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Neither Agouti-Related Protein nor Neuropeptide Y Is Critically Required for the Regulation of Energy Homeostasis in Mice

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Agouti-related protein (AgRP), a neuropeptide abundantly expressed in the arcuate nucleus of the hypothalamus, potently stimulates feeding and body weight gain in rodents. AgRP is believed to exert its effects through the blockade of signaling by α-melanocyte-stimulating hormone at central nervous system (CNS) melanocortin-3 receptor (Mc3r) and Mc4r. We generated AgRP-deficient (*Agrp*$^{-/-}$) mice to examine the physiological role of AgRP. *Agrp*$^{-/-}$ mice are viable and exhibit normal locomotor activity, growth rates, body composition, and food intake. Additionally, *Agrp*$^{-/-}$ mice display normal responses to starvation, diet-induced obesity, and the administration of exogenous leptin or neuropeptide Y (NPY). In situ hybridization failed to detect altered CNS expression levels for proopiomelanocortin, Mc3r, Mc4r, or NPY mRNAs in *Agrp*$^{-/-}$ mice. As AgRP and the orexigenic peptide NPY are coexpressed in neurons of the arcuate nucleus, we generated AgRP and NPY double-knockout (*Agrp*$^{-/-}$;*Npy*$^{-/-}$) mice to determine whether NPY or AgRP plays a compensatory role in *Agrp*$^{-/-}$ or NPY-deficient (*Npy*$^{-/-}$) mice, respectively. Similarly to mice deficient in either AgRP or NPY, *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice suffer no obvious feeding or body weight deficits and maintain a normal response to starvation. Our results demonstrate that neither AgRP nor NPY is a critically required orexigenic factor, suggesting that other pathways capable of regulating energy homeostasis can compensate for the loss of both AgRP and NPY.

Agouti is a 131-amino-acid paracrine hormone normally expressed in skin where it controls hair color through antagonism at the melanocortin-1 receptor (Mc1r) (2). The obesity observed in *A*^{y}*/a* mice results from ectopic expression of agouti in the brain, where it antagonizes the actions of the proopiomelanocortin (POMC)-derived agonist α-melanocyte-stimulating hormone (α-MSH) at Mc3r and Mc4r (10, 17, 18, 21). The 132-amino-acid agouti-related protein (AgRP), which is normally expressed in the brain, and agouti share significant amino acid sequence identity, particularly in the cysteine-rich C-terminal domain. Since both agouti and AgRP are antagonists at Mc3r and Mc4r (11, 21), they are believed to elicit very similar metabolic consequences.

In the central nervous system (CNS), AgRP is expressed exclusively in a subset of hypothalamic arcuate nucleus neurons, which also express the orexigenic peptide neuropeptide Y (NPY) (1, 15, 21, 27). The only peripheral tissue known to express AgRP is the adrenal gland. AgRP is detected at a low level in the plasma of rats and humans. Plasma AgRP levels in these two species are affected by meal consumption, and it has been postulated that plasma AgRP levels may reflect satiety (25a). The relevance of AgRP towards the modulation of appetite was also deduced from experiments in which intracerebroventricular (icv) administration of AgRP was shown to acutely stimulate feeding (20). Finally, transgenic mice overexpressing AgRP develop hyperphagia and obesity, which is similar to the obesity observed in *A*^{y}*/a* mice (12, 21).

Genetic and pharmacological studies with rodents revealed that the adipose tissue-derived hormone leptin exerts its effects on appetite and energy expenditure in part through its action on CNS neurons expressing Mc4r (7, 19, 28). The actions of leptin include the modulation of the activity of arcuate nucleus neurons (7). Leptin reduces the expression of the orexigenic peptides NPY and AgRP and concomitantly increases the neuronal activity of POMC neurons (7). Enhanced neuronal activity of POMC neurons is expected to result in increased release of α-MSH, subsequent agonism at Mc3r and Mc4r, and hence modulation of appetite, energy expenditure, and feed efficiency. These conclusions were corroborated by studies of Mc3r and Mc4r knockout mice (3, 4, 17), which are obese.

To further evaluate the physiological role of endogenous AgRP, we generated *Agrp*$^{-/-}$ mice. To determine whether or not AgRP and NPY can functionally compensate for the absence of one another, we generated *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice. Since *Agrp*$^{-/-}$ and *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice are without obvious phenotypes, we conclude that neither AgRP nor NPY is critically required for the regulation of energy homeostasis, and we suggest that compensatory mechanisms or redundant inputs from non-AgRP and non-NPY pathways contribute to the regulation of energy homeostasis.

