygous mutants by in situ hybridization (Figures 7D–7F). No new sites of POMC gene expression and no consistent change in the levels of POMC mRNA

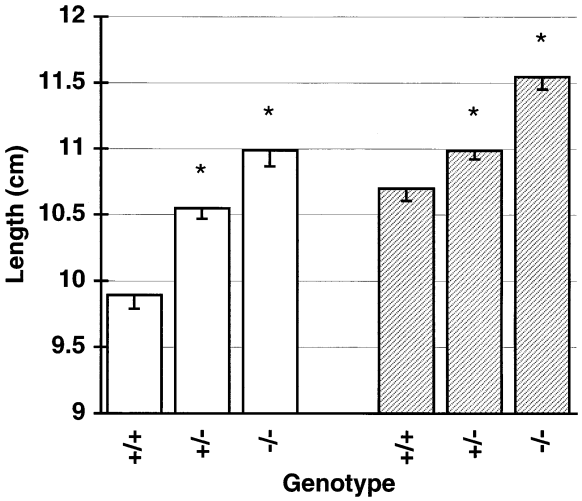

Figure 3. Increased Linear Growth of MC4-R–Deficient Mice

The body length of female (open bars) and male (hatched bars) was measured at approximately 19 weeks of age (between 132 and 138 days). The bars indicate the mean length of 12 wild-type (+/+), 14 heterozygous (+/−), and 9 homozygous mutant (−/−) female F2 mice, and 15 wild-type, 20 heterozygous, and 9 homozygous mutant male F2 mice. Error bars represent the standard error of the mean, and the asterisks denote significant difference ($p < 0.02$ by two-tailed Student t test) compared to the wild-type value within a similar sex.

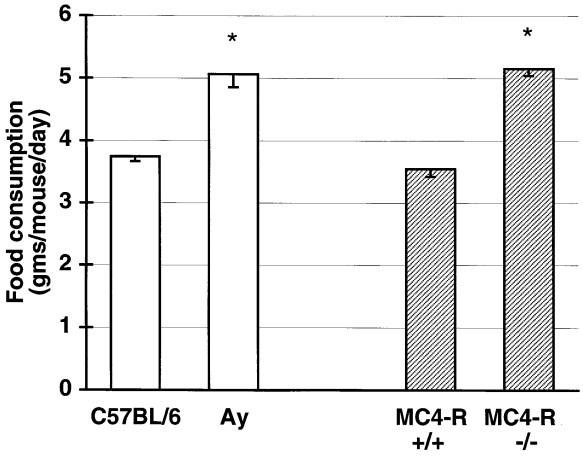

Figure 4. Mice Lacking the MC4-R Are Hyperphagic

The food intake of female mice housed in pairs was measured every weekday over a two-week period. The open bars represent the mean of eight measurements on one cage each of two A^y and two control C57BL/6 mice, at 9 weeks of age. The hatched bars represent the mean of eight measurements on each of two cages of two homozygous mutant mice (−/−) and two F2 wild-type controls (+/+). Two of each of the mutant mice and F2 controls were 15 weeks of age; the remaining two of both genotypes were each 17.5 and 20.5 weeks of age. Error bars represent the standard error of the mean, and the asterisks denote significant difference ($p < 0.01$ by two-tailed Student t test) of either A^y compared to C57BL/6 or MC4-R −/− homozygous mutants compared to MC4-R +/+ wild-type F2 mice.

in its primary site of expression, the arcuate nucleus of the hypothalamus, were detected by this assay. In addition, no gross neuroanatomical defects were observed in thionin-stained brain sections from heterozygous or homozygous mutant MC4-R–deficient animals by histological analysis (Figures 7A–7C).

