Chemogenetic manipulation of parasympathetic neurons (DMV) regulates feeding behavior and energy metabolism

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**Abstract**

Parasympathetic nervous system (PNS) innervates with several peripheral organs such as liver, pancreas and regulates energy metabolism. However, the direct role of PNS on food intake has been poorly understood. In the present study, we investigated the role of parasympathetic nervous system in regulation of feeding by chemogenetic methods. Adeno associated virus carrying DREADD (designer receptors exclusively activated by designer drugs) infused into the target brain region by stereotaxic surgery. The stimulatory hM3Dq or inhibitory hM4Di DREADD was over-expressed in selective population of dorsal motor nucleus of the vagus (DMV) neurons by Cre-recombinase-dependent manners. Activation of parasympathetic neuron by intraperitoneal injection of the M3-muscarinic receptor ligand clozapine-N-oxide (CNO) (1 mg/kg) suppressed food intake and resulted in body weight loss in ChAT-Cre mice. Parasympathetic neurons activation resulted in improved glucose tolerance while inhibition of the neurons resulted in impaired glucose tolerance. Stimulation of parasympathetic nervous system by injection of CNO (1 mg/kg) increased oxygen consumption and energy expenditure. Within the hypothalamus, in the arcuate nucleus (ARC) changed AGRP/POMC neurons. These results suggest that direct activation of parasympathetic nervous system decreases food intake and body weight with improved glucose tolerance.

**Keywords:**

DREADD, CNO, PNS, Food intake, Energy expenditure.

1. Introduction

The number of people with obesity and diabetes has been increasing worldwide. Obesity and diabetes are recognized as common diseases and causing not only healthcare but also economic burdens. Although many various research and treatment methods have been suggested and tested for obesity and diabetes [8,14], it is still far from complete cure of those diseases. Autonomic nervous system plays a crucial role in the regulation of energy homeostasis [7,9,13]. To date, its role of appetite regulation is increasingly recognized because it malfunctions resulted in obese phenotypes. These mechanisms involve a complex interplay between central and peripheral nervous systems including both afferent and efferent vagus nerve fibers [2,21]. Previous works revealed several different hypothalamic regions such as arcuate nucleus (ARC), ventral medial hypothalamus (VMH), lateral hypothalamus (LH) modulate autonomic neural flow to peripheral organs such as liver, pancreas, and fat [10–14,24]. Both efferent central neuronal and afferent peripheral signals converged on dorsal motor nucleus of the vagus. DMV encompasses the nucleus tractus solitaries and modulates autonomic nervous system which regulates feeding behavior and energy metabolism [12,19]. Thus, DMV is a critical node in autonomic nervous system and its downstream VN fibers provide a potential therapeutic target for anti-obesity treatments. In fact, vagus nerve electric stimulator has been approved for human anti-obesity treatment option by the Food and Drug Administration (FDA) [4]. Compared to the role of sympathetic nervous system on feeding and energy metabolism, the role of parasympathetic nervous system on feeding and energy expenditure is unclear. To examine specifically in vivo effects of efferent parasympathetic nervous signals on feeding and energy expenditure, we have generated mouse models of temporal activation or inhibition of
DMV regions by chemogenetic approach using DREADD techniques. DREADD techniques involve an artificial membrane receptor expression on target cells and receptor-specific artificial ligand administration for temporal modulation of the system [16–19]. Our hypothesis was that specific activation/inhibition of the vagus nerve can modulate feeding behavior and energy expenditure. We also used Cre recombinase expressing genetically modified mouse models such as ChAT-Cre to improve the specificity of DREADD receptor expression on parasympathetic motor neurons. ChAT-Cre mice show Cre expression in preganglionic parasympathetic neurons of the DMV, intermediodorsal nucleus of the cholinergic neurons [27]. Here we investigated whether the specific changes in parasympathetic nervous system could be alternative treatment options for obesity and diabetes. Our results provide that possible therapeutic roles of parasympathetic modulation in anti-obesity and diabetes.

