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Melanocortin-3 receptors expressed on agouti-related peptide neurons inhibit feeding behavior in female mice.

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Abstract

Objective—Activation of hypothalamic agouti-related peptide (AgRP)$^{+ve}$ neurons during energy deficit is a negative valence signal, rapidly activating food seeking behaviors. This study examined the roles of MC3Rs expressed by *AgRP*$^{+ve}$ neurons.

Methods—AgRP-MC3R mice expressing MC3Rs selectively in *AgRP*$^{+ve}$ neurons were generated by crossing *AgRP-IRES-Cre* mice with LoxTB*Mc3r* mice containing a "loxP-STOP-loxP" sequence in the 5' UTR. Body weight, body composition and feeding behavior were assessed during *ad libitum* and timed-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/12h, n=6) compared to controls (35.3±1.8 kJ/12h, n=16) and LoxTB*Mc3r* mice (32.1±2.1 kJ/12h, 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 LoxTB*Mc3r* mice was similar, suggesting compensatory mechanisms for reduced calorie intake. Remarkably, AgRP-MC3R mice continued to consume less food during re-feeding after fasting and timed-restricted feeding.

Conclusions—MC3Rs expressed on *AgRP*$^{+ve}$ neurons appear to exert a strong inhibitory signal on hypothalamic networks governing feeding behavior.

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CG and AAB conceived the experiments. CG contributed materials for the collection of data, performed experiments, and analyzed and interpreted data. DLM contributed materials for the collection of data, assisted with data analysis and interpretation, and reviewed the manuscript. AAB contributed materials for data collection, assisted with data interpretation, and prepared the first draft of the manuscript. AAB had full access to the data and takes responsibility for the integrity and accuracy of the analysis.

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Keywords

Appetite Regulation; Hypothalamic arcuate nucleus; Neuropeptide; Energy Balance

INTRODUCTION

The melanocortin receptors are a family of five closely-related 7 transmembrane receptors (MC1–5R) that are coupled to trimeric G protein complexes, β−arrestins, and an inwardly-rectifying potassium channel (K_{ir}7.1) (1, 2). High affinity endogenous ligands for these receptors include the melanocyte-stimulating hormones (MSH): α−, β− and γ−MSH, and adrenocorticotropic hormone (ACTH) (1). These peptides are derived from the post-translational processing of the precursor polypeptide 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 cAMP-dependent pathway (1). Two related secreted peptides, agouti signaling protein (ASIP) 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 timed-restricted feeding protocols (12, 13). Central administration of AgRP produces lasting increases in food intake in laboratory rodent models (14, 15), while over expression using transgenesis causes hyperphagic obesity (16). Co-release of GABA and neuropeptide Y (Npy) from *AgRP*$^{+ve}$ neurons rapidly increases food-seeking behaviors, while actions involving the release of AgRP and MC4R produce a delayed long-acting response (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 requires functional MC4R (17–19). Unlike MSH analogs, acute orexigenic responses to AgRP may involve both MC3R and MC4R (15). In humans, *MC4R* haploinsufficiency 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 non-redundant mechanisms (2).

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The contribution of MC3R signaling to the defense of body weight by the central nervous melanocortin system remains 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*$^{+ve}$ or *POMC*$^{+ve}$) ARC neurons (24–27). This has led to speculation that MC3Rs expressed on first order ARC neurons have autoreceptor (or autoinhibitory) roles, a conclusion supported by early electrophysiological analysis of ARC *POMC*$^{+ve}$ neurons (28). Whether MC3R signaling in *AgRP*$^{+ve}$ neurons affect processes related to the control of energy balance during times when the release of α−MSH from *POMC*$^{+ve}$ neurons is enhanced during situations of positive energy balance, or the release of AgRP is increased during situation of negative energy balance, is not clear.

To investigate functions of neural MC3Rs expressed in neuronal subpopulations, we developed a Cre-inducible rescue model (the LoxTB*Mc3r* mouse). Our data suggest 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.

MATERIALS AND METHODS

Experiments involving mice were performed in accordance to 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*$^{TB/TB}$ mouse model (also known as *LoxTBMc3r* or *Mc3r*^{tm1Butl}/J) was 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'UTR. 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$^{tm1(cre)Lowl}$/J transgenic mice (*AgRP-IRES-Cre*) have an internal-ribosomal-entry-site/Cre construct (IRES-Cre) inserted in exon 3 of the *AgRP* gene (34). These mice were originally derived from electroporated W4-derived 129S6/SvEvTac embryonic stem cells. Chimeric males were bred to either B6 × 129 or FVB females. Offspring carrying the modified *AgRP* allele were then mated with 129S4/SvJae-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/J mice carrying a flp-recombinase gene to delete a kan/neo cassette in the targeting vector; progeny were further crossed to remove the Flp-expressing mutation, producing heterozygous carriers of the *AgRP-Ires-cre* allele. These mice were bred to C57BL/6J inbred

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mice at the Jackson Laboratory to establish the colony. Upon arrival in our facility, the mice were crossed a minimum of 2 generations onto the B6 background. The genetic background of the AgRP-MC3R and littermates (wild type, *AgRP-IRES-Cre*$^{+ve}$, *Mc3r*$^{TB/TB}$) used for the current study is thus considered to be at least 87.5% B6.