Discussion

In this study, we have generated mice lacking expression of the MC4-R by gene targeting, and we report that a maturity onset obesity syndrome associated with hyperphagia, hyperinsulinemia, and hyperglycemia occurs in the absence of the receptor. This syndrome is quite distinct from that elicited by mutations of the *ob* and *db* loci, in which the inability to produce leptin (*ob*/*ob*) or respond to it (*db*/*db*) results in an early-onset obesity and extreme hyperphagia and hyperinsulinemia. In addition, in *ob* and *db* mice, basal- and stress-activated corticosterone levels are significantly elevated (Dubuc et al., 1975; Coleman and Burkart, 1977), and adrenalectomy dramatically decreases obesity (Solomon and Mayer, 1973), whereas no effect on basal corticosterone was detected in MC4-R–deficient mice. Furthermore, whereas *ob* and *db* mice are shorter than their wild-type littermates (Heston and Vlahakis, 1962; Wolff, 1963), mice lacking the MC4-R display increased linear growth.

Consistent with a role in body weight regulation, the MC4-R is expressed in a number of hypothalamic sites, including the ventromedial, lateral, dorsomedial, and paraventricular nuclei (Mountjoy et al., 1994), which play an important role in regulating feeding behavior (reviewed in Bray, 1987). These neurons are also in synaptic contact with neurons from the hypothalamic arcuate nucleus and the nucleus of the solitary tract of the brain stem, the primary sites of expression in the brain of POMC (Jacobowitz and O'Donohue, 1978; Watson et al., 1978). Melanocortin peptides derived from processing of POMC, including adrenocorticotrophin (ACTH) and the α-, β- and γ-melanocyte–stimulating hormones (α, β, γ MSH) (reviewed in O'Donohue, 1982), are the sole known ligands for the melanocortin receptors. The observed loss of weight regulation in mice lacking the MC4-R implies an inhibitory role of MC4-R ligand(s) in body energy balance and metabolism. This is further supported by recent pharmacological studies of the cyclic melanocortin analogues, MTII and SHU9119, a high affinity agonist and antagonist, respectively, of both neural melanocortin receptors MC3 and MC4 (Hruby et al., 1995). Intracerebroventricular (ICV) injection of the agonist MTII in four models of hyperphagia (*ob* mice, fasted C57BL/6J mice, A^y mice, and mice coinjected with NPY) suppressed feeding in a dose-dependent manner, and this suppression could be prevented by coinjection of the antagonist SHU9119 (Fan et al., 1997). Furthermore, injection of the antagonist produced a significant increase in food intake. Together with the data presented here, these results demonstrate a novel role for melanocortinergic neurons in tonic inhibition of feeding and metabolism. The increased somatic growth observed in MC4-R–deficient mice also suggests involvement of neurons expressing MC4 receptors in regulation of growth hormone–releasing hormone.

Expression of melanocortin peptides in the brain have previously been implicated in a number of other biological activities, including increasing retention of learned

