Doctoral thesis (abridged version)

Regulation of glucoprivation-induced carbohydrate selection by NPY-CRH neural axis in the paraventricular nucleus of the hypothalamus

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Introduction

Feeding is one of the most important activities to maintain energy homeostasis in animals. While neural mechanisms responsible for the regulation of calorie intake have been extensively studied, the neural basis of food selection for macronutrients is less studied. My lab previously reported that a subset of corticotropin-releasing hormone (CRH) neurons expressing fasting-responsive AMP-activated protein kinase (AMPK) in the paraventricular nucleus of the hypothalamus (PVH) is necessary for a high carbohydrate diet (HCD) selection over a high fat diet (HFD) under fasting. However, neuronal mechanisms for HCD intake in other conditions such as glucoprivation remain unknown. Glucoprivation induced by a glucopenic reagent, 2-deoxy glucose (2-DG) is known to increase HCD intake, and neuropeptide Y (NPY)-expressing neurons projecting to the PVH might be involved in the food selection. In this study, I investigated the role of NPY-CRH neural axis in the PVH in mice in the glucoprivation-induced change in food selection of HCD and HFD after intraperitoneal (IP) administration of 2-DG.

Materials and Methods

Animals

WT C57BL/6J mice were purchased from Nihon SLC. CRH-Cre mice (JAX:012704) and NPY-Cre mice (JAX:027851) were purchased from the Jackson Laboratory. Mice were housed under a regular 12 h dark/light cycle with lab chow diet (LCD) and water ad libitum. All experiments were performed with 10-20-week-old male mice and carried out in compliance with institutional guidelines for the care and handling of experimental animals and were authorized by the Institutional Animal Care and Use Committee of the National Institutes of Natural Sciences.

AAV injection

Mice that were at least 10 weeks old were anesthetized with 1–2% isoflurane and placed into a stereotaxic apparatus. The skull was exposed via a small incision and a small hole was drilled into the skull. A Hamilton Neuros Syringe was inserted into the brain for AAV delivery. For

chemogenetic inhibition experiments, CRH-Cre mice were bilaterally injected with 200 nl (titer: 4.3×10^{12} vg/ml) of AAV8-hSyn-DIO-hM4Di-mCherry in the PVH. For suppression of mRNA expression of AMPK alpha subunits in PVH CRH neurons, CRH-Cre mice were bilaterally injected with 200 nl (titer: 1.5×10^{10} vg/μl) of AAVDJ-loxP-shAMPK or control AAV2-lox-shEmpty at the PVH. For retrograde tracing, NPY-Cre mice were bilaterally injected with 200 nl (titer: 1.2×10^{13} GC/ml) of AAVrg-FLEX-tdTomato in the PVH. The following coordinates for the PVH were used: from bregma: AP, -0.5 mm; ML, ±0.35 mm; DV, -4.75mm. For optogenetic experiments, NPY-Cre mice were bilaterally injected with 200 nl (titer: 7.7×10^{12} vg/ml) of AAV5-DIO-ChR2-EYFP into the NTS: from bregma: AP, -7.2 mm; ML, ±0.5 mm; DV, -5.0 mm and then optic fibers (400-mm diameter) were bilaterally implanted above the PVH: from bregma: AP, -0.5 mm; ML, -0.4 mm; DV, -4.4 mm at a 0° angle and AP, -0.5 mm; ML, 1.17 mm; DV, -4.55 mm at a 15° angle, respectively. Mice were allowed to recover for at least 3 weeks after AAV injection, and behavioral or staining experiments were performed.

ICV and Intra-PVH injection

For ICV injection, a guide cannula (C315GS-2/SP, 4 mm PED cut 2.5 mm below pedestal) was placed into the lateral ventricle with the following coordinates: from bregma: AP, -0.5 mm; ML, -1.0 mm; DV, -2.0 mm. After 2 week of recovery period, NPY (0.04 nmol dissolved with 1 ml of saline) was injected into the lateral ventricle. For intra-PVH injection, a guide cannula (C315GS-4/SP, 4 mm PED cut 5 mm below pedestal) was placed into the PVH with the following coordinates: from bregma: AP, -0.5 mm; ML, -0.4 mm; DV, -4.3 mm. After 2 weeks of the recovery period, several compounds were injected into the PVH. Following compounds were used for intra-PVH injection: NPY (0.08 nmol dissolved with 400 nl of saline), Y1R antagonist BIBP 3226 trifluoroacetate (0.4 nmol dissolved with 200 nl of saline), Y5R antagonist CGP 71683 hydrochloride (0.4 nmol in 200 nl dissolved with DMSO), a melanocortin receptor agonist Melanotan II (MTII) (0.136 nmol dissolved with 200 nl of saline).

Optogenetics

A 473nm blue light laser was used to deliver light pulses to the brain through fiber optic cables (200–mm diameter) firmly attached to implanted optic fibers. The power intensity of the laser at the optic fiber terminal was adjusted to approximately 10 mW/mm^2. For photostimulation experiments, the following pulse protocol was delivered to the PVH: 10-ms pulses, 50 pulses for 1 s, repeated every 3 s.

IP injection

IP injection of Clozapine-N-oxide (CNO, 1.0 mg/kg) was performed for chemogenetic inhibition experiments, and IP injection of 2-DG (500 mg/kg or 1 mg/kg) was performed to induce glucoprivation.

Food selection assay

Mice were singly housed for at least 2 weeks before food selection assay. Mice were habituated to a high carbohydrate diet (HCD) (D11071540: 69 kcal% carbohydrates, Research Diet) for 5 days a high fat diet (HFD) (D12492: 60 kcal% fat, Research Diet) for 2 days, and a combination of HFD and HCD for 2 days, respectively 1 week before food selection assay.

