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MOLECULAR METABOLISM

Melanocortin-3 receptors in the limbic system mediate feeding-related motivational responses during weight loss

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ABSTRACT

Objective: Appetitive responses to weight loss are mediated by a nutrient-sensing neural network comprised of melanocortin neurons. The role of neural melanocortin-3 receptors (MC3R) in mediating these responses is enigmatic. *Mc3r* knockout mice exhibit a paradoxical phenotype of obesity and reduced feeding-related behaviors in situations of nutrient scarcity. Here we examined whether MC3Rs expressed in mesolimbic neurons regulate feeding-related motivational responses.

Methods: Interactions between *Mc3r* genotype, cognitive function and energy balance on food self-administration were assessed using operant conditioning with fixed- and progressive ratio (FR1/PR1) settings. Inhibition of *Mc3r* transcription by a loxP-flanked transcriptional blocker (TB) in C57BL/6JN mice ($Mc3r^{TB/TB}$) was reversed in mesolimbic neurons using DAT-Cre (DAT-MC3R).

Results: Caloric restriction (CR) caused 10–15% weight loss and increased motivation to acquire food rewards during training sessions. c-Fos-expression in the nucleus accumbens was increased 1 h following food presentation. While exhibiting weight loss, total food self-administration, enhanced motivation to self-administer food rewards in training sessions held during CR and c-Fos-activation in the nucleus accumbens following re-feeding were all markedly attenuated in $Mc3r^{TB/TB}$ mice. In contrast, cognitive abilities were normal in $Mc3r^{TB/TB}$ mice. Total food self-administration during FR1 sessions was not rescued in DAT-MC3R mice, however enhanced motivational responses to self-administer food rewards in PR1 conditions were restored. The nutrient-partitioning phenotype observed with *Mc3r*-deficiency was not rescued in DAT-MC3R mice.

Conclusions: Mesolimbic MC3Rs mediate enhanced motivational responses during CR. However, they are insufficient to restore normal caloric loading when food is presented during CR and do not affect metabolic conditions altering nutrient partitioning.

Keywords Melanocortins; Melanocortin-3 receptors; Dopamine; Motivation; Appetite; Caloric restriction

1. INTRODUCTION

The central nervous melanocortin system forms a focal point for the nutrient-sensing neural networks coordinating appetitive and metabolic responses to internal cues of metabolic state [1]. The core system is comprised of two populations of neurons in the arcuate nucleus of the hypothalamus (ARC) expressing the endogenous melanocortin ligands. Activation of neurons expressing proopiomelanocortin (POMC), a precursor polypeptide processed to produce α− and γ−melanocortin stimulating hormones (α−/γ−MSH), inhibits feeding behaviors and increases metabolic rate. Neurons co-expressing agouti-related peptide (AgRP), neuropeptide Y (Npy) and GABA act oppositely, promoting expression of feeding-related behaviors while activating metabolic programs conserving and storing energy.

The acute feeding and metabolic responses to melanocortin analogs requires melanocortin-4 receptors (MC4Rs), a 7-transmembrane G protein-coupled receptor expressed throughout the brain [2–4]. Clinical relevance is indicated by observation of hyperphagic obesity syndromes in humans inheriting nonsense mutations in the *POMC* or *MC4R* genes [1,5]. Another member of the melanocortin receptor family, the melanocortin-3 receptor (MC3R), is expressed in hypothalamic and limbic structures controlling autonomic function and complex ingestive behaviors [6–8]. Deletion of the *Mc3r* gene results in altered nutrient partitioning that favors accrual of fat mass (FM) in mice [8–13]. *MC3R* mutations may also cause obesity in humans [14]. While MC4Rs have been linked to circuits governing appetite and energy expenditure [15], the exact physiological roles of neural MC3Rs related to weight control have remained enigmatic.

Experiments using *Mc3r* and *Mc4r* knockout (−/−) mice treated with MSH-analogs indicate MC3Rs are neither necessary nor sufficient for inhibiting feeding behaviors [10,16–19] or stimulating energy expenditure [17,19]. Both hyper- and hypophagia have sometimes

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been observed in *Mc3r*-deficient mice, suggesting sensitivity to genetic background and housing conditions [10,13,14]. The obese phenotype of *Mc3r*−/− mice has therefore been widely considered to be metabolic, with altered partitioning of nutrients between lean tissues and lipid reserves. Factors contributing to this metabolic condition have been proposed to include hypercorticosteronemia [20], attenuated fasting-induced lipolysis in white adipose tissue [20], failure to suppress hepatic *de novo* lipogenesis during situations of caloric insufficiency [21,22], and increased differentiation of mesenchymal stem cells to adipocytes [14].

Mc3r-deficiency in mice is associated with a paradoxical phenotype in which obesity is associated with reduced feeding-related anticipatory responses [8,23–26]. MC3Rs are necessary for adaptive feeding responses during fasting [20] and hypocaloric restricted feeding paradigms [8,23,24,26]. MC3Rs may modulate responses to internal cues of metabolic state inducing food anticipatory activity (FAA), a progressive increase in locomotor activity anticipating food presentation when mice are fed a single hypocaloric meal at daily intervals [27]. FAA involves a coordinated phase shift in rhythms of appetitive behaviors and wakefulness to coincide with nutrient consumption [28,29]. Attenuated FAA in *Mc3r*−/− mice may at least partially result from insensitivity of AgRP/NPY neurons to internal cues of metabolic state [20,25]. As observed for MC4Rs [15], MC3R-dependent pathways affecting feeding behavior, peripheral metabolism and protection from obesity may be distributed between discrete populations of cells in the central nervous system and periphery.