2. Material and methods

2.1. Animals

ChAT-Cre mice on a C57BL/6 genetic background were generated as previously described [25,26]. This mouse line was Cre-recombinase expression is controlled via an IRES-Cre sequence was inserted in the genome downstream of the ChAT gene stop codon. The Cre allele was detected using the following primers: 5′-GGTTTGCAGAAGCGGTGGG-3′ (M336), 5′-GATAGATAATGAGGGCTC-3′ (M337), and 5′-AGATAGATAATGAGGGCTC-3′ (M338). All animal Procedures were conducted in accordance with The Institutional Animal Care and Use Committee of the Seoul National University and Seoul National University Hospital Institute of Biomedical Research, Seoul, Korea. Mice weighing 23–25 g were maintained in individual cages under controlled temperature (21–23 °C) and light (light on 8:00, off at 20:00) with free ad libitum access to food and water.

2.2. AAV vectors for hM3dG or hM4di expression

The stimulatory DREADD, designated “hM3dG”, couples through the Gq pathway to depolarize neurons [1,4]. The hM3dGq and hM4dii coding sequences were cloned into a mCherry vector [11,15] upstream of the mCherry sequence to generate C-term mCherry fusion proteins. The hM3dGq-mCherry and hM4dii-mCherry coding sequences were amplified by PCR, and the amplicons and a Cre-inducible AAV vector with a human Synapsin 1 promoter [3]. We were hM3dGq and hM4dii AAV-virus purchased from addgene.

2.3. Stereotaxic surgery and microinjection

Mice were injected anesthetized with ketamine (80 mg/kg, i.p) and xylazine (10 mg/kg), the brain was removed and post-fixed overnight with 4% paraformaldehyde in phosphate buffer saline (PBS). And, the samples were subsequently cryo-protected in 0.1 M PB containing 20% sucrose. The brain embedded in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan), were sectioned into 14μm thickness and were prepared on a freezing microtome (Leica CM3050S; Leica, Nussloch, Germany) chilled at -20°C for immunofluorescence staining, 4 mice per group were analyzed.

2.4. Body weight and food intake

Mice were housed in individual cages, and body weight and food intake were determined dark phase (8 A.M - 8 P.M) and light phase (8 A.M - 8 P.M). Preweighed food was placed in the food hoppers and measured on a per-cage basis. Food intake was determined as grams consumed per day.

2.5. Metabolic cage studies

Mice were placed into an 8-cage Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA) on the experimental day, and given a 48 h to acclimate. On experiment day after IP administration of CNO (1 mg/kg) measured both groups. O2 consumption (V02), CO2 consumption (VCO2), Respiratory exchange ratio (RER), heat production and locomotor activity, food intake, water intake were monitored every 10 min during the 48 h period at room temperature. Cages were opened and calculations were stopped for 1 h between 9:00 and 10:00 am daily for replanning food, measuring body weight, and performing injections. Energy expenditure calculated energy expenditure according to the following formula provided by the manufacturer: energy expenditure (kcal)=(3.815 + 1.232VO2/VCO2) xV02. On final day, Mice were removed from the cages.

2.6. Intraperitoneal glucose tolerance test (GTT)

Glucose tolerance tests were 12–14 wk old male ChAT-Cre mice that were being maintained on a normal Chow diet were obtained from the Seoul National University and Seoul National University Hospital Institute of Biomedical Research. After an overnight fast (16–18 h), On the day of experimentation, Mice were intraperitoneal (i.p) glucose (1 mg/kg) administration. Blood samples were drawn from the tail vein immediately prior to CNO treatment and at 0, 15, 30, 60, and 120 min for plasma glucose measured using a Glucometer (Accuchek). For plasma insulin, blood samples (50 or 100ul) were collected from the tail vein into EDTA-coated tube 10-minute glucose loading, immediately centrifuged, and the plasma was separated and stored at −20 °C until assayed.