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 transgene females (*AgRP-IRES-Cre;Mc3r*$^{TB/+}$) were bred with *Mc3r*$^{TB/+}$ males to produce *AgRP-IRES-Cre;Mc3r*$^{TB/TB}$ mice expressing MC3Rs only on Npy/AgRP/GABA neurons (AgRP-MC3R). All mice studied were littermates obtained from breeding heterozygotes. Genotyping PCR using tail-tip DNA was used to assess germline 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*$^{TB/\Delta}$ (10 male/7 female) were obtained. Animals showing Cre-mediated recombination in the tail (*AgRP-IRES-Cre;Mc3r*$^{TB/\Delta}$), and hence 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, PA). 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 ^{33}P-labeled rat *Mc3r* riboprobe (corresponding to bases 808–1204; GenBank accession number NM_008561.3) (0.2 pmol/ml) was denatured, dissolved in hybridization buffer along with tRNA (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 (1000×) unlabeled antisense probe. Slides were covered with glass coverslips, 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 coverslips.

Analysis of body weight and composition.

Mice were weighed at weaning (25 days of age) and then once a week starting at 5 wk of age until 13 wk of age. Nuclear magnetic resonance (NMR, Bruker Minispec) was used to measure fat mass (FM), fat-free mass (FFM) and free H_2O in 12 wk-old mice.

Analysis of feeding behavior.

Feeding behavior was examined in 18 wk old female mice using an automated system for continuous monitoring of food consumption (BiodaQ 2.3, Research Diets Inc., New Brunswick, NJ) and BiodaQ 2.3 software, as previously described (30). Mice were

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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 wk. This diet has been previously by used in our studies examining feeding behavior of *Mc3r*-deficient mice (2), and was again used for this study for consistency.

After acclimation, mice were transferred to BiodaQ cages. After 3d of acclimation, habitual feeding behavior was established using 2d of recordings. On the 6th day, food access was removed. Starting the following day, mice were then granted food access daily for 4 hours between ZT4 and ZT8 (ZT, zeitgeber time; ZT 0 and ZT12 represent respectively times of dark/light and light/dark transition).

At the end of the experiment, mice were euthanized and their brains 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 vers. 23. The effects of genotype on body composition was assessed by ANCOVA with genotypes (*Mc3r, AgRP-IRES-Cre*) as fixed variables and total body mass as a covariate. FM, FFM and free H_2O 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 2-way ANCOVA with baseline body weight and age used as covariates. Food intake data is presented as kJ per mouse.

RESULTS

Measurement of Mc3r expression in AgRP-MC3R mice.

Subpopulations of ARC melanocortin neurons exhibit *Mc3r* mRNA expression as assessed by ISH (26) and single-cell RNA-seq (35). In the current study, ISH was used to compare expression of *Mc3r* mRNA in nuclei known to exhibit robust expression (ARC, ventromedial hypothalamus and habenula) (25). AgRP-MC3R mice exhibited robust *Mc3r* expression in the ARC (Fig. 1A), but not in the ventromedial hypothalamus (VMH, Fig. 1B) or habenula (Fig. 1C). No signal was observed in *Mc3r*$^{TB/TB}$ mice (Suppl. Fig. S1).

AgRP-MC3R and Mc3r$^{TB/TB}$ mice exhibit similar body composition.

Body weights were recorded weekly after weaning of male (Fig. 2A) and female mice (Fig. 2B). *Mc3r*$^{TB/TB}$ mice exhibited increased weight gain compared to mice with normal Mc3r signaling around 7–8 wk of age. Restoring ARC *Mc3r* expression had no effect on obesity due to *Mc3r*-deficiency (Fig. 2A, B). *Mc3r*$^{TB/TB}$ mice exhibited the expected partitioning phenotype observed with loss of MC3R in both males (Fig. 2C) and females (Fig. 2D). Analysis of body composition within sex used ANCOVA used genotype (*Mc3r*, *AgRP-IRES-Cre*) as fixed variables and total body weight as a covariate. *Mc3r* genotype had a

