Melanocortin-3 Receptors Expressed on Agouti-Related Peptide Neurons Inhibit Feeding Behavior in Female Mice

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Objective: Activation of hypothalamic agouti-related peptide expressing (AgRP)+ve neurons during energy deficit is a negative valence signal, rapidly activating food-seeking behaviors. This study examined the roles of melanocortin-3 receptors (MC3Rs) coexpressed in a subpopulation of AgRP+ve neurons.

Methods: AgRP-MC3R mice expressing MC3Rs selectively in AgRP+ve neurons were generated by crossing AgRP-IRES-Cre mice with LoxTBMc3r mice containing a “loxP-STOP-loxP” sequence in the 5′ untranslated region. Body weight, body composition, and feeding behavior were assessed during ad libitum and time-restricted feeding conditions.

Results: In females, food intake of AgRP-IRES-Cre+ve (n = 7) or AgRP-IRES-Cre-ve (n = 9) mice was not significantly different; these mice were therefore pooled to form the “control” group. Female AgRP-MC3R mice exhibited lower food intake (25.4 ± 2.4 kJ/12 h; n = 6) compared with controls (35.3 ± 1.8 kJ/12 h; n = 16) and LoxTBMc3r mice (32.1 ± 2.1 kJ/12 h; n = 9) in the active phase during the dark period. Food intake during the rest phase (lights on) when mice consume less food (9-10 kJ) was normal between genotypes. Body weight and composition of AgRP-MC3R and LoxTBMc3r mice were similar, suggesting compensatory mechanisms for reduced calorie intake. Remarkably, AgRP-MC3R mice continued to consume less food during refeeding after fasting and time-restricted feeding.

Conclusions: MC3Rs expressed on AgRP+ve neurons appear to exert a strong inhibitory signal on hypothalamic networks governing feeding behavior.

Introduction

The melanocortin receptors are a family of five closely related seven-transmembrane receptors (MC1R-MC5R) that are coupled to trimeric G protein complexes, β-arrestins, and an inwardly rectifying potassium channel (1,2). High-affinity endogenous ligands for these receptors include the melanocyte-stimulating hormones (MSHs), α-, β- and γ-MSH, and the adrenocorticotropic hormone (1). These peptides are derived from the posttranslational processing of the prohormone proopiomelanocortin (POMC); they are classically defined as agonists based on cell-based receptor assays showing coupling to the G proteins containing stimulatory α subunits (Gs) to activate adenylyl cyclase and the cyclic AMP-dependent pathway (1). Two related secreted peptides, agouti signaling protein and agouti-related peptide (AgRP), act as melanocortin receptor inverse agonists/competitive antagonists. Agouti normally regulates pigmentation by regulating MC1R signaling in the dermis (1,2). AgRP expressed in the arcuate nucleus of the hypothalamus (ARC) functions as an inverse agonist/competitive antagonist for MC3R and MC4R and regulates feeding-related behaviors and hypophysiotropic and autonomic circuits that govern metabolism (1-3).

Heterogeneous populations of AgRP+ve and POMC+ve neurons have crucial opposing roles in the defense of body weight by the central nervous melanocortin system. POMC loss-of-function mutations cause hyperphagic obesity syndromes in humans (4), dogs (5), and mice (6,7). Accordingly, feeding behavior is suppressed by administration of MSH analogs to laboratory rodents (8), nonhuman primates (9,10), and humans with POMC deficiency (11). In contrast, genetic ablation of AgRP+ve neurons causes hypophagia and behavioral inflexibility during time-restricted feeding protocols (12,13). Central administration of AgRP produces lasting increases in food intake in laboratory rodents.
MC3Rs in being highly expressed on “first-order” (AgRP) neurons. The role of MC3Rs specifically constituting a heterogeneous population throughout distinct hypothalamic nuclei, including the ARC, yet the role of MC3Rs specifically expressed on POMC+ve neurons (28). It is not clear, however, whether MC3R signaling in neurons affects processes related to the control of energy balance.