Immunohistochemistry

Mice were perfused with 4% paraformaldehyde (PFA). Brain tissue was sectioned on a microtome at 50 μm thick. Brain slices were incubated overnight at 4°C with primary antibodies diluted in PBS. The following primary antibodies were used: goat polyclonal anti-tdTomato (1:800; AB8181-200), guinea pig polyclonal anti-c-fos (1:400; 226004, Synaptic System), FITC-conjugated goat polyclonal anti-GFP antibody (1:800; ab6662, Abcam), and rabbit monoclonal anti-Phospho-AMPKα (Thr172) antibody (1:100, #2535, Cell Signaling Technology). The brain slices were then washed three times and incubated with fluorophore-conjugated secondary antibodies for 2 h at room temperature except in the case of FITC-conjugated goat polyclonal anti-GFP antibody. The following secondary antibodies were used: donkey anti-goat Alexa 488 (1:800; A11055, Thermo Fisher Scientific), donkey anti-guinea pig IgG Alexa Alexa 488 (1:800; 706-

545-148, Jackson Immuno Research), and donkey anti-rabbit Alexa 568 (1:300; A10042, Thermo Fisher Scientific). Fluorescence images were taken with Confocal Laser Scanning Microscope.

Statistical Analysis

The data are presented as means ± standard deviation (SD). Statistical analysis was performed with GraphPad Prism 9 software. Multiple groups were compared using analysis of variance (ANOVA) followed by post hoc Sidak's test, while two groups were compared using paired Student's t-test. Statistical significance was determined by a *P* value less than 0.05.

Results

First, I selectively expressed AAV encoding Cre-dependent hM4Di, an inhibitory DREADD (Designer Receptor Exclusively Activated by Designer Drugs), into the PVH of CRH-Cre mice. I performed the food selection assay for 4 h after IP injection of 2-DG. 2-DG injection increased HCD intake while it decreased HFD intake. By contrast, chemogenetic inhibition of PVH CRH neurons significantly suppressed the food selection of HCD over HFD. I next examined whether inhibition of CRH neurons affects NPY-induced HCD selection, using the inhibitory DREADD system. Intracerebroventricular (ICV) or intra-PVH injection of NPY led to a rapid increase in HCD intake within 1 h and then a slow increase in HFD intake. By contrast, inhibition of PVH CRH neurons suppressed the HCD intake with no effects on HFD intake. To further examine whether 2-DG-induced change in food selection is mediated by direct action of NPY on the PVH, I next investigated the effects of pharmacological blockade of NPY receptors Y1R and Y5R expressed in the PVH. Intra-PVH injection of Y1R and Y5R antagonists blocked the 2-DG-induced HCD intake but not HFD intake. I further suppressed mRNA expression of AMPK alpha subunits in PVH CRH neurons. AAV encoding Cre-dependent shRNA towards AMPK was injected into the PVH of CRH-Cre mice. Both intra-PVH injection of NPY and administration of 2-DG activated AMPK in the PVH, and suppression of mRNA expression of AMPK alpha subunits in PVH CRH neurons inihibited both NPY and 2-DG-induced HCD intake but not HFD

intake. These results suggest that the NPY-CRH neural axis in the PVH is necessary for 2-DG-induced HCD selection.

Melanocortin-4 receptor (MC4R) in the PVH is important for normal feeding. I investigated the possible role of PVH melanocortin-4 receptor (MC4R) in NPY-induced food selection. Injection of a MC4R agonist, MTII, into the PVH preferentially inhibited HFD intake but not HCD intake after the intra-PVH NPY injection, indicating that the food selection of HFD is mediated by a distinct neural circuit in the PVH from that of HCD selection.

To investigate which NPY neurons project to the PVH and are activated by 2-DG, I introduced the retrograde AAV encoding Cre-dependent tdTomato into the PVH of NPY-Cre mice and administered saline or 2-DG. I found that NPY neurons in various brain areas innervate the PVH, and among them, PVH-projecting NPY neurons in the nucleus tractus solitarius (NTS), ventrolateral medulla (VLM), periaqueductal gray (PAG), and arcuate nucleus of the hypothalamus (ARC) are strongly activated by 2-DG. Since NTS NPY neurons have been reported to be involved in the 2-DG-induced increase in total calorie intake, I next examined the effect of optogenetic activation of PVH-projecting NTS NPY neurons on food selection. AAV encoding Cre-dependent ChR2-EYFP was injected into the NTS of NPY-Cre mice and optic fibers were bilaterally implanted above the PVH to activate the PVH-projecting NTS NPY neurons specifically. Optogenetic activation of PVH-projecting NTS NPY neurons increased c-fos expression in the PVH and led to an increase in HCD intake along with a slow increase in HFD intake similar to those observed after ICV or intra-PVH injection of NPY. These results suggest that NTS NPY neurons projecting to the PVH play an important role in 2-DG-induced HCD selection.

Discussion

In this study, my findings indicate that the NPY-CRH neural axis in the PVH is essential for 2-DG-induced HCD selection. I found that 2-DG-induced HCD selection requires 1) NPY neurons

projecting to the PVH, 2) NPY receptors Y1R and Y5R expressing in the PVH, and 3) PVH CRH neurons that activate AMPK in response to NPY. The study with melanocortin-4 receptor (MC4R) agonist, Melanotan II (MTII), also indicated that food selections of HFD is mediated by a distinct neural circuit in the PVH from that for HCD intake. Furthermore, my results show that NPY neurons in the nucleus tractus solitarius (NTS) projecting to the PVH are one of the important neurons that mediate 2-DG-induced HCD selection. Collectively, my results suggest that the NPY-CRH neural axis in the PVH is necessary for 2-DG-induced HCD selection and food selection of HFD is mediated by a distinct neural circuit in the PVH.