2.7. Immunohistochemistry

I.p. administration was performed 60 min before perfusion in overnight (16–18 h) fasted mice. Mice were anesthetized by an intraperitoneal administration of sodium ketamine (80 mg/kg) with xylazine (10 mg/kg). The brain was removed and post-fixed overnight with 4% paraformaldehyde in phosphate buffer saline (PBS). And, the samples were subsequently cryo-protected in 0.1 M PB containing 20% sucrose. The brain embedded in Tissue-Tek OCT compound (Sakura Finetek Japan, Tokyo, Japan), were sectioned into 14μm thickness and were prepared on a freezing microtome (Leica CM3050S; Leica, Nussloch, Germany) chilled at -20°C. For immunofluorescence staining, sections were blocked in 3% normal donkey serum (Sigma Aldrich, CA, USA) for 1 h incubated at room temperature, and then incubated 48 h at 4 °C with goat AGRP antibody (1:1000; Abcam, UK), rabbit β-endorphin (a product of POMC) antibody (1:3000; Phoenix Pharmaceuticals, Inc. CA). Slices were washed with PBS and incubated with Alexa Fluor 488-labeled anti-rabbit or Alexa Fluor 555-labeled anti-goat (1:500; Invitrogen, Carlsbad, CA) at room temperature for 1 h. Nuclei in brain sections were identified by staining four, 6-diamidino-2-phenylindole (DAPI) (Molecular Probes, Eugene, OR). For fluorescent section images, an Olympus fluorescence microscope (Olympus, Tokyo, Japan) was used. For quantitative histological analysis of POMC and AGRP neurons was manually counted using Image J software (NIH) at a magnification of 40X for three sections. For the immunofluorescence staining, 4 mice per group were analyzed.

2.8. Electrophysiology study

ChAT-Cre mice injected with pAAV- hSyn-hM3D(Gq)-mCherry viruses were used activation of DMV neurons upon CNO administration. Changes in DMV neuron firing rates were recorded from DMV-mCherry + neurons in the brain stem DMV were measured by silicon neural probe.
2.9. Statistical analysis

Data are expressed as means ± s.e.m. The level of statistical significance was determined using paired t-test when the difference between the means of two populations was considered or a two-way repeated ANOVA (Drug x Time as repeated measures) analysis of variance was used to study the effect of hM3Dq and hM4Di injection of CNO in ChAT-Cre mice.

3. Results

3.1. Selective activation/inhibition of parasympathetic neurons

We used a Cre-recombinase-dependent adeno-associated virus (AAV) to express either hM3Dq, hM4Di receptor for inhibition of DMV complex neurons in ChAT-Cre mice (Fig. 1A, B). The receptors are fused to mCherry fluorescent protein so that virus-mediated the receptor expression could be monitored. We have confirmed that mCherry fluorescent protein expression was detected exclusively in the DMV regions in both ChAT-Cre (Fig. 1C). Additionally, I.p. injection of clozapine-N-Oxide, a specific ligand for DREADD receptor, activates the receptor and initiates the neuronal firing rate and generate action potentials for hM3dq receptor and it has been recorded by multichannel recording silicon probe. (E) I.p administration of CNO in vivo induces c-fos immunoreactivity in DMV neurons. The brain was collected for c-fos analysis 60 min after i.p injected of saline or CNO (1 mg/kg).

3.2. Effect of parasympathetic modulation on feeding, blood glucose levels

We investigated the effect of acute modulation of parasympathetic nervous system on food intake. The activation of parasympathetic nervous system decreased food intake in ChAT-Cre mice (Fig. 2A–C). The reduction in food intake was significantly different compared to the control group and last up to 4 h after CNO i.p injection (Fig. 2A). In contrast, the inhibition of parasympathetic nervous system significantly but only briefly increased food intake in ChAT-Cre (Fig. 2E). We next examined the effect of parasympathetic nervous system on glucose homeostasis. After activation or inhibition of parasympathetic neurons, we measured blood glucose levels during intraperitoneal glucose tolerance test in ChAT-Cre. The activation of parasympathetic nervous system in ChAT-Cre mice shows improved glucose tolerance (Fig. 2G) while the inhibition of parasympathetic nervous system results in impaired glucose tolerance (Fig. 2H).