To investigate functions of neural MC3Rs expressed in neuronal subpopulations, we developed a Cre-inducible rescue model (the LoxTB/Mc3r strain). Our data suggest that MC3Rs expressed in hypothalamic and limbic structures regulate behavioral adaptation to energy deficit (2). In Mc3r-deficient mice, AgRP+ve neurons exhibit a blunted increase in the expression of orexigenic neuropeptides (AgRP, NPY) during energy deficit (2). Rescuing Mc3r expression in dopaminergic neurons partially restores appetitive responses to energy deficit (29). MC3Rs expressed in Nkx2.1+ve neurons restore normal regulation of AgRP+ve neurons and motivational responses to energy deficit. Mc3r+ve;Nkx2.1+ve neurons constitute a heterogeneous population throughout distinct hypothalamic nuclei, including the ARC, yet the role of MC3Rs specifically expressed on AgRP+ve neurons has not been explored. Here, we report findings from a rescue of Mc3r expression specifically in AgRP+ve neurons that indicate an inhibitory role in feeding behavior.

The contributions of MC3Rs signaling to the defense of body weight remain poorly understood (2). In the rodent brain, Mc3r expression is confined to hypothalamic and limbic structures and also differs from MC4Rs in being highly expressed on “first-order” (AgRP) neurons rapidly increase feeding behaviors, while the release of AgRP produces a delayed long-acting response by acting on MC4R (3). Recent observations using deep-brain calcium imaging suggest that activation of AgRP+ve neurons is a negative valence signal during energy deficit, with activity rapidly suppressed by food-related cues (3).

Acute suppression of food intake and weight loss in mice induced by melanocortin analogs require functional MC4R (17-19). Unlike MSH analogs, acute orexigenic responses to AgRP may involve both MC3R and MC4R (15). In humans, MC4R haplinsufficiency associates with an early-onset hyperphagic obesity syndrome and is the most common monogenic obesity syndrome observed (20). Partially inactivating mutations in the MC3R gene also appear to associate with obesity (21-23). Genetic deletion of either Mc3r or Mc4r genes in mice produces obesity, albeit involving distinct nonredundant mechanisms (2).

Obesity models (14,15), whereas overexpression using transgenesis causes hyperphagic obesity (16). Gamma-aminobutyric acid (GABA) and neuropeptide Y (NPY) coreleased from AgRP+ve neurons rapidly increase food-seeking behaviors, while the release of AgRP produces a delayed long-acting response by acting on MC4R (3). Recent observations using deep-brain calcium imaging suggest that activation of AgRP+ve neurons is a negative valence signal during energy deficit, with activity rapidly suppressed by food-related cues (3).

Methods
Experiments involving mice were performed in accordance with the guidelines and regulations provided by the Institutional Animal Care and Use Committee of the Scripps Research Institute, which reviewed and approved the studies.

Transgenic mouse models
The development and characterization of the C57BL/6J (B6) Mc3r^{TB/+} mouse model (also known as LoxTB/Mc3r or Mc3r^{tm1Butl/J}) have been described previously (29-31). In this strain, Mc3r expression is inhibited by insertion of a loxP-flanked transcription block (loxP-STOP-loxP) cassette into the 5’ untranslated region. Homozygous carriers of the null allele (Mc3r^{TB/TB}) exhibit a nutrient-partitioning phenotype reported in earlier experiments in which the Mc3r locus was replaced with a neomycin-selection cassette (31-33).