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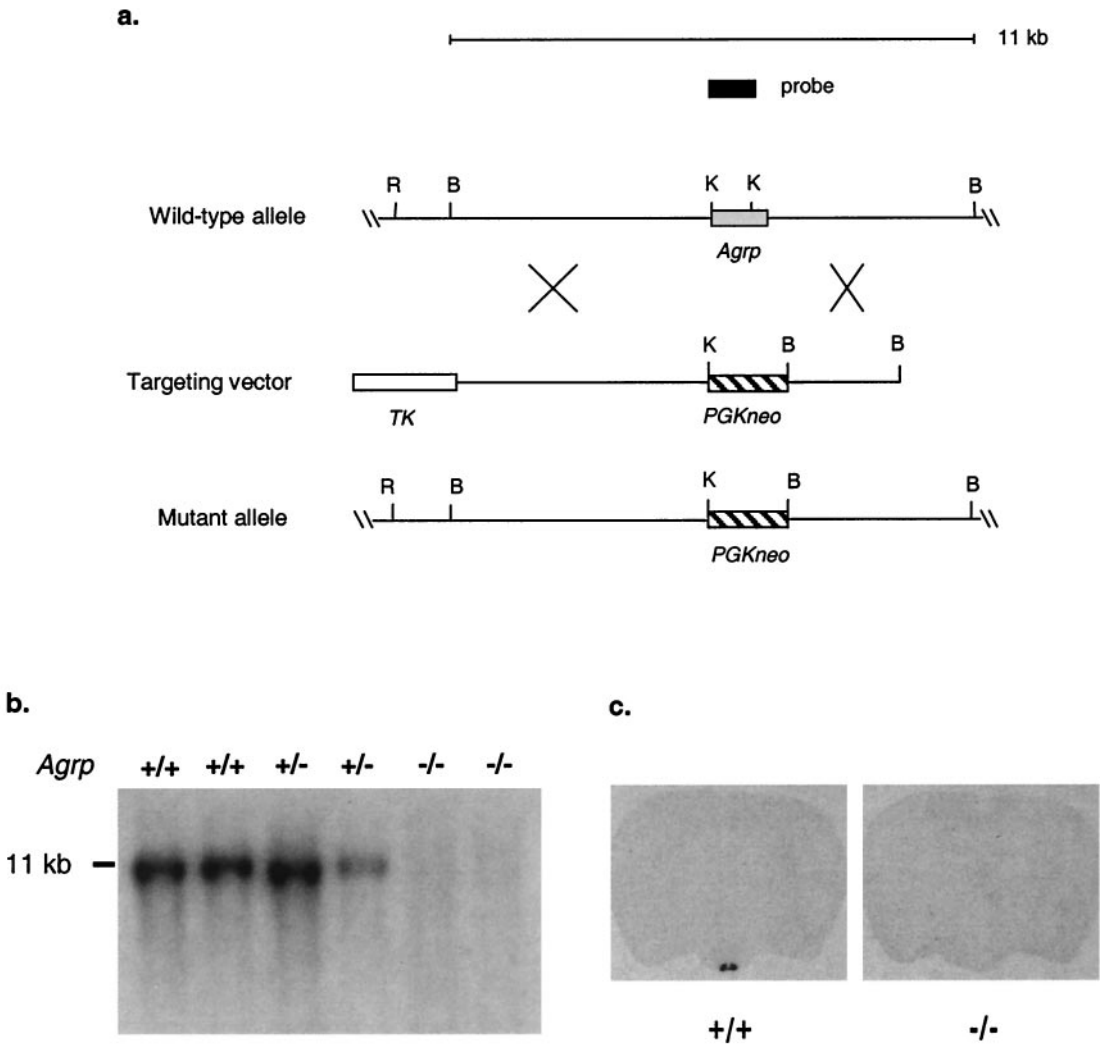

FIG. 1. Generation and validation of *Agrp*$^{-/-}$ mice. (a) Schematic diagrams of the murine wild-type *Agrp* allele, targeting vector, and mutant allele. The mutant allele arises from a double reciprocal homologous recombination between the wild-type allele and targeting vector. R, *Eco*RI; B, *Bam*HI; K, *Kpn*I; TK, thymidine kinase gene; PGKneo, PGK-driven neomycin resistance gene. (b) Southern blot analysis of *Bam*HI-digested tail DNAs. The coding-region probe (depicted in panel a) detects an 11-kb restriction fragment from wild-type and *Agrp*$^{+/-}$ mice but not from *Agrp*$^{-/-}$ mice. (c) In situ hybridization with a mixture of two oligonucleotide probes to *Agrp* reveals AgRP mRNA in the arcuate nucleus of wild-type mice but not in the same brain region of *Agrp*$^{-/-}$ mice.

MATERIALS AND METHODS

Animal care. All animal protocols used in these studies were approved by the Merck Research Laboratories Institutional Animal Care and Use Committee in Rahway, N.J. We housed mice in microisolator cages (Labproducts) in a barrier facility with an air shower entrance or in a specific-pathogen-free facility.

Generation of *Agrp*$^{-/-}$ mice. A mouse 129SvEv genomic library (Lambda FIX II vector; Stratagene) was screened with a 300-bp mouse *Agrp* cDNA probe, which was generated by PCR with primers derived from the rat *Agrp* (27). Two positive clones containing 23-kb overlapping mouse *Agrp* genomic sequences were isolated. *Agrp* coding and surrounding regions were separately subcloned into pBluescript vector (Stratagene). An *Agrp* targeting vector was generated by inserting a 5.2-kb 5′ *Bam*HI to *Kpn*I restriction fragment between the *Bgl*II and *Kpn*I sites and a 2.2-kb 3′ *Spe*I restriction fragment into the *Eco*RI sites of pKO Scrambler 1901 (Stratagene), flanking the neomycin resistance cassette. The targeting vector was linearized by *Sal*I digestion and transformed into AB2.2 ES cells by electroporation. We cultured transfected cells with G418 and 2′-fluoro-2′-deoxy-1β-D-arabinofuranosyl-5-iodo-uracil for positive and negative selections, respectively. Approximately 800 clones were selected, and 10 correctly targeted ES cell clones were identified by Southern blot analysis. We injected the targeted ES clones into C57BL/6 blastocysts and implanted these into pseudopregnant female mice (22). Several chimeric progenies gave germ line transmission of the mutant *Agrp* allele, and two independent *Agrp*$^{+/-}$ lines were established.

Genotyping. We performed Southern blot analysis using a 3′ probe and a coding-region probe (25). The 3′ flanking probe is a 0.75-kb *Bgl*II-*Not*I restriction fragment located 3′ of the short arm of the targeting vector. Upon *Bam*HI digestion, the probe detects a 11.0-kb band from the wild-type *Agrp* allele and a 4.0-kb band from the mutant allele. The coding-region probe is a 0.78-kb *Kpn*I restriction fragment of *Agrp*, spanning a region from bp 53 5′ to the initiation codon to bp 97 5′ of the stop codon. The coding-region probe detects a 11.0-kb wild-type band from *Bam*HI-digested genomic DNA but detects no signal from the mutant allele (Fig. 1b). A PCR method was also employed for genotyping. This protocol is available upon request.

Generation of *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice. To generate *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice, we crossed *Agrp*$^{+/-}$;*NPY*$^{+/+}$ mice with *Agrp*$^{+/+}$;*Npy*$^{-/-}$ mice. The resulting *Agrp*$^{+/-}$;*Npy*$^{+/-}$ mice were backcrossed with *Agrp*$^{+/+}$;*Npy*$^{-/-}$ mice to generate *Agrp*$^{+/-}$;*Npy*$^{-/-}$ mice which were then interbred to produce *Agrp*$^{-/-}$;*Npy*$^{-/-}$ and *Agrp*$^{+/+}$;*Npy*$^{-/-}$ mice. In parallel, *Agrp*$^{+/+}$;*Npy*$^{+/-}$ mice were crossed with

Agrp$^{+/+}$;*Npy*$^{+/+}$ mice to generate *Agrp*$^{+/+}$;*Npy*$^{+/+}$ wild-type control mice with a similar genetic background. All study groups were of a similar genetic background of 12.5% C57BL/6 and 87.5% 129sv.