3.3. Effect of parasympathetic modulation on energy expenditure

Food intake and energy expenditure is a critical factor for body weight maintenance. To examine metabolic phenotypes including energy expenditure, individual mouse is acclimatized and housed in a single metabolic cage from Comprehensive Laboratory Animal Monitoring System (CLAMS; Columbus Instruments, Columbus, OH, USA). We measured oxygen consumption (VO2), Energy expenditure (EE) to study the effect of parasympathetic nervous system (Fig. 3). Energy expenditure calculated energy expenditure according to the following formula provided by the manufacturer: energy expenditure (kcal) = (3.815 + 1.232VO2/VCO2) xVO2. In Chat-Cre mouse models, modulation of parasympathetic nervous system significantly increased oxygen consumption and energy expenditure (Fig. 3C,D). Thus we concluded body weight reduction in Chat-Cre mouse model is mainly because of less food intake.

3.4. Effect of modulation of parasympathetic nervous system on AgRP/POMC neurons in arcuate nucleus of the hypothalamus

We also investigated the cellular mechanisms underlying the regulation of food intake in the arcuate nucleus (ARC) of the hypothalamus. Co-staining with c-Fos, early neuron activation marker, and either POMC or AgRP revealed that chemogenetic activation of DMV (hM3Dq) increased POMC neurons in ChAT-Cre mice (Fig. 4A, B). However, inhibition of DMV (hM4Di) significantly decreased the number of not only POMC neurons but also POMC and c-fos double-positive neurons in ChAT-Cre mouse (Fig. 4A, C). These results show
that reduced food intake due to increased POMC expressing neurons upon activation of parasympathetic nervous system. We next investigated AGRP expression in the ARC of the hypothalamus. Chemogenetic activation of DMV (hM3Dq) decreased AGRP neurons in ChAT-Cre. In contrast, chemogenetic inhibition of DMV (hM4Di) highly increased AGRP neurons in ChAT-Cre mice (Fig. 4D, F). These results suggest that specific chemogenetic modulation of parasympathetic neurons mediates food intake through altering the number of AGRP and POMC expression neurons in ARC of the hypothalamus.

4. Discussion

The parasympathetic nervous system plays a key role in the control of both food intake and energy expenditure and results in body weight changes [19,20]. Our results suggest that DMV modulates the parasympathetic nervous system and modulation of DMV affects food intake and energy expenditure. Altered food intake and energy expenditure result in body weight changes. The efferent parasympathetic autonomic signal is conveyed via preganglionic cell in DMV brain stem [9]. These mechanisms play a role in the control of energy expenditure [11,22]. The preceding discussion provides two main evidences. Parasympathetic nervous system regulates 1) energy expenditure by innervating with peripheral tissue and 2) interplay with central neurons in ARC of the hypothalamus. Hypothalamic arcuate nucleus (ARC) contains a various population of neurons expressing the orexigenic factor neuropeptide Y (NPY) and AGRP and the anorexigenic factors POMC and cocaine- and amphetamine-regulated transcript (CART) [16]. However, not all neural networks that calibrate energy status require signaling from the hypothalamus, and a solely hypothalamus centric view offers only an incomplete picture of homeostatic energy regulation [23]. Possible mechanisms by which VNS affects energy expenditure have been suggested in several studies. From an anatomical viewpoint, it is interesting to note that the majority of fibers present within the vagus nerve are afferent fibers (74%) and only a minority