AgRP-IRES-Cre^{+ve} mice were crossed onto the B6(Cg)-Mc3r^{tm1Butl/J} (LoxTB/Mc3r) strain. Animal husbandry and genotyping followed established protocols (34). For breeding, heterozygous carriers of the null Mc3r allele (Mc3r^{TB/+}) and the AgRP-IRES-Cre transgenic females (AgRP-IRES-Cre;Mc3r^{TB/+}) were bred with Mc3r^{TB/+} males to produce AgRP-IRES-Cre;Mc3r^{TB/+} mice expressing MC3Rs only on NPY/AgRP/GABA neurons (AgRP-MC3R). All mice studied were littersmates obtained from breeding heterozygotes. Genotyping by polymerase chain reaction using tail-tip DNA also allowed us to assess germine recombination. Out of 187 pups (88 male, 99 female) generated, 20 Mc3r^{WT/WT} (9 male, 11 female), 28 AgRP-IRES-Cre;Mc3r^{WT/WT} (18 male, 10 female), 23 Mc3r^{TB/TB} (12 male, 11 female), 9 AgRP-IRES-Cre;Mc3r^{TB/TB} (AgRP-MC3R, 3 male, 6 female), and 17 AgRP-IRES-Cre;Mc3r^{TB/Δ} (10 male, 7 female) were obtained. Animals showing Cre-mediated recombination in the tail (AgRP-IRES-Cre;Mc3r^{TB/Δ}), and thus exhibiting recombination outside the ARC, were removed from the study.

In situ hybridization
Targeting of Mc3r expression in the ARC was confirmed using in situ hybridization (ISH) as previously described (29). Briefly, coronal sections (20 μm) cut on a cryostat were thaw-mounted onto Superfrost Plus slides (VWR Scientific, West Chester, Pennsylvania). Hypothalamic sections were collected in a 1:6 series from the diagonal band of Broca (bregma −0.50 mm) caudally through the mammillary bodies (bregma −5.00 mm). Antisense 33P-labeled rat Mc3r riboprobe (corresponding to bases 808-1,204; GenBank accession number NM_008561.3) (0.2 pmol/mL) was denatured, dissolved in hybridization buffer along with transfer RNA (1.7 mg/mL), and applied to slides. Controls used to establish the specificity of the Mc3r riboprobe included slides incubated with an equivalent concentration of radiolabeled sense Mc3r riboprobe or radiolabeled antisense probe in the presence of excess (1,000x) unlabeled antisense probe. Slides were covered with glass cover slips, placed in a humid chamber, and incubated overnight at 55°C. The following day, slides were treated with RNase A and washed under conditions of increasing stringency. Slides were dipped in 100% ethanol, air-dried, and then dipped in NTB-2 liquid emulsion (Eastman Kodak Co.). Slides were developed 16 days later and covered with glass cover slips.
Analysis of body weight and composition
Mice were weighed at weaning (25 days of age) and then once a week starting at 5 weeks of age until 13 weeks of age. Nuclear magnetic resonance (Bruker Minispec; Bruker, Billerica, Massachusetts) was used to measure fat mass (FM), fat-free mass (FFM), and free H2O in 12-week-old mice.

Analysis of feeding behavior
Feeding behavior was examined in 18-week-old female mice using an automated system for continuous monitoring of food consumption (BioDAQ version 2.3; Research Diets, Inc., New Brunswick, New Jersey), as previously described (30). Mice were acclimated to single housing on bedding with no caloric value (alpha cellulose) and a refined diet (Research Diets 12450; 70% kJ carbohydrates, 10% kJ fats, and 20% kJ protein) for 2 weeks. This diet has been previously used in our studies examining feeding behavior of Mc3r-deficient mice (2) and was again used for consistency.

After acclimation, mice were transferred to BioDAQ cages. After 3 days of acclimation, habitual feeding behavior was established using 2 days of recordings. On day six, food access was removed. Starting the following day, mice were then granted food access daily for 4 hours between zeitgeber time (ZT)4 and ZT8 (ZT0 and ZT12 represent, respectively, times of dark/light and light/dark transition).

At the end of the experiment, mice were euthanized, and their brains were collected, frozen on dry ice, and stored at −80°C until further processing for ISH.