In situ hybridization. *Agrp*$^{-/-}$ and age- and sex-matched wild-type control mice were decapitated under CO_2 anesthesia. Brains were quickly removed, frozen in −40°C isopentane, and stored at −80°C until use. Coronal brain sections (14 μm) were cut at −17°C with a cryostat microtome and thaw mounted onto baked microslides. Following fixation in ice-cold 4% phosphate-buffered paraformaldehyde, the tissue sections were stored in 95% ethanol at 4°C until use. The mouse Agrp probe consists of an equal molar mixture of two nonoverlapping, antisense oligonucleotides against the coding region of Agrp, with the following sequences: oligonucleotide 296, 5′-TGCAGCAGAACTTCT TCTGCTCGGTCTGCAGTTGTCTTCTTGAGG-3′, and oligonucleotide 297, 5′-AGCTTGCGGCAGTAGCAAAAGGCATTGAAGAAGCGGCAGTAGCAC -3′. For expression surveys, 6-month-old male *Agrp*$^{-/-}$ mice and their littermate-matched wild-type controls were used. Hybridization was performed with one to three antisense oligonucleotide probes for each target. In cases where multiple probes were used, an equal molar mixture of nonoverlapping probes was applied. The oligonucleotide sequences used to detect NPY, melanin-concentrating hormone (MCH), POMC, MC3r, and MC4r are available upon request. The probes were terminally labeled with [α-^{33}P]dATP and terminal transferase, and hybridization and washing conditions were as described previously (13).

Quantitative analysis for mRNA levels was carried out as described previously (13). Briefly, autoradiographs were captured and analyzed with the MCID/M2 image analyzer (Imaging Research Inc., Ontario, Canada). Density measurement was performed blindly on coded images. The optical density of each region in multiple adjacent brain sections was quantified and compared against a ^{14}C-labeled autoradiographic standard (Amersham, Arlington Heights, Ill.), which was calibrated to a series of brain paste containing various amounts of [^{33}P]dATP to represent tissue mRNA levels (nCi/g of brain tissue). An average value for each neuropeptide and receptor at each brain region was then obtained for each mouse, and the results were analyzed statistically by using a two-tailed, paired *t* test.

Due to the heterogeneous nature of MCH mRNA distribution in the lateral hypothalamic area (LHA), brain sections from different mice were carefully matched. Fourteen to 16 adjacent brain sections encompassing the entire MCH-expressing regions in the LHA were quantified, and from the numbers obtained a single average value was calculated for each mouse. The average values were then subjected to the statistical analysis mentioned above.

Growth curves. We housed the mice two to three per cage and provided them with ad libitum access to regular chow and water. Body weight and food intake were measured weekly.

Evaluation of appetitive behavior. *Agrp*$^{+/+}$, *Agrp*$^{+/-}$, and *Agrp*$^{-/-}$ mice were individually housed at approximately 1 month of age and at least 7 days prior to the experiments. Regular mouse chow (Teklad 7012, 5.67% kcal from fat and 3.41 kcal/g; Harlan Teklad) was provided to 9.5- to 10-week-old male mice as pellet food in wire cage tops containing food hoppers and weighed daily for 3 days. For studies involving the high-fat diet (Teklad 97070, 60% kcal from fat and 5.07 kcal/g), we provided ground food in a glass jar located in the cage and weighed the jar weekly. Food intake is reported as the average food consumed per animal per day over the course of 3 consecutive days.

For taste preference studies involving a high-fat diet, we provided both regular mouse chow and a pellet form of high-fat diet (Teklad TD88137, 42% kcal from fat and 4.54 kcal/g). For sucrose preference studies, one bottle of sucrose solution and one bottle of water were placed in the wire cage top in addition to regular chow. Bottle positions were alternated daily to reduce bottle position preferences. Both bottles were weighed daily for 5 consecutive days.

For measurement of food intake after fasting, the animals were weighed and the chow was removed at approximately 7 a.m. (light cycle, 3 a.m. to 3 p.m.) on the morning of fasting. The animals were weighed 8 h later (before lights out) and weighed again the following morning and afternoon. Twenty-four or 48 h after the initiation of fasting, the animals were given a weighed amount of chow, and the latency to refeed was measured. The animals and the feed were weighed at 1, 2, 4, 8, 24, 36, and 48 h postfeeding.

Assessment of locomotor activity and fine movements. We evaluated locomotor activity and fine movements of individually housed mice with a cage rack Photobeam Activity System (San Diego Instruments) as described previously (5).

Body composition determination by DEXA. We analyzed whole-body composition by dual energy X-ray absorptiometry (DEXA) (QDR 4500; Hologic, Inc.), using the QDR 4500 Small Animal Studies software version 9.0 as described elsewhere (5).

Plasma measurements. We measured plasma leptin, insulin, and total T4 levels as described previously (5). Plasma glucose, triglyceride levels, and free fatty acids were measured by enzyme-colorimetric assays (Sigma and Roche).

icv cannulations and injections. We anesthetized mice with an intramuscular injection of ketamine (75 mg/kg of body weight) and domitor (1 mg/kg) and placed them in a sterotaxic device (Kopf Instruments). A 26-gauge single acute guide cannula (Plastics One) was implanted into the dorsal third ventricle (0.22 mm posterior, 0.3 mm lateral, and 3.3 mm ventral to bregma). Following surgery, a 33-gauge dummy cannula (Plastics One) was inserted into each guide cannula and mice were given an intramuscular injection of antisedan (1 mg/kg). All cannulated mice were given 1 week of postoperative recovery, during which time they were handled daily to minimize nonspecific stress. All substances were administered to conscious mice with a repeating dispenser (Hamilton) equipped with a 50-μl Hamilton syringe and a 33-gauge needle designed to extend 0.1 to 0.2 mm beyond the tip of the guide cannula. We dissolved NPY (Peninsula Laboratories) in artificial cerebrospinal fluid (Harvard Apparatus) and injected a total volume of 2 μl. Mice were given at least a 48-h recovery period between treatments. All icv injection studies were of crossover design.

Upon completion of the icv injection studies, we verified cannula placement in every animal by injecting 2 μl of a 1% cresyl violet solution and subsequently examining coronal brain slices. Only those animals exhibiting dye distributed throughout the cerebroventricular system were included in the data analysis.