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highly significant effect (p<0.001) on relative FM (increased) and FFM (reduced) in males (Fig. 2C) and females (Fig. 2D). Interestingly, expression of the *AgRP-IRES-Cre* transgene appears to affect nutrient partitioning (Fig. 2C, D), with highly significant differences in relative FM (p<0.001) and FFM (p=0.001). There was however no interaction between *Mc3r* and *AgRP-IRES-Cre* genotype in either sex. Restoring *Mc3r* expression in 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 MC3R 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 collected in *ad libitum* feeding condition. The average age of the mice at the start of the experiment was 18 wk (mean, 17.7wk; std. deviation, 0.9 wk), with a 3.7 wk range (minimum 15.7wk; maximum, 19.4 wk). Analysis of total body weight used ANCOVA to compare *AgRP-IRES-Cre* genotype and *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 *AgRP-IRES-Cre* genotype (estimate marginal means for age-adjusted body weight for $AgRP\text{-}IRES\text{-}Cre^{-ve}$ mice, 23.5±0.7g, n=18; for $AgRP\text{-}IRES\text{-}Cre^{+ve}$ mice, 23.6±0.8g, n=13, p=0.896). As predicted, there was a highly significant (p<0.001) effect of $Mc3r^{TB}$ genotype ($Mc3r^{WT/WT}$, 21.0±0.7g, n=16; $Mc3^{TB/TB}$, 26.1±0.7g, n=15). There was no interaction between *AgRP-IRES-Cre* and *Mc3r* genotype (p=0.548) (wild type, 21.3±1.0g, n=9; $Mc3^{TB/TB}$, 25.7±1.0,n=9; *AgRP-IRES-Cre*, 20.7±1.1, n=7; AgRP-MC3R, 26.5±1.2, n=7).

AgRP-MC3R mice exhibited a feeding phenotype in the *ad libitum* feeding condition (Fig. 3). Food intake was not significantly affected by *AgRP-IRES-Cre* genotype (Fig. 3A), we therefore pooled data from “wild type” 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) (Fig 3B). *Post hoc* analysis indicated that intake in kJ/d was significantly lower in AgRP-MC3R mice compared to 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 70–80% of their daily intake (Fig. 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), while there was a tendency (p=0.069) for intake to be lower in AgRP-MC3R compared to $Mc3r^{TB/TB}$ 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 (Fig. 4A,C,E), but was different during the dark period (Fig. 4B, D, F). Meal frequency was not affected by genotype, irrespective of the time of day (Fig. 4A, B). However, meal size was significantly affected by genotype in the dark period (p<0.05), with AgRP-MC3R mice

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exhibiting significantly smaller meals compared to controls (Fig 4D). Meal duration was not significantly affected by genotype, irrespective of the time of day (Fig. 4E, F).

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

We next subjected mice to a timed restricted feeding protocol, limiting food access to a 4h window in the lights-on period. Again, there was no significant effect of the *AgRP-IRES-Cre* genotype on feeding (Fig. 5A), so the control group includes wild type and *AgRP-IRES-Cre*$^{+ve}$ mice. The data recorded on the first day RF is equivalent to the initial phase of a fasting-refeeding study. A marked reduction of food intake was evident in AgRP-MC3R mice on day 1 (Fig. 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 being to gorge (Suppl. Figure S2A).

As previously observed (36), *Mc3r*$^{TB/TB}$ mice exhibit impaired adaptation resulting in less calorie intake during the 4h during which food is available during the later days of the timed restricted feeding protocol (Fig. 5B). AGRP-MC3R mice exhibit a more severe phenotype (Fig. 5B). Repeated measures analysis with genotype (control, *Mc3r*$^{TB/TB}$ or AgRP-MC3R) as fixed variables indicated a significant effect of time ($p<0.001$), with mice adapting by increasing food consumption during the 4h period over the 4 days of the study. There was a significant interaction between time and genotype ($p<0.01$); pairwise comparisons indicate 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 (Suppl. Figure S2B–D).

All mice lost weight during timed-restricted feeding (grand mean of weight loss in g adjusted for baseline body weight and age, 1.9±0.1g). There was a significant effect of *Mc3r* genotype (estimated marginal means adjusting for baseline body weight and age for weight loss of *Mc3r*$^{WT/WT}$, 1.4±0.2g; *Mc3r*$^{TB/TB}$, 2.3±0.2g, $p<0.005$), but not of *AgRP-IRES-Cre* genotype (*AgRP-IRES-Cre*$^{-ve}$ mice, 1.8±0.2g, *AgRP-IRES-Cre*$^{+ve}$ mice, 2.0±0.2g). AgRP-MC3R appeared to lose more weight compared to *Mc3r*$^{TB/TB}$ mice (2.5±0.3 vs. 2.1±0.2g), consistent with lower food intake of the former. However, there was no statistically significant interaction between *AgRP-IRES-Cre* and *Mc3r* genotype when compared using a 2-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 LoxTB*Mc3r* mouse model indicated MC3Rs expressed in neuronal subpopulations found in hypothalamic and limbic structures regulate behavioral adaptation to energy deficit (2). There are two primary outcomes for the current study. First, selectively restoring MC3R signaling in *AgRP*$^{+ve}$ neurons in the ARC is not sufficient to support behavioral adaptation to energy deficit. Second, the current data strongly suggest that MC3R signaling in *AgRP*$^{+ve}$ neurons has an *inhibitory* impact on