The studies presented herein had two objectives. We first assessed whether deficits in food anticipatory behaviors observed in *Mc3r*-deficient mice manifest as reduced feeding-related motivational responses, using operant conditioning to measure self-administration of food rewards in situations of nutrient sufficiency (*ad libitum* feeding) and caloric restriction (CR). We report that MC3Rs are required for the full expression of feeding-related motivational responses, but only in situations of caloric insufficiency. Second, we assessed whether MC3Rs expressed in the mesolimbic dopaminergic system receiving inputs from AgRP and POMC neurons [6,7,30,31], regulate feeding-related motivational responses. Injection of melanocortin agonists into the ventral tegmental area (VTA) alters reward-related behaviors towards food and addictive substances, and affects changes in dopamine transmission to the ventral striatum/nucleus accumbens (NAc) [31,32]. We report that rescuing MC3Rs in the mesolimbic dopaminergic system enhances some feeding-related motivational responses during situations of caloric insufficiency, but has no impact on the obese phenotype.

2. MATERIALS AND METHODS

2.1. Animals

All studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Scripps Research Institute in Jupiter, FL. Animals were maintained in a 12 h light/dark cycle, at standard room temperature (23 °C) and were singly housed during the experimental procedure. All studies were performed in male mice during the light phase of the cycle. Training sessions and feeding times were kept consistent every day in each group of animals.

The role of MC3Rs in regulating motivational responses was assessed using $Mc3r^{tm1But}$ ($Mc3r^{TB/TB}$) mice. The expression of *Mc3r* gene in this strain is suppressed by insertion of a loxP-flanked transcription blocker (TB) into the genes 5′ UTR [8]. The initial studies of food self-administration used male $Mc3r^{TB/TB}$ and littermate controls produced from $Mc3r^{TB/+}$ breeding pairs on a mixed C57BL/6J;C57BL/6N background. While the *LoxTBMc3r* strain was developed using Bruce 4 embryonic stem cells derived from C57BL/6J mice [8], contamination with C57BL/6N is presumed to have occurred at some stage during colony maintenance. The ratio of genes derived from the 6J to 6N substrains in the mice used for these experiments is therefore unclear. The role of MC3Rs and MC4Rs in regulating motivational responses to melanocortin agonists administered icv was further assessed using B6;129S4-$Mc4r^{tm1Lowl}$/J mice ($Mc4r^{TB/TB}$) purchased from the Jackson Laboratory. In this strain, *Mc4r* expression is suppressed by insertion of the loxP-flanked TB into the genes 5′ UTR [33].

To assess the role of Mc3rs expressed by dopaminergic neurons in regulating feeding-related behaviors, we used Cre-mediated recombination to selectively restore transcription in $Mc3r^{TB/TB}$ mice. B6.SJL-Slc6a3$^{tm1.1(cre)Bkmn}$/J mice (DAT-Cre) were used to excise the TB in mesolimbic neurons in the ventral tegmental area (VTA) [34], generating *DAT-cre;*$Mc3r^{TB/TB}$ mice (hereafter referred to as DAT-MC3R). *DAT-cre*(-ve);$Mc3r^{TB/+}$ females were mated with *DAT-cre(+ve);*$Mc3r^{TB/+}$ males. DAT-cre and 'true' wild type controls (WT: negative for both the Cre and TB-modified *Mc3r* alleles), $Mc3r^{TB/TB}$ and DAT-MC3R were derived from the same litters.

To visualize Mc3r-expressing neurons, we used Tg(Mc3r-EGFP) BX153Gsat mice (hereafter referred to as Mc3r-eGFP) purchased from the Mutant Mouse Regional Resource Center [7,35]. This strain carries a bacterial artificial chromosome containing a modified *Mc3r* gene; an enhanced green fluorescent protein (eGFP) followed by polyadenylation signal were inserted into the ATG transcription initiation codon. Cre activity in Mc3r-positive neurons in the VTA was verified using a reporter strain expressing red fluorescent protein variant (tdTomato) (B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J) [36] crossed with the DAT-Cre and Mc3r-eGFP strains.

2.2. Behavioral testing

Self-administration experiments were performed in operant chambers housed in sound-attenuating cubicles (Coulbourn Instruments). Each chamber was equipped with two levers, a food pellet hopper and a house light. One lever was predetermined to be the "active" lever (lever pressing resulted in a positive outcome/food reward), while the other lever was "inactive" (lever pressing had no outcome). Total active lever pressings (not only the pressings that lead to reward, but also pressings during the time-out period after pellet acquisition) are presented. An initial evaluation indicated similar results when only the pressings that lead to reward were analyzed (data not shown). Inactive lever pressings were assessed. Active and inactive levers were counterbalanced between animals.

Adult mice aged 3.5–4 months were initially subjected to caloric restriction (CR) for 14 d before the start of behavioral testing. During this period, mice were provided 2.7 g of standard rodent chow (Teklad Global 19.1% Protein Rodent Diet, Harlan Laboratories) at the time coinciding with the completion of self-administration sessions later in the study. This feeding protocol resulted in a 10–15% reduction of body weight, and is compatible with previously described protocols used to study anticipatory behaviors in Mc3r-deficient mice [37,38]. During training, chow was provided once per day after the training session (2.5 g/d). The first two days of habituation involved mice undergoing a "magazine schedule" in the chambers, a 30 min session during which a 20 mg chocolate flavored food pellet (sucrose; Dustless Precision Pellets 20 mg, Bioserv) was released from the food hopper every minute coinciding with activation of a light cue over the active lever (total of 30 pellets). The mice were then trained for 7 d in a Fixed Ratio 1 (FR1) schedule; pressing the active lever resulted in presentation of a sucrose pellet and activation of a light cue. Pressing the