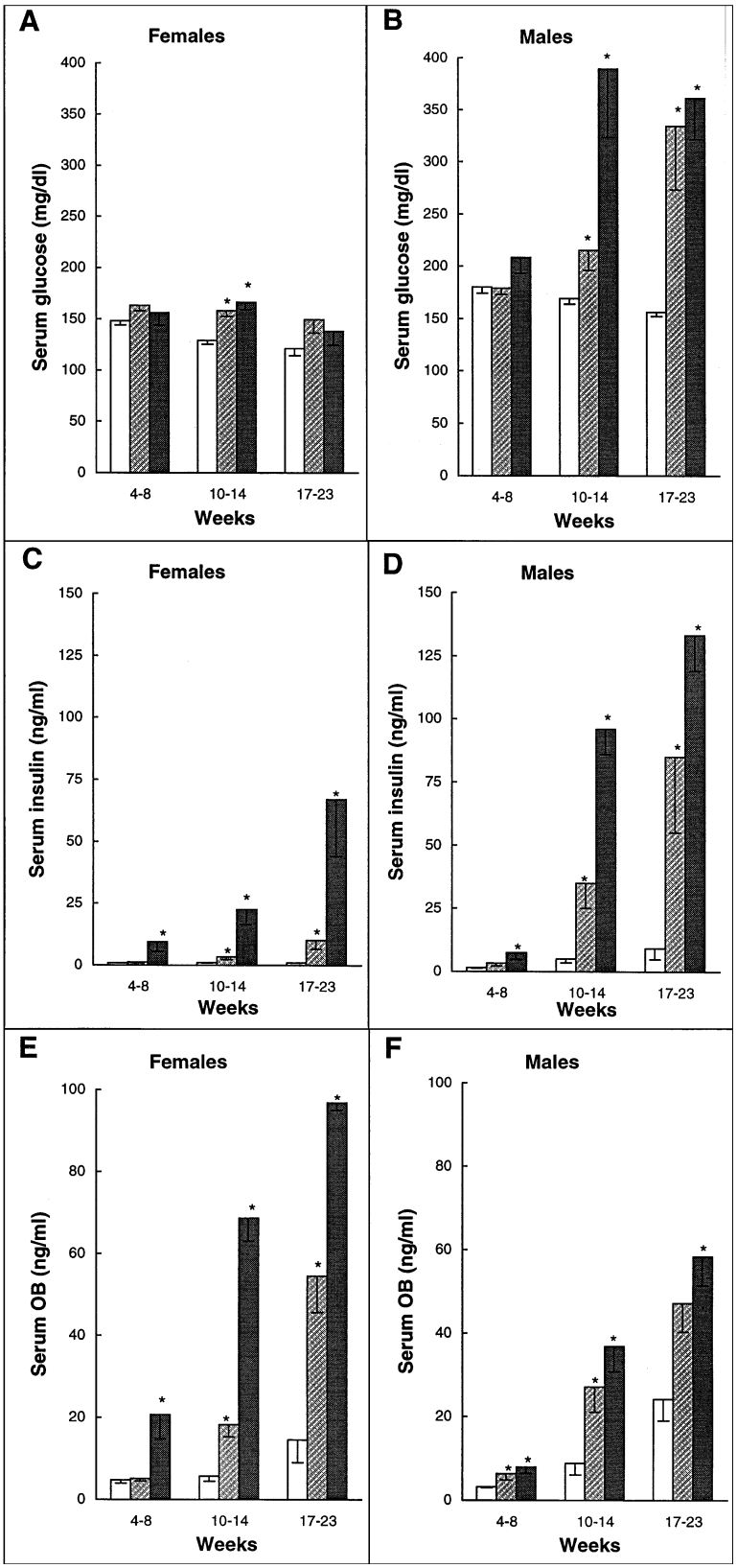

Figure 5. Serum Glucose, Insulin, and Leptin Levels in Mice Lacking the MC4-R

Glucose, insulin, and leptin were each measured on the same serum samples. Open bars represent mean values for wild-type F2 controls, hatched bars represent heterozygotes, and shaded bars represent homozygous mutant mice. Error bars indicate the standard error of the mean. Asterisks denote significant difference ($p < 0.05$ by two-tailed Student t test) compared to control within the same sex and age group. For female mice, the n for wild-type mice at 4–8 weeks, 10–14 weeks, and 17–23 weeks was 11, 14, and 7, respectively; for heterozygotes, 17, 17, and 7, respectively; and for homozygous mutants, 7, 11, and 3, respectively. For male mice, the n for wild-type mice at 4–8 weeks, 10–14 weeks, and 17–23 weeks was 14, 14, and 6, respectively; for heterozygotes, 14, 14, and 5, respectively; and for homozygous mutants, 8, 8, and 9, respectively.

(A and B). Serum glucose levels of female and male mice, respectively. Five μl of serum was analyzed using a glucose oxidase assay.

(C and D). Serum insulin levels of female and male mice, respectively, were assayed by radioimmunoasay using rat insulin as the standard.

(E and F). Serum leptin levels of female and male mice, respectively, were measured by radioimmunoassay.