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feeding behavior in female mice during situations when food is freely available, and in situations of acute and chronic negative balance.

MC3R signaling in *AgRP*$^{+ve}$ neurons may regulate feeding behavior via direct or indirect mechanisms. MC3Rs signaling could prevent activation *of AgRP*$^{(+ve)}$ neurons by signals of energy balance observed in response to internal signals of energy deficit (3). Alternatively, MC3R signaling in *AgRP*$^{+ve}$ neurons could alter the response of midbrain reward circuits to energy deficit. Ablation of *AgRP*$^{+ve}$ neurons or deficits in energy sensing by AGRP neurons influences the setting of dopamine neurons 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 drug (DREADD) suppresses sympathetic nervous activity, reduces energy expenditure and increases food intake (38, 39). On the other hand, activation of the stimulatory subunit of the g protein complex (Gs) in AgRP neurons results in lasting increases in food intake (40). While AgRP-MC3R mice consumed less food, no significant difference in body weight or adiposity were observed. If MC3R signaling suppressed the activity of *AgRP*$^{+ve}$ neurons, then increased energy expenditure might have been predicted. The absence of an effect of ARC 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 ventromedial hypothalamic 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 agonsits (43). It is however also important to consider that studies using DREADD or optogenetics are indiscriminate in affecting the activity of *AgRP*$^{+ve}$ neurons, and their exact physiological significance thus open for debate. ARC *AgRP*$^{+ve}$ 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. 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 are also required.

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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 does not appear to contribute directly to hypothalamic obesity syndromes.

Overall, a functional divergence appears to exist between MC3Rs expressed on "2nd order" neurons in the ventral tegmental area (VTA) (29) and VMH (31). VTA 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 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 has not been established. MC3R signaling in steroidogenic 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 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*$^{+ve}$ neuron appears to be have an inhibitory role in regulating feeding behavior. When compared to previous studies using this model (2, 29–31), there appears to be functional divergence between MC3Rs expressed on "1st order" and "2nd order" neurons of the central nervous melanocortin system. MC3Rs expressed on 1st order *AgRP*$^{+ve}$ neurons are inhibitory. On the other hand, MC3Rs expression on 2nd order neurons receiving inputs from *AgRP*$^{+ve}$ and *Pomc*$^{+ve}$ neuronal projections appear to support expression of feeding behaviors. further studies are needed to examine the functions of MC3Rs expression on 1st order *POMC*$^{+ve}$ neurons, and to identify *Mc3r*$^{+ve}$ neurons that are critical for supporting the expression of appetite responses to negative energy balance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is already known about this subject?

- Activation of *AgRP*$^{+ve}$ neurons in the arcuate nucleus of the hypothalamus instigates food-seeking behaviors and increases food intake.
- Melanocortin-3 receptors (MC3R) are expressed in a subpopulation of *AgRP*$^{+ve}$ neurons.

What does this study add?

- A strain of C57BL/6J mice with MC3Rs expressed selectively in *AgRP*$^{+ve}$ neurons was developed (AgRP-MC3R mice).
- Restoring MC3R signaling in *AgRP*$^{+ve}$ neurons has no effect on the nutrient-partitioning defect observed in *Mc3r*-deficient mice.
- In females, food intake during the active phase in the dark period in *ad libitum* feeding conditions and situations of energy deficit is significantly reduced in AgRP-MC3R mice compared to *Mc3r*-deficient and control animals.