For meal structure, “bouts” indicate disturbance of the hopper and instability in scale readings suggesting approach and investigation; actual changes in food weight were used to estimate meal size. Meals were defined as bouts occurring within 5 minutes of each other resulting in the consumption of ≥ 0.02 g of food.

Statistical analysis
Data were analyzed in SPSS Statistics version 23 (IBM Corp., Armonk, New York). The effect of genotype on body composition was assessed by analysis of covariance (ANCOVA), with genotypes (Mc3r, AgRP-IRES-Cre) as fixed variables and total body mass as a covariate. FM, FFM, and free H2O are presented as estimated marginal means adjusted for total body mass unless stated otherwise. The impact of genotype on weight loss during time-restricted feeding was also assessed using two-way ANCOVA, with baseline body weight and age used as covariates. The Mc3r genotype had a highly significant effect (P < 0.001) on relative FM (increased) and FFM (reduced) in males (Figure 2C) and females (Figure 2D). Interestingly, expression of the AgRP-IRES-Cre transgene appears to affect nutrient partitioning (Figure 2C-2D), with highly significant differences in relative FM (P < 0.001) and FFM (P = 0.001). There was, however, no interaction between Mc3r and the AgRP-IRES-Cre genotype in either sex. Restoring Mc3r expression in

AgRP-MC3R and Mc3r<sup>TB/TB</sup> mice exhibit similar body composition
Body weights were recorded weekly after weaning of male (Figure 2A) and female mice (Figure 2B). Mc3r<sup>TB/TB</sup> mice exhibited increased weight gain compared with mice with normal MC3R signaling around 7 to 8 weeks of age. Restoring ARC Mc3r expression had no effect on obesity due to Mc3r deficiency (Figure 2A-2B). Mc3r<sup>TB/TB</sup> mice exhibited the expected partitioning phenotype observed with loss of MC3Rs in both males (Figure 2C) and females (Figure 2D). Body composition within sex was analyzed using ANCOVA, with genotypes (Mc3r, AgRP-IRES-Cre) as fixed variables and total body mass as a covariate. The Mc3r genotype had a highly significant effect (P < 0.001) on relative FM (increased) and FFM (reduced) in males (Figure 2C) and females (Figure 2D). Interestingly, expression of the AgRP-IRES-Cre transgene appears to affect nutrient partitioning (Figure 2C-2D), with highly significant differences in relative FM (P < 0.001) and FFM (P = 0.001). There was, however, no interaction between Mc3r and the AgRP-IRES-Cre genotype in either sex. Restoring Mc3r expression in
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ARC AgRP+ve neurons thus does not appear to be sufficient to rescue the nutrient-partitioning phenotype associated with Mc3r deficiency.

**AgRP-MC3R mice exhibit reduced food intake during the dark period**

We next examined the response of female AgRP-MC3R mice to restricted feeding paradigms. The goal of the experiment was to determine whether MC3Rs expressed on AgRP+ve neurons rescue impaired adaptation to restricted feeding previously observed in global Mc3r-deficient mice (2). Females were used for the experiment owing to the small numbers of males obtained during breeding.

Mice were first acclimated to single housing in the BioDAQ, and 2 days of baseline data were collected in the ad libitum feeding condition. The average age of the mice at the start of the experiment was 18 weeks (mean 17.7 weeks, SD 0.9 weeks), with a 3.7-week range (minimum 15.7 weeks, maximum 19.4 weeks). Analysis of total body weight used ANCOVA to compare the AgRP-IRES-Cre genotype and the Mc3r genotype and controlled for differences in age. Age as a covariate was a significant predictor of body weight (P < 0.05). There was no significant effect of the AgRP-IRES-Cre genotype (estimate marginal means for age-adjusted body weight for AgRP-IRES-Cre-ve mice, 23.5 ± 0.7 g, n = 18; for AgRP-IRES-Cre+ve mice, 23.6 ± 0.8 g, n = 13; P = 0.896). As predicted, there was a highly significant (P < 0.001) effect of the Mc3r genotype (Mc3rWT/WT, 21.0 ± 0.7 g, n = 16; Mc3rTB/TB, 26.1 ± 0.7 g, n = 15). There was no interaction between the AgRP-IRES-Cre and Mc3r genotype (P = 0.548) (WT, 21.3 ± 1.0 g, n = 9; Mc3rTB/TB, 25.7 ± 1.0 g, n = 9; AgRP-IRES-Cre, 20.7 ± 1.1 g, n = 7; AgRP-MC3R, 26.5 ± 1.2 g, n = 7).