Statistical analysis. We reported all values as means ± standard errors of the means and analyzed data by the two-tailed, unpaired Student *t* test unless stated otherwise. *P* values of <0.05 were reported as significant.

RESULTS

Mouse AgRP is a 132-amino-acid peptide encoded by three exons. We replaced a 1.03-kb region of murine *Agrp* that spans the entire peptide-coding sequence with a neomycin resistance cassette (Fig. 1a). Two lines of *Agrp*$^{+/-}$ mice were established from independent embryonic stem cell clones. Mating of *Agrp*$^{+/-}$ mice produced progeny of all three genotypes in the expected Mendelian ratio (*Agrp*$^{+/+}$ mice, $n = 310$; *Agrp*$^{+/-}$ mice, $n = 662$; *Agrp*$^{-/-}$ mice, $n = 345$). Southern blot analysis (Fig. 1b) and in situ hybridization (Fig. 1c) confirmed the absence of the *Agrp* gene and mRNA in *Agrp*$^{-/-}$ mice, respectively. Heterozygous F_1 *Agrp*$^{+/-}$ mice descended from germ line-transmitting chimeras were interbred to generate F_2 wild-type, *Agrp*$^{+/-}$, and *Agrp*$^{-/-}$ littermate groups for behavioral analyses. All study groups have similar genetic backgrounds of 50% C57BL/6 and 50% 129SvEv.

Agrp$^{-/-}$ mice of both genders were fertile and exhibited no gross histological or pathological abnormalities. Group-housed wild-type, *Agrp*$^{+/-}$, and *Agrp*$^{-/-}$ littermate mice of both genders exhibited similar growth rates (Fig. 2a), and DEXA revealed that both male and female 8-month-old *Agrp*$^{-/-}$and wild-type littermate mice have similar body compositions (Fig. 2b). A survey of serum parameters revealed normal levels of plasma glucose, leptin, insulin, triglyceride, and free fatty acids in female *Agrp*$^{-/-}$ mice (Table 1).

In wild-type mice, AgRP83-132 potently stimulated food intake when administered to the CNS, and the level of endogenous AgRP increased dramatically when mice were subjected to fasting (20). These data suggest that AgRP is involved in the regulation of appetite and fasting-induced hyperphagia. Surprisingly, we found that both male (Fig. 2c) and female (data not shown) *Agrp*$^{-/-}$ mice fed ad libitum had levels of food intake similar to those of wild-type littermate mice and did not exhibit any deficits in reflex hyperphagia following 48 h of food deprivation (Fig. 2d). Changes in the body weights of male (Fig. 2d) and female (data not shown) *Agrp*$^{-/-}$ mice during