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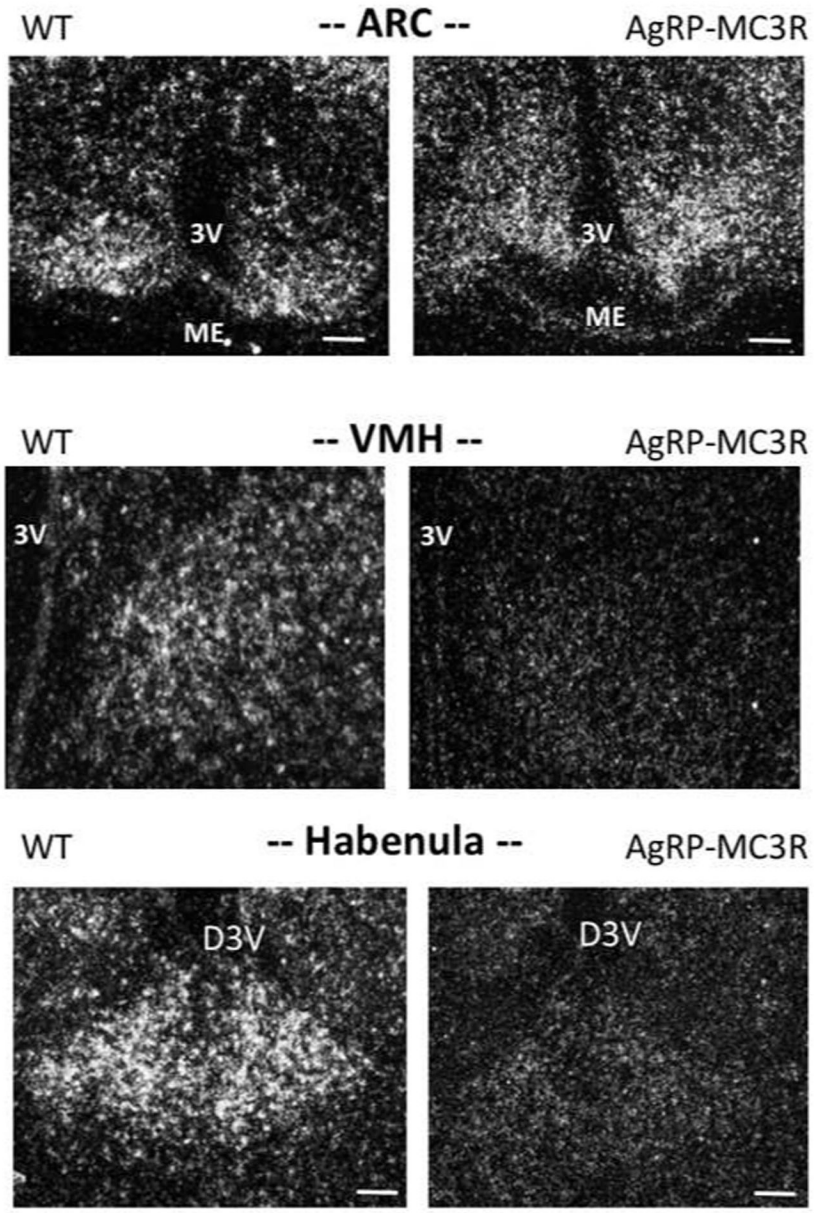

Figure 1.

Comparison of *Mc3r* expression in selected areas of the nervous system of wild-type (WT) and AGRP-MC3R mice using *in situ* hybridization. *Mc3r* expression is similar in the ARC of WT and AgRP-MC3R mice (A). In contrast, *Mc3r* expression normally observed in the VMH (B) and habenula (C) is only observed in WT mice. Scale bars are 100 microns.

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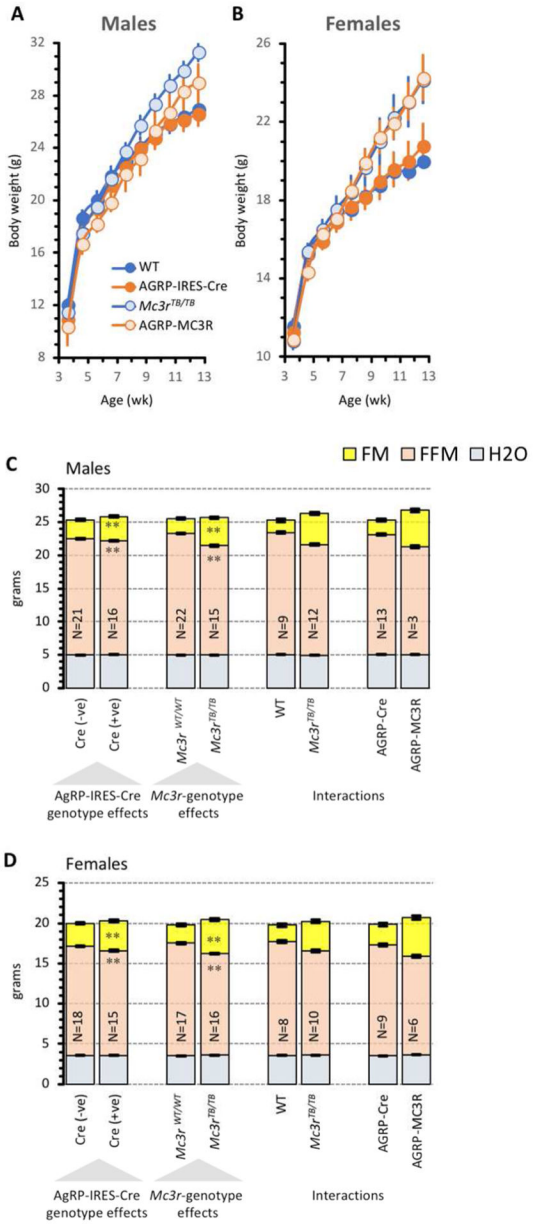

Figure 2.