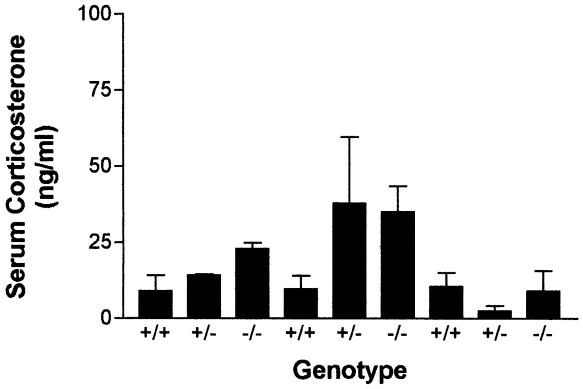

Figure 6. MC4-R Gene Deletion Does Not Affect Basal Serum Corticosterone

Serum corticosterone levels were measured in three sets of sex-matched littermates containing a representative animal of each genotype: +/+ wild-type control, +/− heterozygote, −/− homozygous mutant. Sets are, from left to right, male, female, and male. Males were 15 weeks of age, females were 18 weeks. Data indicate the means of measurements performed using two serum samples obtained on different days. Measurement on each day was performed in duplicate. Bars indicate standard deviation. Analysis of data by two-way ANOVA indicated no significant difference in corticosterone levels as a function of genotype.

behaviors (Murphy and Miller, 1955; DeWied and Jolles, 1982), antipyresis and regulation of temperature control (Lipton and Glyn, 1980; Murphy et al., 1983; Feng et al., 1987), elevation of heart rate, blood pressure, and natriuresis (Klein et al., 1985; Lin et al., 1987). The involvement of MC4-R–dependent signaling in mediating these various biological effects can now be addressed in mice lacking expression of the receptor.

An alternative to the conclusion that signaling by the MC4-R directly influences feeding and metabolism is that MC4-R gene deletion could result in a defect in hypothalamic brain development resulting in hyperphagia and hypometabolism. For example, defects in functioning of the ventromedial hypothalamus in humans and rodents are associated with obesity (reviewed in Bray, 1984). This argument is formally difficult to exclude; however, no gross neuroanatomical defects were observed in brain sections from mice lacking the MC4 receptor. Furthermore, direct evidence for a pharmacological etiology is provided by the modulation of feeding behavior affected by the cyclic melanocortin analogues, MTII and SHU9119, as described above. In addition, the MC4-R–deficient obesity phenotype is strikingly similar to the *agouti* obesity syndrome, consistent with a specific common mode of action of the two mutations (i.e. interference with MC4-R signaling) rather than a developmental defect in MC4-R–deficient mice.

The agouti protein is a pigmentation factor normally expressed in the skin where it regulates the synthesis of pigment by antagonism of the MC1-R on melanocytes. Dominant mutations of the *agouti* locus that result in widespread ectopic expression give rise to the pleiotropic *agouti* obesity syndrome (Bultman et al., 1992; Michaud et al., 1993; Miller et al., 1993; Duhl et al., 1994; Michaud et al., 1994a; 1994b). The demonstration that agouti in vitro is a competitive antagonist of not only the melanocyte MC1-R but also the neural MC4-R led to the hypothesis that agouti induces obesity by chronic antagonism of the MC4-R (Lu et al., 1994). In this study we have observed noteworthy similarities between the MC4-R–deficient and *agouti* obesity syndromes. Mice lacking the MC4-R develop obesity and hyperinsulinemia with a time course very similar to that described for A^y and A^{vy} mice (Dickie and Woolley, 1946; Dickerson and Gowen, 1947; Yen et al., 1976a; Frigeri et al., 1983; Warbritton et al., 1994; reviewed in Yen et al., 1994). The MC4-R–mutant mice also exhibit increased linear growth, a feature unique to the *agouti* syndrome among rodent obesity models. Furthermore, as is also the case for the *agouti* obesity syndrome, the obesity of MC4-R–deficient mice is not dependent on elevation of basal adrenocortical steroids (Wolff and Flack, 1971). Analysis of the expression of neuropeptide Y (NPY, a potent stimulator of feeding) in A^y and MC4-R–deficient mice has uncovered a further common and unique feature of the