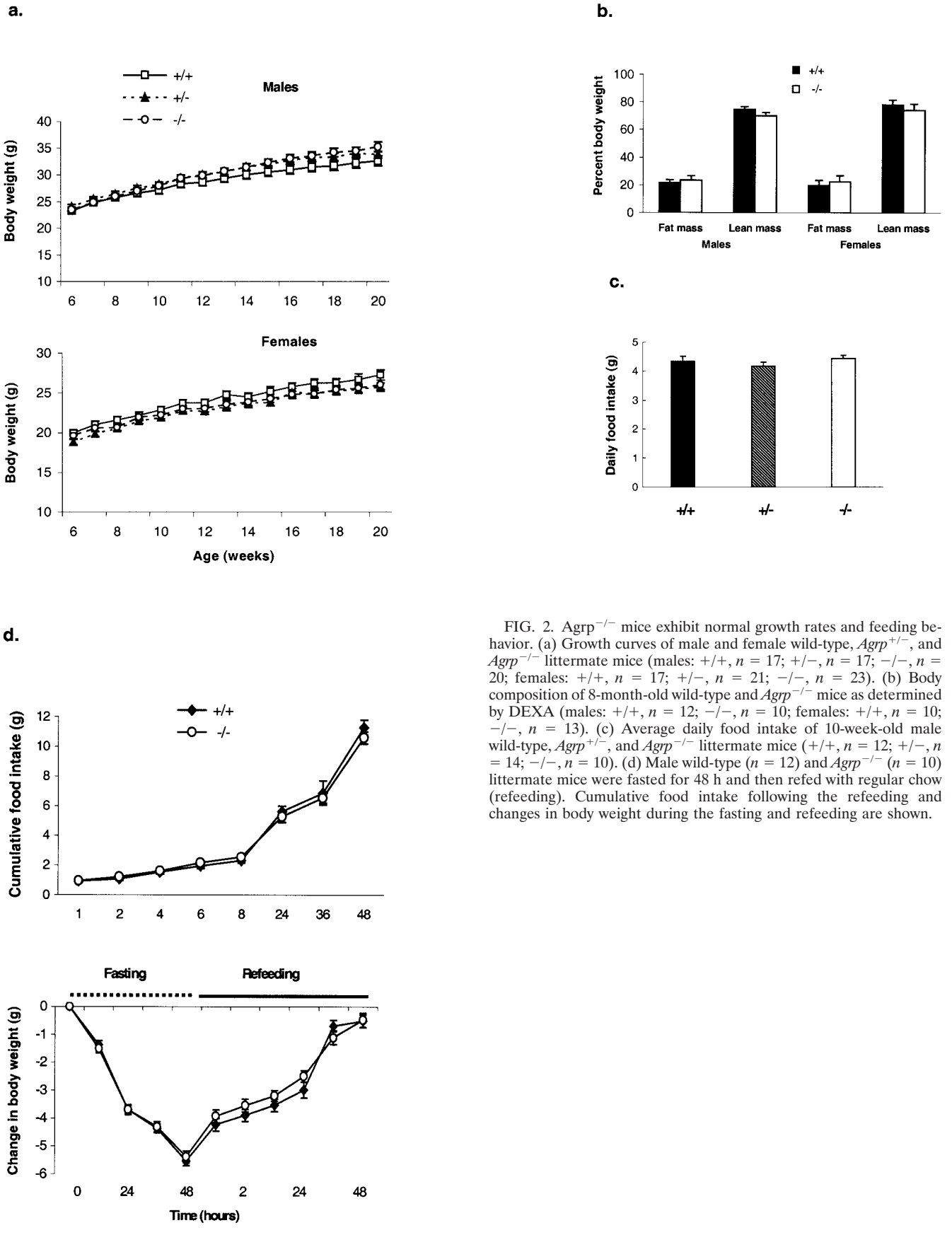

FIG. 2. Agrp$^{-/-}$ mice exhibit normal growth rates and feeding behavior. (a) Growth curves of male and female wild-type, *Agrp*$^{+/-}$, and *Agrp*$^{-/-}$ littermate mice (males: +/+, $n = 17$; +/−, $n = 17$; −/−, $n = 20$; females: +/+, $n = 17$; +/−, $n = 21$; −/−, $n = 23$). (b) Body composition of 8-month-old wild-type and *Agrp*$^{-/-}$ mice as determined by DEXA (males: +/+, $n = 12$; −/−, $n = 10$; females: +/+, $n = 10$; −/−, $n = 13$). (c) Average daily food intake of 10-week-old male wild-type, *Agrp*$^{+/-}$, and *Agrp*$^{-/-}$ littermate mice (+/+, $n = 12$; +/−, $n = 14$; −/−, $n = 10$). (d) Male wild-type ($n = 12$) and *Agrp*$^{-/-}$ ($n = 10$) littermate mice were fasted for 48 h and then refed with regular chow (refeeding). Cumulative food intake following the refeeding and changes in body weight during the fasting and refeeding are shown.

TABLE 1. Serum parameters of 8-month-old female mice[a]

Genotype (no. of mice)	Mean ± SEM of:				
	Glucose (mg/dl)	TG (mg/dl)	FFA (mM)	Insulin (ng/ml)	Leptin (ng/ml)
$Agrp^{+/+}$ (10)	118.48 ± 8.80	47.69 ± 4.67	0.782 ± 0.063	0.338 ± 0.057	3.125 ± 0.662
$Agrp^{-/-}$ (12)	126.05 ± 3.36	46.62 ± 5.29	0.721 ± 0.060	0.254 ± 0.038	3.764 ± 1.139

[a] Mice were fasted for 4 h prior to blood collection. TG, triglyceride; FFA, free fatty acids.

fasting and refeeding were also closely parallel to those of wild-type controls.

Locomotor activity was examined to evaluate its contribution to total energy expenditure. $Agrp^{-/-}$ mice maintained in an environment with a cycle of 12 h of light and 12 h of dark exhibited normal levels of daily ambulatory activity (distance traveled by $Agrp^{-/-}$ males, 178.1 ± 28.8 m; by wild-type males, 126.3 ± 15.6 m [$n = 8$ per genotype, $P > 0.05$]; distance traveled by $Agrp^{-/-}$ females, 113.7 ± 26.2 m; by wild-type females, 98.9 ± 13.0 m [$n = 7$ to 8 per genotype, $P > 0.05$]) and daily fine motor movements (data not shown). Core body temperatures were evaluated as a gross measure of metabolic rate. Both male and female $Agrp^{-/-}$ possessed core body temperatures similar to those of wild-type littermate mice (data not shown). Performance on a rotarod and grip strength were assessed to evaluate motor coordination. The two genders of $Agrp^{-/-}$ and wild-type littermate mice performed comparably on the rotarod and exhibited similar forelimb and hindlimb grip strengths (data not shown).

In rats, centrally administered AgRP83-132 selectively increased the consumption of a high-fat diet but not of a low-fat diet, suggesting that AgRP influences not only food intake but food selection as well (14). When given free access to both a low-fat diet and a high-fat diet, both male (Fig. 3a) and female (data not shown) wild-type and $Agrp^{-/-}$ littermate mice preferred the high-fat diet to similar degrees, indicating that AgRP is not essential for high-fat dietary preferences. To determine if the absence of AgRP has any effect on taste preference for sweets, we employed a two-bottle paradigm in which animals were given ad libitum access to both water and a sucrose solution. In 5-day studies, we found that male and female $Agrp^{-/-}$ and wild-type littermate mice all exhibited a threefold-greater preference for a 0.05 or a 0.5% sucrose solution over water (data not shown). These data suggest that AgRP is not required for sweet taste preferences.

$Agrp^{-/-}$ and wild-type littermate mice were exposed to a high-fat diet to determine if the absence of AgRP alters susceptibility to diet-induced obesity. We compared changes in body weights of $Agrp^{-/-}$ and wild-type littermate mice following 10 weeks of maintenance on a high-fat diet. Increases in body weights of $Agrp^{-/-}$ and of wild-type littermate mice were comparable, and both were greater than the weight gains of control groups maintained on regular chow (Fig. 3b). We conclude that AgRP deficiency does not alter susceptibility to diet-induced obesity.

The synthesis and release of AgRP are subject to inverse regulation by leptin, suggesting that AgRP is an essential mediator of leptin signaling (29). We examined if $Agrp^{-/-}$ mice exhibit a reduced response to the anorectic actions of leptin. Figure 4a shows that the peripheral administration of leptin resulted in the same degree of suppression of food intake in $Agrp^{-/-}$ and in wild-type littermate mice. Additionally, leptin administration resulted in similar reductions in the body weights of $Agrp^{-/-}$ and wild-type littermate mice (data not shown). These results indicate that AgRP is not required for normal leptin signaling.

Given the diversity of parameters examined above, the lack of AgRP does not appear to significantly compromise the normal regulation of energy homeostasis. This contrasts sharply with the pronounced effect of AgRP overexpression (12, 21). The absence of detectable energy homeostasis phenotypes in $Agrp^{-/-}$ mice was remarkably similar to that of $Npy^{-/-}$ mice, which are also free of obvious feeding and body weight deficits (8). Compensation by AgRP has been proposed to explain the