AgRP-MC3R mice exhibited a feeding phenotype in the ad libitum feeding condition (Figure 3). Food intake was not significantly affected by the AgRP-IRES-Cre genotype (Figure 3A); therefore, we pooled data from WT B6 mice with AgRP-IRES-Cre mice into a single control.
group. Food intake averaged over 2 days was significantly affected by genotype ($P<0.05$) (Figure 3B). Post hoc analysis indicated that intake in kilojoules per day was significantly lower in AgRP-MC3R mice compared with controls ($P<0.01$) and $Mc3r^{TB/TB}$ mice ($P<0.05$). This difference was due primarily to differences of food intake during the dark period, when mice consumed 70% to 80% of their daily intake (Figure 3B). Food intake in the dark was significantly affected by genotype ($P<0.05$). AgRP-MC3R mice consumed significantly less than controls ($P<0.01$), whereas there was a tendency ($P=0.069$) for intake to be lower in AgRP-MC3R compared with $Mc3r^{TB/TB}$ mice. Food intake during the light period was not significantly affected by genotype.

Analysis of meal structure suggests that genotype had no effect during the lights-on period (Figure 4A, 4C, 4E) but was different during the dark period (Figure 4B, 4D, 4F). Meal frequency was not affected by genotype, irrespective of the time of day (Figure 4A-B). However, meal size was significantly affected by genotype in the dark period ($P<0.05$), with AgRP-MC3R mice exhibiting significantly smaller meals compared with controls (Figure 4D). Meal duration was not significantly affected by genotype, irrespective of the time of day (Figure 4E-F).

Reduced food intake in AgRP-MC3R mice during time-restricted feeding

We next subjected mice to a time-restricted feeding protocol, limiting food access to a 4-hour window in the lights-on period. Again, there was no significant effect of the AgRP-IRES-Cre genotype on feeding (Figure 5A), so the control group includes WT and AgRP-IRES-Cre$^{+ve}$ mice. The data recorded on the first day of time-restricted feeding are equivalent to the initial feeding phase of a fasting–refeeding study. A marked reduction of food intake was evident in AgRP-MC3R mice on day one (Figure 5B). This was mostly due to low intake during the latter stages of feeding, with intake being normal in the first 30 minutes when mice are beginning to gorge (Supporting Information Figure S2A).

As previously observed (36), $Mc3r^{TB/TB}$ mice exhibited impaired adaptation resulting in less calorie intake during the 4 hours food was available during the later days of the time-restricted feeding protocol (Figure 5B). AGRP-MC3R mice exhibited a more severe phenotype (Figure 5B). Repeated-measures analysis with genotypes (control, $Mc3r^{TB/TB}$, or AgRP-MC3R) as fixed variables indicated a significant effect of repeated exposure to time-restricted feeding ($P<0.001$), with mice adapting by increasing food consumption during the 4-hour period over the 4 days of the study. There was a significant interaction between time and genotype ($P<0.01$); pairwise comparisons indicated that all three genotypes differed significantly (control vs. $Mc3r^{TB/TB}$, AgRP-MC3R: $P<0.001$; $Mc3r^{TB/TB}$ vs. control, AgRP-MC3R: $P<0.01$). Lower food intake of AgRP-MC3R mice appears to be due to reduced meal size, with no significant differences in frequency or duration (Supporting Information Figure S2B-S2D).