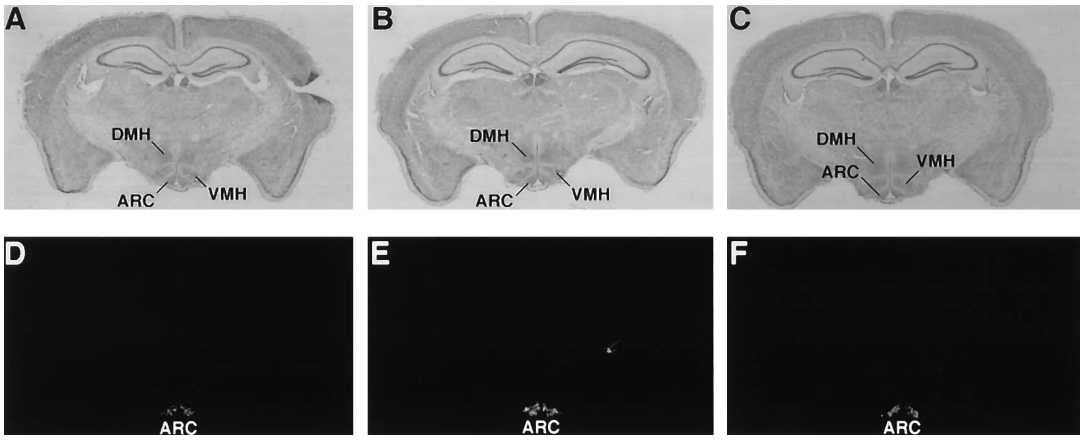

Figure 7. MC4-R Gene Deletion Does Not Affect Brain POMC mRNA Levels

(A–C) Thionin-stained brain sections from wild-type, heterozygous, and homozygous mutant MC4-R–deficient mice, respectively.
(D–F) Autoradiographs of brain sections from wild-type, heterozygous, and homozygous mutant MC4-R–deficient mice, respectively, hybridized with a ^{35}S-POMC antisense cRNA probe.

two melanocortinergic obesity models (R. C. and R. K., personal communication). NPY expression was unchanged in the arcuate nucleus, but dramatically elevated in the dorsal medial hypothalamic (DMH) nucleus in both obese A^y and MC4-R–deficient mice. Elevation of NPY in the arcuate nucleus is found in several obesity models, including *ob* mice (Sanacora et al., 1990; Stephens et al., 1995; Schwartz et al., 1996), but expression of NPY in the DMH has not previously been reported in association with obesity. This finding shows a common change in hypothalamic gene expression in the agouti- and MC4-R–deficient mice, and may imply involvement of the DMH in the melanocortinergic obesity syndrome.