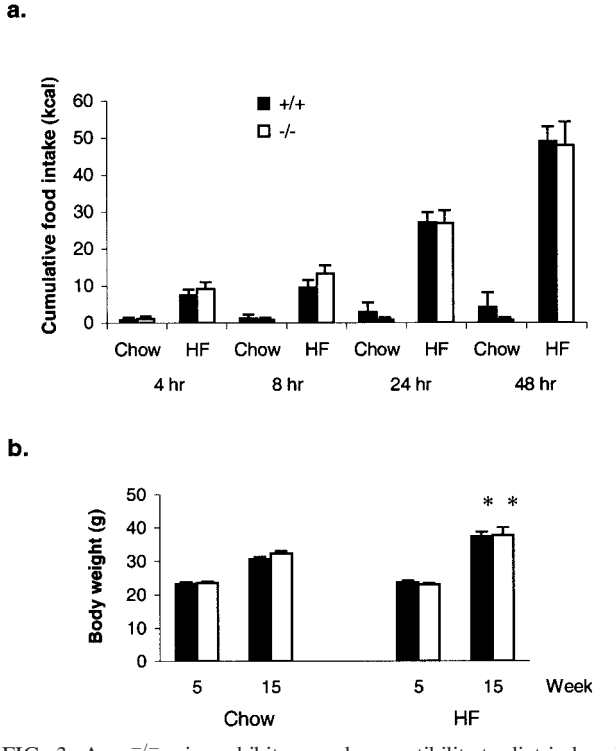

FIG. 3. $Agrp^{-/-}$ mice exhibit normal susceptibility to diet-induced obesity. (a) Male $Agrp^{-/-}$ mice and wild-type littermates were given equal access to regular chow (Chow) and a high-fat diet (HF). $Agrp^{-/-}$ and wild-type mice exhibited comparable preferences and consumption of the high-fat diet during a 48-h exposure. (b) Both wild-type and $Agrp^{-/-}$ male littermates gained more body weight after 10 weeks of maintenance on a high-fat diet (HF) than after 10 weeks of maintenance on chow (Chow) (HF groups: +/+, $n = 10$; −/−, $n = 10$) (Chow groups: +/+, $n = 17$; −/−, $n = 20$). *, $P < 0.05$ versus corresponding chow-fed groups at week 15.

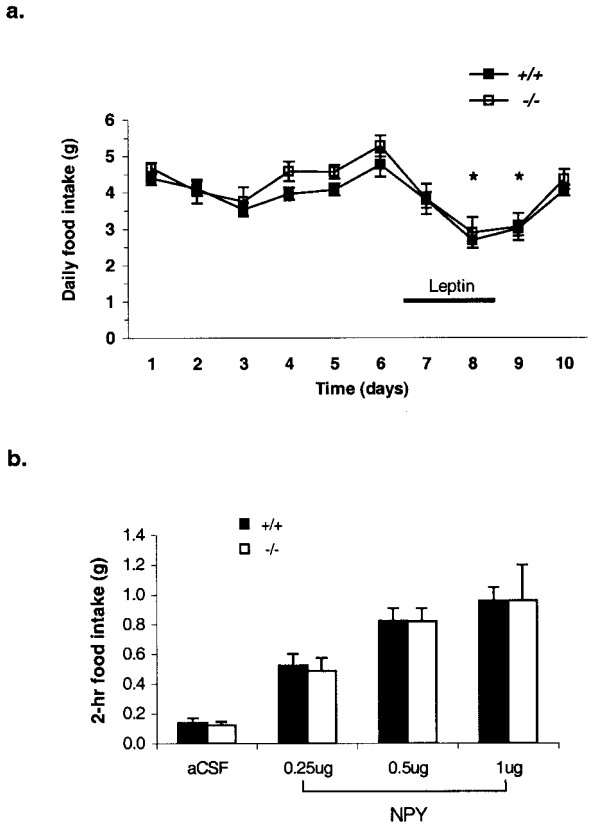

FIG. 4. Agrp$^{-/-}$ mice exhibit normal responses to exogenous leptin and NPY. (a) Twice daily intraperitoneal dosing of 1.5 mg of leptin/kg on days 7 and 8 suppressed food consumption to similar extents in both male *Agrp*$^{-/-}$ ($n = 10$) and wild-type ($n = 12$) littermate mice, relative to that observed with vehicle injections on days 1 to 6. On days 8 and 9 the daily food intake of both *Agrp*$^{-/-}$ and wild-type mice was significantly lower than their corresponding average daily food intake from days 1 to 7. *, $P < 0.005$. (b) Injection of 0.25, 0.5, and 1.0 μg of NPY into the dorsal third cerebroventricle of male *Agrp*$^{-/-}$ ($n = 6$ to 7) and wild-type ($n = 9$ to 12) littermate mice elicited similar dose-dependent increases in food intake. For both genotypes, all three doses of NPY significantly ($P < 0.05$) stimulated 2-h food intake relative to aCSF treatment.

lack of a feeding phenotype in *Npy*$^{-/-}$ mice (6, 20). Evidence supporting this hypothesis included (i) the nearly exclusive coexpression of NPY and AgRP in a class of arcuate nucleus neurons (1, 15, 21, 27), (ii) increases in the levels of both peptides and their corresponding mRNAs in response to starvation, (iii) an enhanced sensitivity to centrally administered AgRP83-132 in *Npy*$^{-/-}$ mice (20), and (iv) the finding that NPY can functionally antagonize signaling by MTII, an agonist of Mc3r and Mc4r (6). Based on these observations, we considered NPY to be the primary candidate capable of compensating for the loss of AgRP.

Plausible mechanisms of compensation include, but are not limited to, an increase in the release of NPY, enhanced responsiveness to the orexigenic actions of NPY, and an attenuation of melanocortin signaling. To examine if there is an increased response to feeding stimulation by NPY, we measured changes in food intake in response to icv administration of exogenous NPY. Figure 4b demonstrates comparable dose-dependent increases in food intake in *Agrp*$^{-/-}$ and wild-type

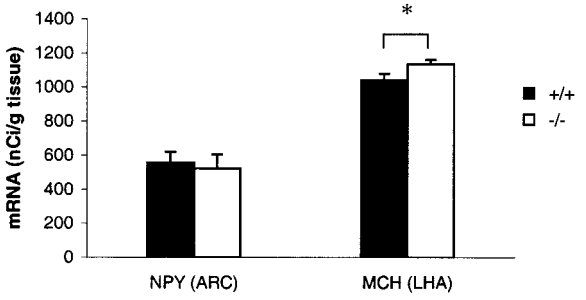

FIG. 5. Expression levels of NPY and MCH mRNAs in the hypothalamus of the wild-type and *Agrp*$^{-/-}$ littermate mice ($n = 5$ per genotype). *, $P < 0.05$ versus wild-type mice. ARC, arcuate nucleus.

littermate mice in response to NPY administration. These data imply that it is unlikely that a heightened response to NPY is compensating for AgRP deficiency.