Growth curves and body composition data for male (A, C) and female mice (B, D). (A, B) Growth curves are actual data. (C, D) Body composition data (fat mass, FM; fat-free mass, FFM; free H_2O) are estimated marginal means adjusted for total body weight in 12 wk old mice. Body composition data were analyzed using 2-way ANCOVA. The first set of two columns in C and D are body composition data for *AgRP-IRES-Cre*$^{-ve}$ and *AgRP-IRES-Cre*$^{+ve}$, irrespective of *Mc3r*-genotype. The second set of two columns are *Mc3r*$^{wt/wt}$ or *Mc3r*$^{TB/TB}$, irrespective of AgRP-IRES-Cre genotype. The last 4 columns are 4 groups (WT, *Mc3r*$^{TB/TB}$, *AgRP-IRES-Cre*$^{+ve}$ and AgRP-MC3R mice). The 2-way analysis examines for the effects of *AgRP-IRES-Cre* genotype, *Mc3r* genotype, and then for interactions between the two genotypes. The analysis identified statistically significant effects of each genetic

modification, but no significant interaction. Sample sizes are provided within the columns.
** significant effect of *AgRP-IRES-Cre* or *Mc3r* genotype, $p<0.001$.

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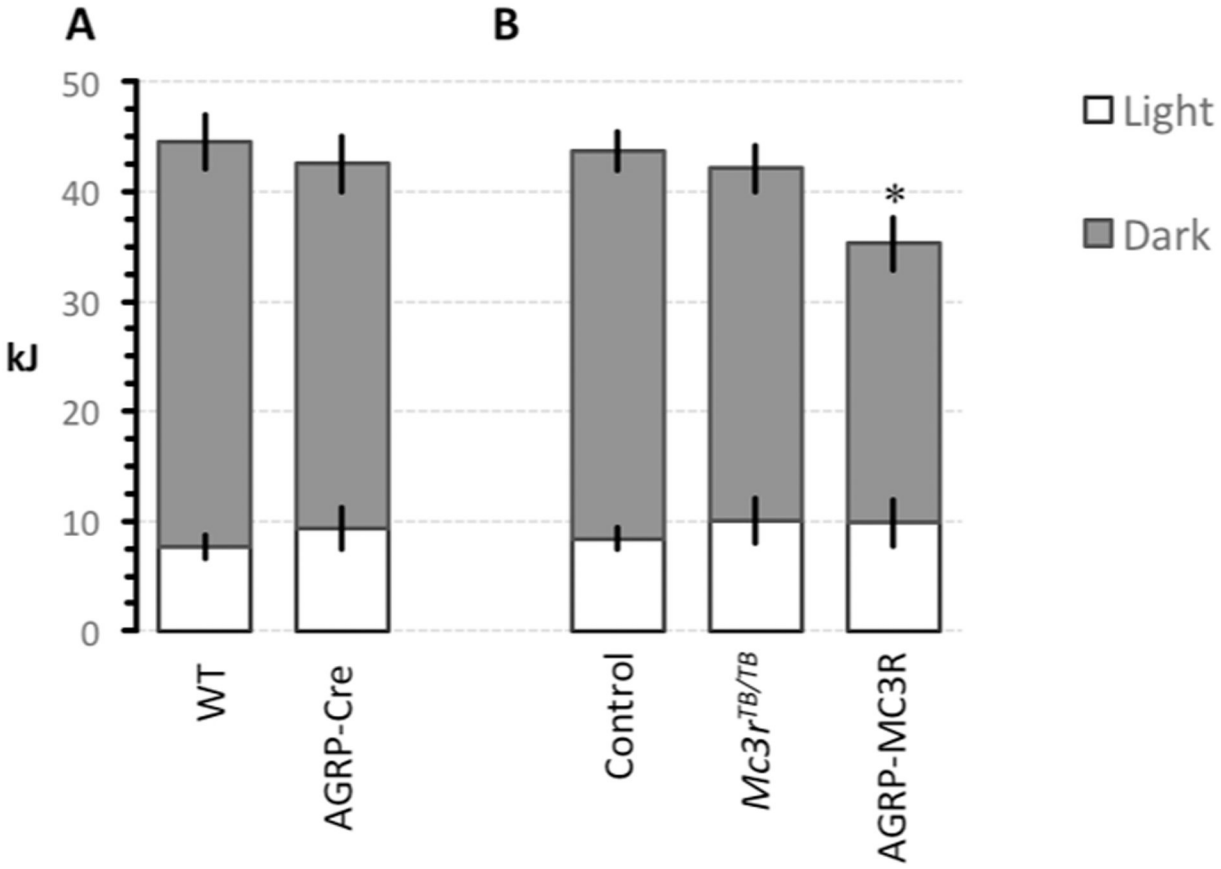