All mice lost weight during time-restricted feeding (grand mean of weight loss in grams adjusted for baseline body weight and age, $1.9 \pm 0.1$ g). In a two-way analysis of variance (ANOVA) with the $Mc3r$ and AgRP-IRES-Cre genotype as fixed variables, there was a significant effect of the $Mc3r$ genotype (estimated marginal means adjusting for baseline body weight and age for weight loss of $Mc3r^{WT/WT}$, 1.4 ± 0.2 g, n = 17; $Mc3r^{TB/TB}$, 2.3 ± 0.2 g, n = 16, $P<0.005$) but not

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**Figure 4** Analysis of meal structure in female wild-type (WT), $Mc3r^{TB/TB}$, and AgRP-MC3R mice under ad libitum feeding. Meal-pattern data shown are 12-hour day and 12-hour night averages; baseline data are averaged over 2 days after 3-day acclimation. (A,B) Meal frequency, (C,D) meal size, and (E,F) meal duration were averaged from ad libitum feeding data recorded on (A,C,E) days four and five and (B,D,F) nights four and five, presented as mean ± SEM (black bars = WT mice, n = 16; orange bars = $Mc3r^{TB/TB}$ mice, n = 9; green bars = AgRP-MC3R, n = 6). One-way ANOVA indicated significant effects on nighttime food intake and meal size (all $P<0.05$). Differences between groups were assessed by Dunn post hoc analysis. *$P<0.05$ compared with controls.
of the AgRP-IRESCre genotype (AgRP-IRESCre mice, 1.8 ± 0.2 g, n = 18, AgRP-IRESCre mice, 2.0 ± 0.2 g, n = 15). When grouped by genotype (WT, AgRP-IRESCre mice, Mc3rTB/TB, and AgRP-MC3R), AgRP-MC3R appear to lose more weight compared with Mc3rTB/TB mice (2.5 ± 0.3 vs. 2.1 ± 0.2 g, n = 6 and 10, respectively), consistent with lower food intake of the former. However, there was no statistically significant interaction between the AgRP-IRESCre and Mc3r genotype when compared using a two-way ANCOVA.

Discussion

It is widely accepted that fasting instigates changes in the internal milieu that are powerful stimuli for appetite and that these responses hinder our ability to voluntarily lose weight. Previous research by our laboratory using the LoxTBMc3r mouse model indicates that MC3Rs expressed on "second-order" neurons in the ventral tegmental area (29) represent a subset with unique physiological functions, are needed. Also required are studies examining whether similar phenotypes are observed with restoring MC3R signaling on body weight and composition is not consistent with this outcome. Compensatory mechanisms involving reduced energy expenditure must therefore be considered.

MC3R signaling in AgRP+ve neurons may affect feeding through other signaling pathways not involving Gs. For example, MC3Rs are coupled to the β-arrestin signaling pathway (41). Another example is the inhibition of excitatory VMH neurons by AgRP that may involve a Gi-coupled mechanism (42). Indeed, recent data suggest that biased agonism plays an important role in defining the actions of MC4R agonists (43). It is, however, also important to consider that studies using Designer Receptors Exclusively Activated by Designer Drugs or Designer Receptors Exclusively Activated by Designer Drugs or optogenetics are indiscriminate in affecting the activity of AgRP+ve neurons, and their exact physiological significance is therefore open for debate. ARCAgRP+ve neurons are a functionally heterogeneous population. When clustered according to their area of projection, not all subpopulations are able to elicit feeding following optogenetic stimulation (44). It is therefore important to consider the possibility that MC3Rs are expressed by a subset of AGRP neurons that primarily influence feeding behavior. Further studies to define the population of AgRP+ve;Mc3r+ve neurons, and determining whether they represent a subset with unique physiological functions, are needed. Also required are studies examining whether similar phenotypes are observed with restoring MC3R signaling in AgRP+ve neurons early in development or in mature mice using inducible systems.