We evaluated brain mRNA levels for NPY and several other neuropeptides and receptors implicated in the regulation of energy homeostasis in 5-month-old male animals by in situ hybridization. Quantitative analyses revealed no significant change in the level of NPY mRNA in *Agrp*$^{-/-}$ mice (Fig. 5). Interestingly, there is a small (9%) but consistent increase in MCH mRNA ($P < 0.05$; $n = 5$) in the lateral hypothalamic area of *Agrp*$^{-/-}$ mice relative to the wild-type littermate mice (Fig. 5). Although less potent than AgRP and NPY at stimulating feeding, MCH is an orexigenic peptide generally believed to act downstream of AgRP and NPY in the regulation of appetite (7, 23, 26). Although small, the observed increase in MCH mRNA may represent a form of compensation for AgRP deficiency. Significant changes in the expression level of POMC in arcuate nucleus could not be detected (1,592.3 ± 91 and 1,684.8 ± 40 nCi/g of tissue for the wild-type and *Agrp*$^{-/-}$ mice, respectively; $n = 5$ per genotype). Expression levels of Mc3r and Mc4r mRNAs in the hypothalamus and other brain regions were not altered either (data not shown).

In light of the normal response to icv administration of NPY and the lack of a detectable up-regulation in NPY mRNA in *Agrp*$^{-/-}$ mice, we generated a strain of *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice. *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice are overtly normal without discernible feeding and body weight deficits. We monitored the growth and food intake of 3-month-old *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice maintained on regular chow for a 7-week period. The growth rates and food consumption of *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice were comparable to those of *Npy*$^{-/-}$ or wild-type mice (Fig. 6).

Finally, we examined the response of *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice to 48 h of food deprivation. Similarly to *Agrp*$^{-/-}$ mice, *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice mounted a normal hyperphagic response to fasting (Fig. 7). Weight changes of *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice were also similar to those of wild-type mice during fasting and refeeding (Fig. 7). We conclude that AgRP and NPY can be abolished simultaneously without a detrimental effect on appetitive behavior.

DISCUSSION

The seemingly normal phenotype of *Agrp*$^{-/-}$ and *Agrp*$^{-/-}$;*Npy*$^{-/-}$ mice defies predictions drawn from the potent orexigenic activities of centrally administered AgRP and NPY, as

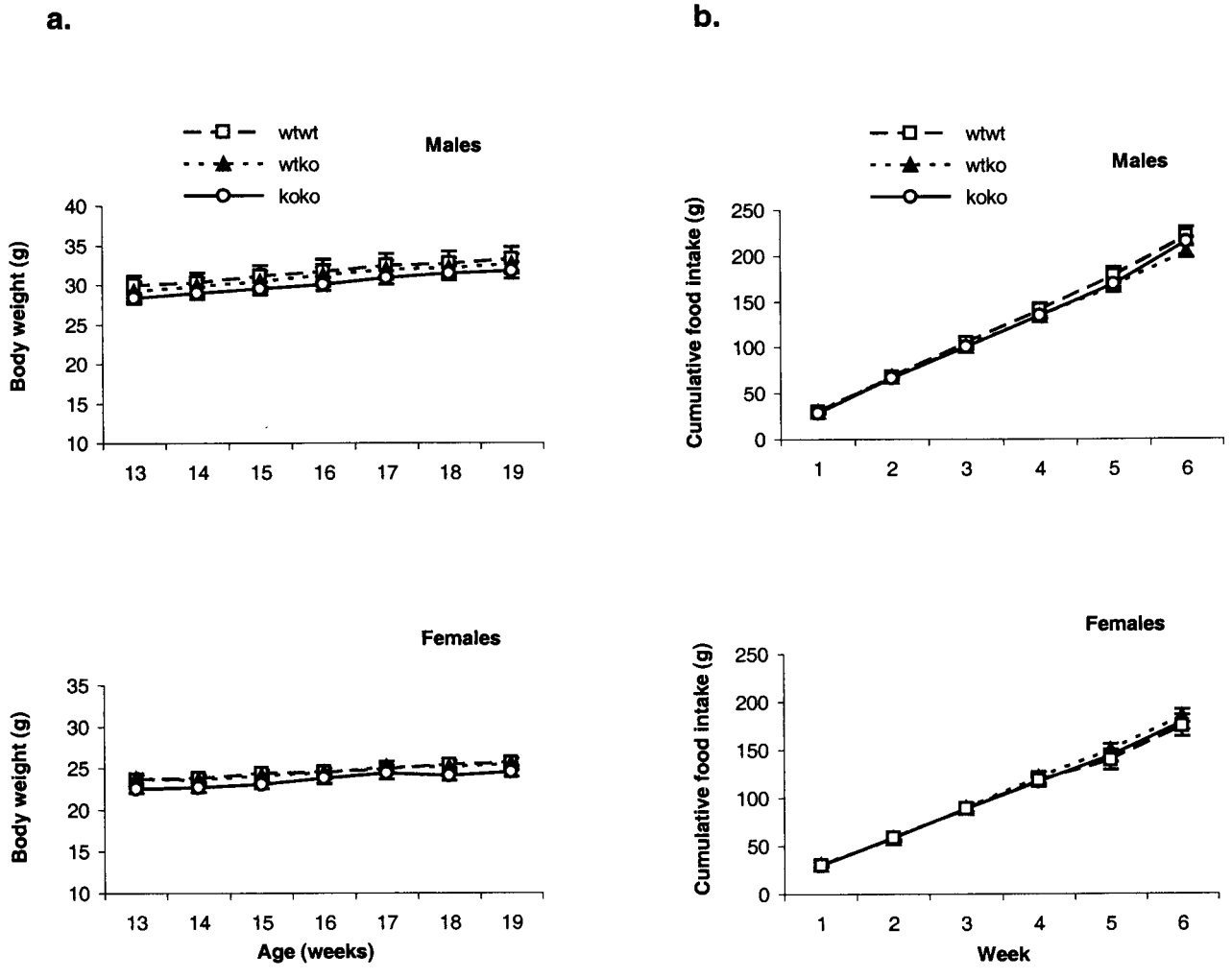

FIG. 6. Growth curves of $Agrp^{-/-}$;$Npy^{-/-}$ mice. (a) Growth curves of male and female wild-type (wtwt), $Agrp^{+/+}$;$Npy^{-/-}$ (wtko), and $Agrp^{-/-}$;$Npy^{-/-}$ (koko) mice (females, $n = 9$ to 11 per genotype; males, $n = 10$ per genotype). All mice were 3 months of age at the beginning of the study. (b) Cumulative food consumption of mice from panel a over the 7-week duration of the study.

well as from the phenotypes of agouti- and AgRP-overexpressing mouse models. Our present observations together with previously drawn conclusions from $Npy^{-/-}$ mice (8) demonstrate that AgRP and NPY are not critically required endogenous orexigenic factors. These findings raise the possibility that the behavioral activities observed in experimental settings where neuropeptides are injected icv are the result of artificial perturbations of the CNS regulation of energy homeostasis. We thus assume that it is possible that under these conditions the behaviors observed may not reflect the absolute physiological functions of the peptides administered.

