Brief Communication

MOLECULAR METABOLISM

Functional analysis reveals differential effects of glutamate and MCH neuropeptide in MCH neurons

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ABSTRACT

Objectives: Melanin-concentrating hormone (MCH) neurons in the lateral hypothalamus (LH) regulate food intake and body weight, glucose metabolism and convey the reward value of sucrose. In this report, we set out to establish the respective roles of MCH and conventional neurotransmitters in these neurons.

Methods: MCH neurons were profiled using Cre-dependent molecular profiling technologies (vTRAP). MCHCre mice crossed to Vglut2$^{fl/fl}$mice or to DTR$^{fl/fl}$were used to identify the role of glutamate in MCH neurons. We assessed metabolic parameters such as body composition, glucose tolerance, or sucrose preference.

Results: We found that nearly all MCH neurons in the LH are glutamatergic and that a loss of glutamatergic signaling from MCH neurons from a glutamate transporter (VGlut2) knockout leads to a reduced weight, hypophagia and hyperkinetic behavior with improved glucose tolerance and a loss of sucrose preference. These effects are indistinguishable from those seen after ablation of MCH neurons. These findings are in contrast to those seen in mice with a knockout of the MCH neuropeptide, which show normal glucose preference and do not have improved glucose tolerance.

Conclusions: Overall, these data show that the vast majority of MCH neurons are glutamatergic, and that glutamate and MCH signaling mediate partially overlapping functions by these neurons, presumably by activating partially overlapping postsynaptic populations. The diverse functional effects of MCH neurons are thus mediated by a composite of glutamate and MCH signaling.

Keywords MCH; Sucrose preference; Body weight; Glucose metabolism; Neuropeptide; Glutamate

1. INTRODUCTION

Melanin concentrating-hormone expressing (MCH) neurons located in the lateral hypothalamus (LH) play an important role in the regulation of energy balance metabolism, and other functions [1–3]. Both an MCH knockout (KO) and ablation of MCH neurons result in reduced food intake and leanness [2,4]. MCH ablation also enhances glucose disposition in a glucose tolerance test. Recently, MCH neurons have been also implicated in sucrose preference, a reward related process, via projections to dopaminergic neurons in the striatum and midbrain [5]. In previous studies we have noted that there are differences between the effects of MCH neural ablation compared to an MCH knockout, raising the possibility that additional transmitters besides MCH mediate the effects of these neurons.

In this report, we set out to establish the molecular phenotypes of MCH neurons by profiling these neurons. A previous study suggested that a subset of MCH neurons are GABAergic while a different study suggested they are nearly exclusively glutamatergic [6,7]. Our data indicated that the great majority of MCH neurons are glutamatergic. We then compared the physiologic effects of ablating glutamate signaling in these neurons to either an MCH knockout or MCH neural ablation. We found that the effects of knocking out the glutamate transporter and MCH were different, suggesting that the function of MCH neurons is a composite of the function of a classic neurotransmitter and a neuropeptide.

2. METHODS

2.1. Mice and diets

C57BL/6 mice were purchased from Jackson (Jaxmice). The generation of *MCH-Cre* mice has been previously reported [5]. *VGlut2$^{loxp/loxp}$* mice were purchased from Jackson (Jaxmice). To generate MCH-

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specific *VGlut2* knock-out mice (*MCHCre;VGlut2fl/fl and MCHCre;V-GATfl/fl*, respectively), *MCH-Cre* mice were crossed with *VGlut2*$^{loxp/loxp}$. Colonies were maintained by breeding *MCH-Cre*; *loxp/loxp* mice with *loxp/loxp* mice. MCHKO mice were purchased from Jackson (Jaxmice), and MCH neuron-ablated mice were generated as described previously [5]. Mice were maintained on a 12:12 h light—dark cycle with free access to water and standard chow or high-fat diet (60% Kcal fat; Research Diets) for 12 weeks (starting at 6 weeks of age). *In vivo* studies were performed following the National Institutes of Health Guidelines on the Care and Use of Animals and approved by the Rockefeller University Institutional Animal Care and Use Committee (Protocols 15797-H and 15818-H).

2.2. Physiological measurements

Body weights were determined weekly. Blood samples were collected via tail vein or trunk bleeds using a capillary collection system (Sarstedt). Blood glucose was measured using an Ascensia Elite XL glucometer (Bayer Health-Care, Tarrytown, NY). Plasma leptin levels were analyzed by ELISA techniques (Alpco immunoassays). Glucose tolerance tests were performed on overnight fasted mice. D-glucose (2 g/kg) was injected intraperitoneally (i.p.) and blood glucose determined at the indicated time points. Locomotion activity was measured using beam breaks in metabolic cages from TSE systems.

2.3. Construction of AAV-IV-GFPL10

For construction of AAV-IV-GFPL10, see [8]. Briefly, we generated the plasmid for AAV-IV-GFPL10 by synthesizing an inverted EGFP sequence flanked by synthetic introns, lox sites, and forward Rpl10a. This fragment was then subcloned into pAAV-DIO-ChR2-mCherry to establish Cre-dependent expression, and it was then packaged into AAV pseudotype 5 (UNC Vector Core).