The striking recapitulation of many features of the *agouti* obesity syndrome in MC4-R–deficient mice strongly supports the conclusion that the syndrome derives from agouti-mediated disruption of an MC4-R–dependent signaling pathway involved in the regulation of body weight. Our data are not consistent with an alternative hypothesis that the primary site of agouti action in eliciting the obesity syndrome is peripheral, mediated by elevation of intracellular levels of free calcium in skeletal muscle (Zemel et al., 1995) and/or adipocytes and pancreatic β-cells (Klebig et al., 1995; Manne et al., 1995). Those phenotypic differences we have detected between mice lacking the MC4-R and dominant *agouti* mutants have been quantitative rather than qualitative. For example, the absolute weight gain of MC4-R mutants appears to be greater than that of *agouti* mutants. Keeping in mind that there are considerable variations in the weight of obese yellow mice on different genetic backgrounds and there is no data for body weight of dominant agouti mutants on the 129/Sv × C57BL/6 background of the MC4-R–deficient mice, this weight increase tends to be higher than that reported for age and sex-matched A^y or A^{vy} mice on a variety of backgrounds (Castle, 1941; Dickie and Woolley, 1946; Dickerson and Gowen, 1947; Carpenter and Mayer, 1958; Yen et al., 1976a; Yen et al., 1976b; Frigeri et al., 1983; Wolff, 1987; Frigeri et al., 1988). Similarly, the levels of serum insulin in both males and females homozygous for MC4-R deletion, as well as glucose levels in MC4-R–deficient males, are higher than those reported for age- and sex-matched A^{vy} mice (Frigeri et al., 1983). Lastly, inactivation of the MC4-R results in a greater increase in the length of mice than that observed as a result of ectopic expression of agouti (Castle, 1941; Carpenter and Mayer, 1958). These differences may reflect a dose dependency of MC4-R antagonism on the severity of the obesity syndrome, with deletion of the receptor equivalent to absolute antagonism as opposed to less complete antagonism of the receptor by ectopic agouti expression. This model is supported by 1) data reported here of a gene dosage effect of heterozygosity for receptor deletion on the extent of obesity, hyperinsulinemia, hyperglycemia, and linear growth; by 2) the observation that the level of ectopic agouti expression in agouti transgenics correlates with the severity of obesity, hyperinsulinemia and hyperglycemia (Klebig et al., 1995; Perry et al., 1995); and by 3) dominant agouti mutations resulting from intracisternal A particle (IAP) insertions in which expressivity of the obese phenotype is positively correlated with the levels of agouti expression (Wolff et al., 1986; Duhl et al., 1994; Michaud et al., 1994b). Although agouti is a secreted protein, it acts in a localized fashion and does not appear to circulate at significant levels (Wolff, 1963; Silvers, 1979). Thus, the relevant concentration of agouti for mediating antagonism of the MC4-R is likely that which is expressed in the immediate milieu of the receptor in the brain.

Targeted deletion of the MC4-R has revealed a novel signaling pathway in the brain controlling nutrient intake and energy balance. Further analysis of the regulation and consequences of melanocortinergic signaling should prove instructive in the elucidation of the physiological control of body weight and may provide novel therapies for treatment of obesity. The obesity syndrome resulting from abrogation of MC4-R signaling convincingly mirrors that generated by ectopic agouti expression, demonstrating central antagonism of melanocortin signaling via the MC4-R as the primary cause of the *agouti* obesity syndrome.

Experimental Procedures

Construction of the MC4-R Targeting Vector

Murine MC4-R gene sequences were isolated from a mouse strain 129/Sv genomic phage library (Stratagene) using a probe generated by PCR amplification of human MC4-R coding sequences.

Construction of the MC4-R targeting construct was carried out in the vector pJN2 that was produced in the following manner. The 1.4 kb EcoRI–AvaI fragment of pBR322 was replaced with the synthetic oligonucleotides AAT TAG CGG CCG CAG TAT GCA AAA AAA AGC CCG CTC ATT AGG CGG GCT and CCG AAG CCC GCC TAA TGA GCG GGC TTT TTT TTG CAT ACT GCG GCC GCT. The resulting plasmid, pJN1, was digested with NotI, and the following oligonucleotides were ligated into the NotI site to generate pJN2: GGC CGG CAT GCA TCA AGC TTA TCT CGA GAT CGT CGA CTA CCA TGG TAC ATC GAT CAG GTA CCA TCC CGG GGC and GGC CGC CCC GGG ATG GTA CCT GAT CGA TGT ACC ATG GTA GTC GAC GAT CTC GAG ATA AGC TTG ATG CAT GCC.