On the other hand, one must consider the possibility of developmental compensation from other orexigenic pathways, which may obscure the phenotypes of knockout mice. The observed up-regulation in MCH mRNA may represent a form of such compensation. This hypothesis can be addressed by generating conditional knockout strains that inactivate *Agrp* or *Npy* at an appropriate postdevelopmental age or by generating a triple knockout for AgRP, NPY, and MCH.

An alternative form of functional compensation is an altered GABAergic tone within the AgRP-NPY neuronal circuits. $GABA_A$ receptors are expressed on arcuate AgRP-NPY neurons and interneurons projecting from the arcuate nucleus to other hypothalamic nuclei (16). The melanocortin agonists α-MSH and MTII have been shown to potentiate GABA-mediated inhibition by paraventricular hypothalamic interneurons, and AgRP and NPY can block this potentiation (6). It is conceivable that a decreased GABAergic tone could offset the loss of attenuation by AgRP and NPY on these GABA neurons. In the analysis of appetite signaling networks, $Agrp^{-/-}$ and $Agrp^{-/-}$;$Npy^{-/-}$ mice could serve as useful models in the search for compensatory pathways that might be important in regulating food intake and body weight. $Agrp^{-/-}$ mice might also be useful in identifying unforeseen physiological functions of AgRP in peripheral tissues.

Syndecan-3, a cell surface heparan sulfate proteoglycan, was recently implicated in the regulation of energy homeostasis (24). Syndecan-3 could potentiate the antagonist actions of AgRP at Mc4r, and food deprivation leads to a fourfold increase in the level of hypothalamic syndecan-3, analogous to

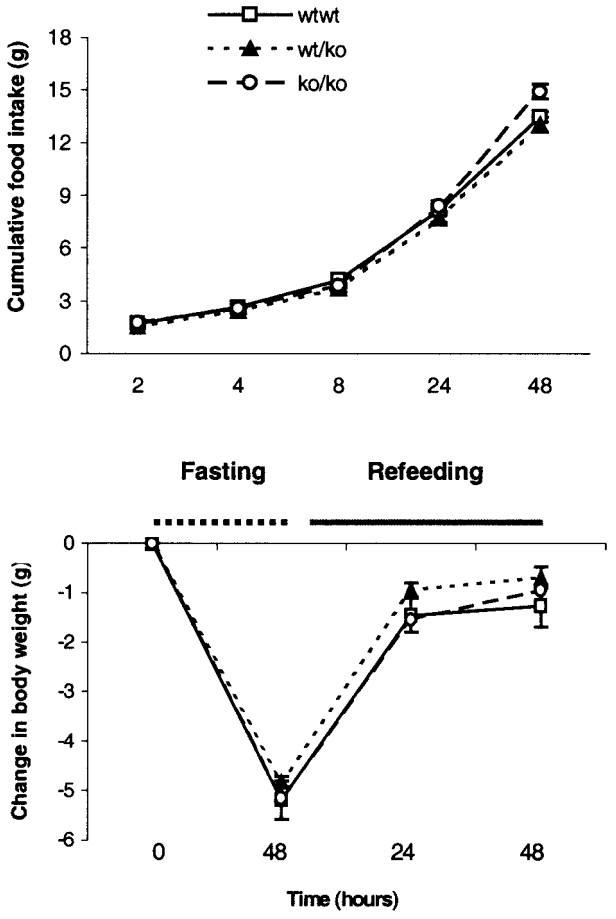

FIG. 7. $Agrp^{-/-}$;$Npy^{-/-}$ mice display a normal hyperphagic response to fasting. Six- to 7-month-old female wild-type (wtwt), $Agrp^{+/+}$;$Npy^{-/-}$ (wtko), and $Agrp^{-/-}$;$Npy^{-/-}$ (koko) mice (n = 14 per genotype) were fasted for 48 h and then refed with regular chow. Cumulative food intake following refeeding and changes in body weight during the fasting and refeeding are shown.

the fasting-induced increase in AgRP. Moreover, syndecan-3 null mice were unable to mount a rigorous reflex hyperphagia following an overnight fast (24), suggesting that a decrease in AgRP activity might underlie this phenotype. Syndecan-3 was proposed to facilitate the antagonistic actions of AgRP at Mc4r and Mc3r. The normal hyperphagic response to starvation exhibited by $Agrp^{-/-}$ mice suggests that AgRP potentiation does not account for the activity of syndecan-3 in the regulation of fasting-induced hyperphagia.

Relative to the number of reported obese mouse strains, there is a paucity of lean-mouse models resulting from inactivation of orexigenic neuropeptides. In fact, among the generally accepted orexigenic factors, only targeted deletion of pro-MCH results in hypophagia, reduced body weight, and leanness (26). The lack of anticipated feeding phenotypes when orexigenic factors are inactivated may reflect a greater degree of redundancy in pathways responsible for stimulating and sustaining feeding behavior than in pathways signaling satiety. It is possible that animals were programmed to feed continuously through early stages of evolution when food supplies were scarce. Satiety pathways may have evolved more recently with less redundancy and consequently are easier to perturb either genetically or pharmacologically.

Despite the apparent normality, it is likely that subtle changes in the $Agrp^{-/-}$ mice exist under select conditions. This is indicated by a detectable up-regulation of MCH gene expression in $Agrp^{-/-}$ mice. Our growth studies on either the $Agrp^{-/-}$ mice or $Agrp^{-/-}$;$Npy^{-/-}$ mice were moderate in duration (up to 6 months of age for $Agrp^{-/-}$ mice and up to 4.5 months of age for $Agrp^{-/-}$;$Npy^{-/-}$ mice). Subtle differences not easily identified in the present study could become apparent in long-term growth studies, or in combination with other mutations, as in the case of mice lacking both NPY and leptin (9). Nevertheless, it can be concluded that neither AgRP nor NPY is critically required for the normal regulation of energy homeostasis under the physiological conditions tested herein.

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