2.4. Immunoprecipitation and RNA-seq analyses

Details on GFP immunoprecipitations can be found in [9].

2.5. Stereotaxic surgeries

MCH Cre transgenic mice of 12—16 weeks of age were induced and maintained on isofluorane before being bilaterally injected with 0.5uL of AAV IV-GFPL10 in the LHA (LHA coordinates: −1.4 AP, −/+ 0.7 L and −5.2 DV). After viral injection, the needle was left in place for 10 min before slowly retracting. The skin was closed with a surgical clip.

2.6. Sucrose preference

Animals were acclimated to the chambers until side preference for either bottle was even. When preference was even, mice were studied as follows: first, sucrose in one bottle and water on the other, then, sucralose in one bottle and water in the other, and, lastly, sucrose in one bottle and sucralose in the other. During the acclimation and exposure periods, mice were water-deprived for 16—23 h and then given water through the bottles inside the chamber for half an hour. Two-bottle preference was calculated as the ratio: preference for 1 = number of licks on bottle 1/(number of licks on bottle 1 + number of licks on bottle 2) and expressed as percentage values, with 50% representing the indifference ratio. Behavioral data was analyzed with Excel and Prism and is expressed, as mean ± SEM. Significance tests comparing groups were *t* tests. The size of each animal group is represented by 'n', and each animal was tested three times. The investigator was blind to the genotype. In all cases, concentration of sucrose was 0.4 M, and concentration of sucralose was 1.5 mM. These concentrations were based on previous literature [10]. Briefly, the differences in molarity of sucrose and sucralose reflect differences in ligand-binding affinity of either sweetener to taste receptors and were chosen among the plateau values of behavioral dose—response curves (preference for either sweetener versus water in [10]). Volume dispensed by the lickometers averages 2 μl/lick [10].

2.7. Immunohistochemistry

Mice were first anesthetized with isofluorane followed by transcardial perfusion with PBS and then 10% formalin. Brains were dissected, incubated in 10% formalin overnight at 4 °C, and 40 μm sections were prepared using a vibratome. Free floating sections were blocked for 1 h at room temperature in blocking buffer (PBS, 0.1% Triton-X, 2% goat serum, 3% BSA) and then incubated overnight at 4 °C with primary antibody for rabbit anti-MCH (1:5000; Phoenix Pharmaceuticals). The next day, sections were washed in washing buffer (PBS, 0.1% Triton-X) 3 times for 20 min, incubated with dye-conjugated secondary antibody (1:1000; Alexa Fluor 488 or 594 goat anti-rabbit) for 1 h at room temperature, washed in washing buffer, and then mounted.

2.8. Microscopy and quantification

Stained brain sections were imaged using Zeiss LSM 510 laser scanning confocal microscope. All parameters were kept constant for quantification purposes and were conducted with ImageJ.

2.9. Statistics

Results are expressed as mean ± SEM. Student t test, one-way ANOVA, or two-way ANOVA was used to assess the significance of difference of the mean or the comparative analyses. Sidak post-hoc tests were used for multiple comparisons. Differences were considered significant at $p < 0.05$.

3. RESULTS

3.1. MCH neuron profiling confirms glutamatergic identity of these neurons

We first set out to establish the phenotypes of MCH neurons in an unbiased manner by molecular profiling using viral TRAP (vTRAP) (short for viral Translating Ribosome Affinity Purification). We injected an adeno-associated virus (AAV) expressing the ribosomal fusion protein L10 flanked by *loxp* sites (AAV-GFPL10) into the LH of MCH Cre mice. After three weeks, polysomes from hypothalamic blocks were precipitated using an anti-GFP antibody followed by high-throughput RNA sequencing (RNA-seq) of both the precipitated polysomal RNA (IP RNA) and total Input RNA (Figure 1A). We plotted FPKM (fragment per kilobase of transcript per million mapped reads) values for the IP RNA vs Input RNA on a log—log scale (Figure 1B) and found that *Pmch* itself was 121 Fold differentially enriched (DE is IP/Input) and that *Tacr3* (19 Fold DE) and *Cartp* (40 Fold DE), two RNAs known to be co-expressed in MCH cells, were also highly enriched (Figure 1B—D) [9,11,12]. As expected, we also found enrichment of GFP (14 Fold DE). Finally, we plotted the number of differentially enriched genes in a histogram as a function of the fold-enrichment in the IP samples (Figure 1E) and found that in addition to *Pmch*, which was the most enriched gene, an additional ~2000 genes were also highly enriched including *Mup6* (78.3 fold), *Klrg1* (53.91 fold), and *Iapp* (30.8 fold). The fold enrichment for all of the enriched genes is listed in Supplemental Table 1. We next replicated the RNA-seq data for a subset of genes by performing qPCR on the IP and total RNA and reconfirmed highly significant enrichment of *Pmch (102.5 Fold), Tacr3* (18.4 Fold), and *Cartp* (29.9 Fold) (Figure 1D). As expected, we did not see enrichment of the arcuate markers *Agrp* and *Pomc* or glial markers (*Gfap, Mal* and *Mbp*) (Figure 1B—D).