A 1.2 kb genomic fragment extending from the HindIII site located approximately 0.6 kb 3′ of the MC4-R gene to the SphI site located approximately 1.8 kb 3′ of the MC4-R gene, representing the 3′ region of genomic homology, was subcloned into SphI–HindIII–digested pJN2. A 3.4 kb genomic fragment extending from the NcoI site located at the 5′ end of the MC4-R gene to the HindIII site located approximately 3.4 kb 5′ of the gene, representing the 5′ region of genomic homology, was also ligated into this plasmid, 5′ of the 1.2 kb HindIII–SphI fragment, and in the same orientation.

The PGK–*neo* expression cassette from the plasmid pKJ1 (Tybulewicz et al. 1991), containing the *neo* gene under the transcriptional control of the mouse phosphoglycerate kinase (PGK-1) promoter and the PGK-1 poly(A) addition site, was ligated between the 5′ and 3′ fragments of MC4-R genomic homology to generate the MC4-R targeting vector. The vector was linearized with NotI digestion prior to electroporation.

ES Cell Transfection

The RF-8 ES cell line (Huang et al., 1996) was cultured on SNL76/7 mitotically inactive feeder cells. For electroporation, cells were trypsinized and resuspended at a concentration of 1.1×10^7/ml in PBS (Ca^{2+}- and Mg^{2+}-free; Gibco). A 0.9 ml aliquot (1×10^7 cells) was mixed with 20 μg of the linearized targeting vector and pulsed at 250V, 500 μF (Bio-Rad Gene Pulser). The cells were then diluted in culture medium, plated at 1–2 × 10^6 per 100mm plate containing feeder cells, and placed under selection 24 hr later in G418 sulfate (400 μg/ml powder, Gibco). G418-resistant clones were picked, dissociated with trypsin, and divided into one well each of two 96-well plates. Upon confluence, ES cells were frozen in one of the 96-well plates as described (Ramirez-Solis et al., 1993) and expanded into a 24-well plate from which DNA was prepared, upon confluence, for Southern blot analysis.

Southern Blot Hybridization

Genomic DNA was prepared in situ from ES cells in 24-well plates and from tail biopsies by the procedure of Laird et al. (1991). Approximately 20 μg of genomic DNA was digested with the indicated restriction endonuclease, electrophoresed through a 1% agarose gel, transferred to Hybond N+ membrane (Amersham), and hybridized with the ^{32}P-radiolabeled probes indicated in the text.

Generation of MC4-R–Deficient Mice

The targeted ES clone was injected into C57BL/6J blastocysts as described (Bradley, 1987) to generate chimeras. Male chimeras were bred with C57BL/6J females, and agouti offspring (representing germline transmission of the ES genome) were screened for the presence of the targeted MC4-R gene by Southern blot hybridization of ApaI- and NcoI-digested tail DNA with the flanking probe shown in Figure 1A. Offspring heterozygous for the mutation were bred together, and mice homozygous for the MC4-R deletion were identified by Southern blot hybridization of ApaI- or NcoI-digested tail DNA with the flanking probe.

Weight and Length Measurements

Weight gain was regularly measured, beginning at 3–4 weeks of age, using a Sartorius model #14800 P balance. Length was measured by manual immobilization and extension of the mouse to its full length, always by the same individual, and measurement of the nose-to-anus distance in centimeters.

Food Consumption

Food intake was measured for two A^y, two C57BL/6J, four MC4-R homozygous mutants, and four wild-type F2 controls, each housed two to a cage. The mice were housed for at least a week before any measurements were taken. Over a two-week period, a sufficient amount of food for the week was then weighed and provided to the mice ad libitum. Each weekday morning, the remaining food was measured, for a total of eight measurements. Cages were carefully monitored for spillage, which was negligible. The A^y and C57BL/6J mice were 9 weeks of age at the time measurement of food consumption was initiated; both the four MC4-R–deficient mice and the F2 controls were each 15, 15, 17.5, and 20.5 weeks of age.