Figure 3.

Reduced food intake during the dark period in female AgRP-MC3R mice (n=6) compared to controls (n=16) and *Mc3r*$^{TB/TB}$ (n=8). (A) Food intake during the light and dark periods of *AgRP-IRES-Cre*$^{+ve}$ and wild-type mice. Food intake was not significantly different between wild type B6 mice (n=9) and AgRP-IRES-Cre mice (n=7). (B) Food intake during the light and dark periods for control (AgRP-IRES-Cre$^{+ve\ or\ -ve}$, n=16), *Mc3r*$^{TB/TB}$ (n=8) or AgRP-MC3R (n=6). * p<0.05 vs. *Mc3r*$^{TB/TB}$, p<0.01 vs. control.

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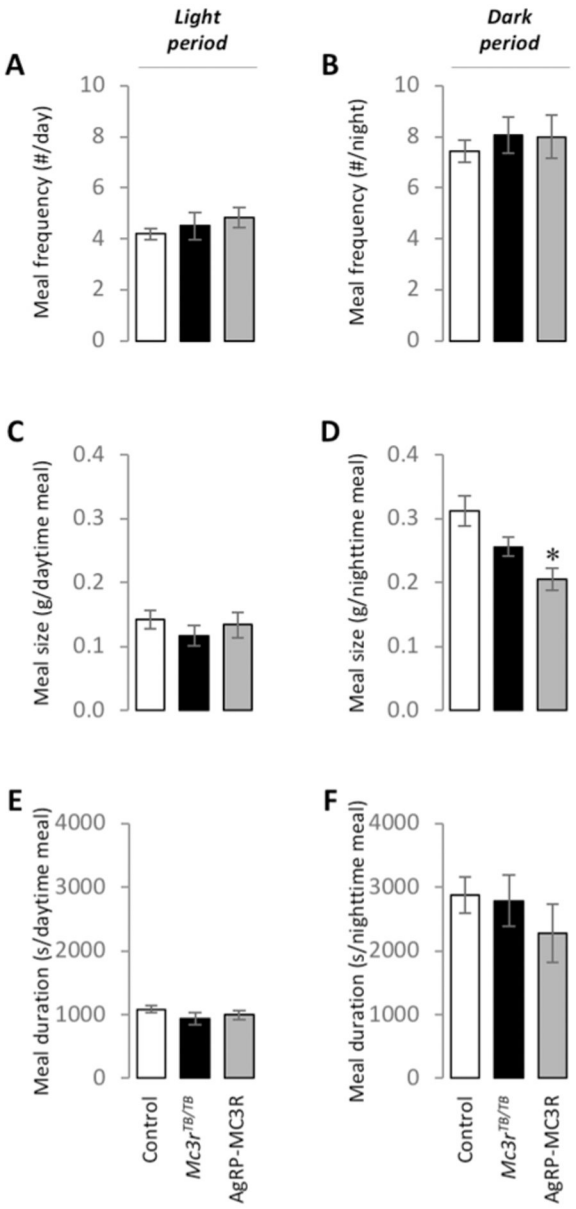

Figure 4.

Analysis of meal structure in female WT, *Mc3r*$^{TB/TB}$ and AgRP-MC3R mice under *ad libitum* feeding. Meal pattern data shown are as 12h day and 12h night averages; baseline data are averaged over 2 d after 3d acclimation. **(A–B)** meal frequency, **(C–D)** meal size and **(E–F)** meal duration were averaged from *ad libitum* feeding data recorded on day 4 and 5 **(A, C, E)** and night 4 and 5 **(B, D, F)**, 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 night-time food intake and meal size (all $p<0.05$). Differences between groups were assessed by Dunn's post hoc analysis. *$p<0.05$ compared to controls.