The central nervous melanocortin system is a crucial focal point in the neural networks that regulate feeding behavior, energy expenditure, and the partitioning of nutrients between lean and adipose tissues (2). Lesions in the basal hypothalamus induce hyperphagia and the preferential partitioning of nutrients into adipose tissue (45). Loss of MC3Rs partially recapitulates this phenotype, producing a nutrient-partitioning phenotype (2). However, our recent results suggest that the actions of neural MC3Rs do not appear to contribute directly to hypothalamic obesity syndromes.

Overall, a functional divergence appears to exist between MC3Rs expressed on “second-order” neurons in the ventral tegmental area (29) and VMH (31). Ventral tegmental area MC3Rs support the expression of feeding-related motivational responses during situations of energy deficit (29). MC3R signaling in Nkx2.1+ve neurons in the hypothalamus also supports behavioral adaptation to energy deficit (30). MC3R signaling in the midbrain (37). Further studies examining responses of AgRP+ve neurons in AgRP-MC3R mice to energy deficit are needed. It is worth noting that the phenotype of AgRP-MC3R mice is remarkable given that AGRP neurons in the ARC of Mc3r-deficient mice already exhibit suppressed activity (2).

It is also important to point out another limitation to this study, which is the small sample size for studies using male mice. Results from males should therefore be viewed with caution, with further studies needed to examine whether a similar phenotype in AgRP-MC3R mice is observed.

Activation of ARC AgRP+ve neurons using optogenetics or Designer Receptors Exclusively Activated by Designer Drugs or Designer Receptors Exclusively Activated by Designer Drugs or Designer Receptors in the ARC have an inhibitory impact on feeding behavior in female mice during situations in which food is freely available and in situations of acute negative balance. Ablation of AgRP+ve neurons or deficits in energy signaling by AgRP+ve neurons influence the setting of dopamine neurons in the ARC (2). Overall, a functional divergence appears to exist between MC3Rs expressed on “second-order” neurons in the ventral tegmental area (29) and VMH (31). Ventral tegmental area MC3Rs support the expression of feeding-related motivational responses during situations of energy deficit (29). MC3R signaling in Nkx2.1+ve neurons in the hypothalamus also supports behavioral adaptation to energy deficit (30). MC3R signaling in the midbrain (37). Further studies examining responses of AgRP+ve neurons in AgRP-MC3R mice to energy deficit are needed. It is worth noting that the phenotype of AgRP-MC3R mice is remarkable given that AGRP neurons in the ARC of Mc3r-deficient mice already exhibit suppressed activity (2).

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in the nervous system thus appears to be predominantly orexigenic, particularly during situations of energy deficit. This is in marked contrast to MC4Rs, which appear to be required for satiety (17-19). The identity and mechanisms of hypothalamic MC3R+ve neurons that support behavioral adaptation to energy deficit have not been established. MC3R signaling in steriodogenic factor-1 neurons in the VMH improves peripheral glucose and lipid metabolism but does not restore food-seeking behaviors in situations of negative energy balance (31). The current study indicates that MC3R signaling in AgRP+ve neurons is also not sufficient to drive food-seeking behaviors in situations of negative energy balance. In summary, the current results indicate that MC3R signaling in AgRP+ve neurons appears to have an inhibitory role in regulating feeding behavior. When compared with previous studies using this model (2,9,31), there appears to be functional divergence between MC3Rs expressed on first-order and second-order neurons of the central nervous melanocortin system. MC3Rs expressed on first-order AgRP+ve neurons are inhibitory. On the other hand, MC3R expression on second-order neurons receiving inputs from AgRP+ve and POMC+ve neuronal projections appears to support expression of feeding behaviors. Further studies are needed to examine the functions of MC3R expression on first-order POMC+ve neurons and to identify MC3R+ve neurons that are critical for supporting the expression of appetite responses to negative energy balance.

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