Serum Glucose, Insulin, and Leptin Measurements

For glucose, insulin, and leptin measurements, blood was collected by retroorbital sinus puncture from animals provided with food and water ad libitum. Mice were handled regularly (three times per week for several weeks) prior to bleeding to minimize stress, and cages were singly moved to a separate location at the time of bleeding. For measurement of glucose levels, 5 μl of serum was analyzed in a YSI Model 27 glucose analyzer (Yellow Springs Instrument Company, Inc.) using a glucose oxidase assay. Results are expressed as mg/dl. The range of detection is 0–500 mg/dl, with a coefficient of variation of $< 1\%$. Serum insulin concentration was measured in duplicate in a 10 μl volume by a specific competitive protein binding assay using rat insulin as the standard. Results are expressed as ng/ml. The range of detection is 0.1–25.0 ng/ml with a coefficient of variation of $< 10\%$. Leptin was measured in duplicate in 20 μl of serum using a radioimmunoassay kit to mouse leptin with recombinant mouse leptin as the standard (Linco Research Inc.).

For serum corticosterone measurements, mice were housed at three animals per cage with food and water ad libitum. Male mice were tested at 15 weeks of age, females at 18 weeks. To prevent stress-mediated elevation of corticosterone levels, mice were handled 2–3 times/day for three days prior to drawing blood. Cages were brought one at a time into a separate room, and mice were weighed and then held as if blood were to be drawn. On the fourth day, mice were handled similarly, and blood was drawn between 8:00 and 9:00 A.M. within 30 s of handling. Cages were not returned to the housing room until all of the samples had been obtained. Blood was obtained by snipping the tail tip and collecting blood into a Multivette S Gel tube (Sarstedt). Tubes were placed on ice for 20–40 min and centrifuged for 3–4 min at 14,000 rpm to separate the serum. Two one-μl aliquots of serum from each sample were then assayed for corticosterone levels using an ImmuChem Double Antibody Corticosterone ^{125}I RIA kit (ICN Biomedicals, Inc.).

Histology

For in situ hybridization analysis of POMC gene expression, wild-type heterozygous, and homozygous mutant mice were maintained under a 12 hr light, 12 hr dark cycle at constant temperature. Food (Purina mouse chow) and water were provided ad libitum. Anesthetized (avertin) animals were sacrificed between 1500 and 1700 hr, before lights out via cardiac puncture and perfusion with saline (20 ml) and then 50 ml of ice-cold fixation buffer (4% paraformaldehyde in borate buffer [pH 9.5]). Whole brains were rapidly removed and then postfixed overnight in 10% sucrose/fixative buffer. Blocked hypothalamic sections were frozen in powdered dry ice and then stored at −80°C prior to sectioning.

Antisense POMC probe was prepared by linearizing the plasmid mPOMCE3ribo (kindly provided by Dr. Malcolm Low), containing exon 3 of the mouse POMC gene, with NcoI. [^{35}S] cRNA probes were prepared by transcribing 1 μg of each linearized DNA with T7 DNA polymerase for 1 hr at 37°C as described (Promega). Hypothalamic brain blocks were mounted on a frozen stage and serially sectioned into 4 series of 20 μM slices with a sliding microtome. Sections were prepared and hybridized as previously described (Arriza et al., 1988). Sections were hybridized for 20 hr at 58°C with ^{35}S-labeled probes (5×10^6 cpm/ml) in 65% formamide, 0.26 M NaCl, 1.3× Denhardt's solution, 1.3 mM EDTA, 13% dextran sulfate, and 13 mM Tris (pH 8). Sections were then digested with RNase (20 μg/ml) for 30 min at 37°C and then desalted in a series of washes from 4× SSC/1mM DTT to a final stringency of 0.1× SSC/1mM DTT at 65°C for 30 min. Sections were dehydrated in ascending ethanol, vacuum dried at room temperature for 30 min, and then exposed to Dupont Cronex film for several days. Dried slides were then dipped in NTB-2 emulsion (Kodak) and developed after 6 days.

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