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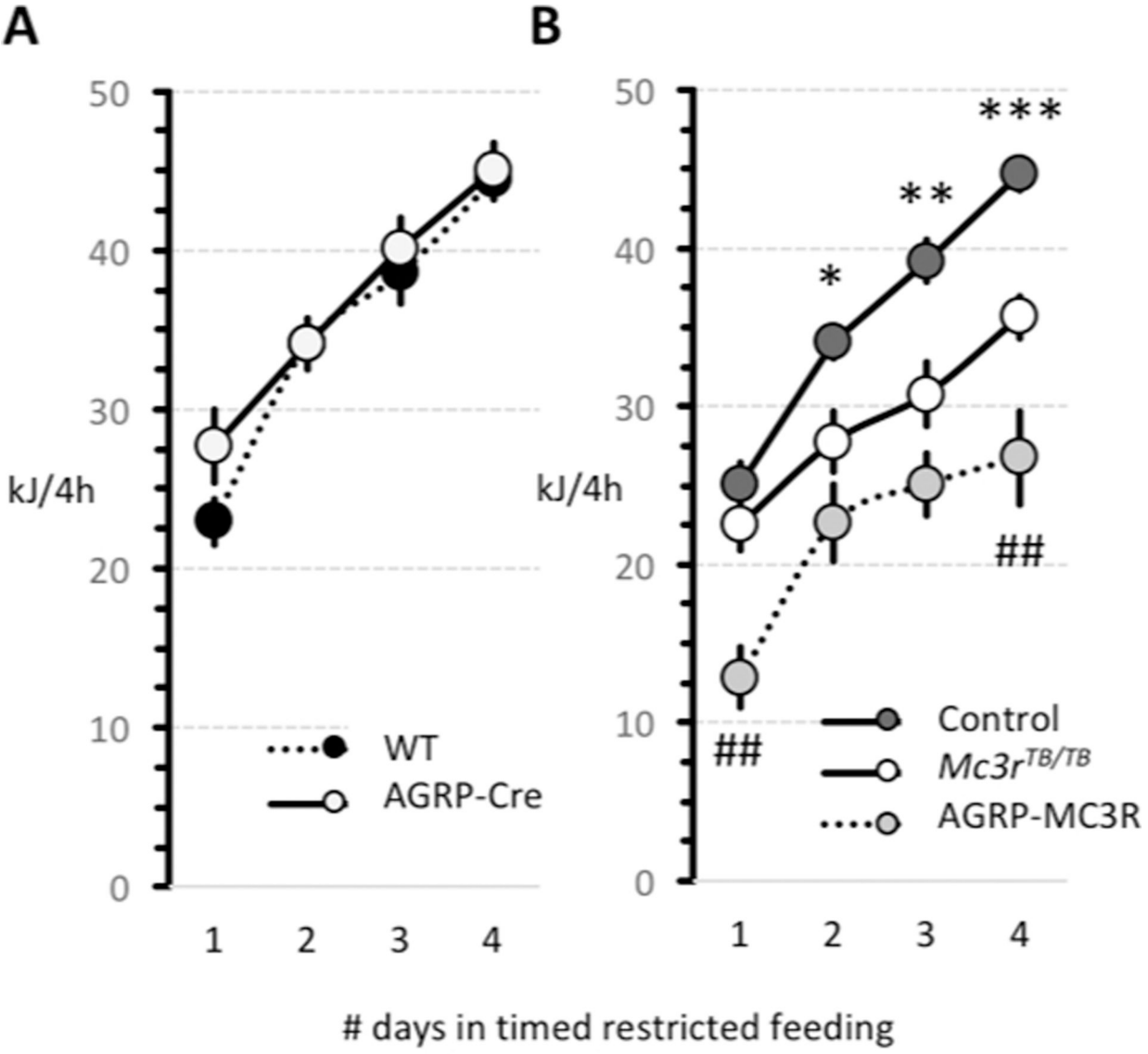

Figure 5.

In female mice, food intake of AGRP-MC3R mice during timed-restricted feeding is reduced compared to both controls and *Mc3r*$^{TB/TB}$ mice. Food access was limited to a 4h period (ZT4–8) for 4 days. (A) Food intake during timed-restricted feeding (kJ/4h) is not significantly affected by *AgRP-IRES-Cre* genotype (n=9 for wild type B6 mice, black circles/dotted line; n=7 for AgRP-IRES-Cre mice, white circles/solid line). (B) Comparison of food intake timed-restricted feeding between controls (*AgRP-IRES-Cre*$^{+ve}$ and WT, n=16, black circles/solid line), *Mc3r*$^{TB/TB}$ (n=8, white circles/solid line) and AGRP-MC3R mice (n=6, gray circles/dotted line). Following two-way ANOVA, differences between groups were assessed by Tukey's post hoc analysis. WT vs *Mc3r*$^{TB/TB}$: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; *Mc3r*$^{TB/TB}$ vs AgRP-MC3R: ## $p < 0.01$.