Fig. 2. Parasympathetic activation of DMV neurons regulates food intake and glucose. (A–C) stimulatory DREADD (hM3Dq). (D–F) inhibitory DREADD (hm4Di). (I) Body weight, CNO (1 mg/kg,i.p) or saline was injected at the start of the 12-h light phase, and food intake was assessed between 1 h and 24 h. (G) CNO activation of DMV (hM3Dq) decreased blood glucose level compared with Saline. (H) Inhibition of parasympathetic DMV neurons increases blood glucose level. Blood glucose level during 0, 15, 30, 60, 90, 120 and IPGTT in ChAT-Cre mice overnight fasting. A) Data are means ± SEM from n = 8 mice per group; F4,60 = 2.581392, P = 0.0461. D) F4,70 = 0.02543797, P = 0.9987 by two-way repeated measures analysis of variance (ANOVA). I) Data are means ± SEM from n = 8 mice per group; by unpaired t-test, ***p < 0.001. G) Data are means ± SEM from n = 8 mice per group; F5,84 = 3.866, P = 0.01. H) Data are means ± SEM from n = 8 mice per group; F5,30 = 250.7165, P = 0.0001 by two-way repeated measures analysis of variance (ANO).
(26%) are efferent, including that the vagus nerve is both an afferent nerve and efferent nerve as well [27]. Vagal sensory information plays a crucial role in the mechanism of satiation but the underlying circuitry in the caudal brainstem and higher up in the brain is not defined. In this study, specific modulation of the parasympathetic nervous system by chemogenetic methods causes changes in the number of proopiomelanocortin (POMC) and agouti-related protein (AGRP) neurons in ARC of the hypothalamus. Our results support that DMV is a node of parasympathetic nervous system including both afferent and efferent signals. The signal from DMV reaches to ARC of the hypothalamus and activates the number of AGRP and POMC expressing neuron. C-Fos and either POMC or AGRP double co-staining show that the parasympathetic nervous system activation affects both POMC and AGRP expression in ARC of the hypothalamus. Interestingly, while chemogenetic activation of DMV neurons simultaneously is increasing peripheral blood glucose, the serum insulin contents are not increased followed by increased blood glucose levels. These results suggest that modulation of DMV may cause an error in central glucose sensing or another common pathway. Previously studies showed that vagus nerve stimulation affects energy expenditure, demonstrated that cephalic phase of digestion induced gastric acid secretion and motility [28]. These cholinergic pre-ganglion also travels to the pancreas within the bulbar outflow tract and the hepatic and gastric nerves of the vagus. In addition to peripheral organs, arcuate nucleus is particularly reciprocal connected with the dorsal vagal complex and integration of endocrine and behavioral aspects of food intake satiety [2]. The activation of

Fig. 3. Activation of DMV neurons regulates oxygen consumption (VO2), energy expenditure (EE). (A–H) ChAT-Cre mice were acclimated in Comprehensive Lab Animal Monitoring System (CLAMS) cage (2day) and injected with Saline (black) or CNO (red) at 10:00 a.m in ChAT-Cre mice. (A) Oxygen consumption (VO2). (B) energy expenditure (EE). Following chemogenetic activation of dorsal vagal complex in ChAT-Cre mice. (C) VO2 (hM3Dq), B) EE (hM3Dq). C) Average light and dark phases (VO2). D) Whole body energy expenditure between Saline and hM3Dq (CNO) group. G) VO2 (hM4Di). H) Whole body energy expenditure between Saline and 4Di (CNO) group. Data are means ± SEM from n = 8 mice per group; by unpaired t-test, *p < 0.05 and **p < 0.01.
preganglionic parasympathetic neurons in the DMV generates action potentials (AP) and the AP travels through the vagus nerve. Parasympathetic neurons innervate with peripheral organs. When AP arrives on the target organs, acetylcholine is released into a synapse and it binds to the receptor expressed on the target organs [16]. For example, when the VN is stimulated, the terminals of the preganglionic nerves in the intra-pancreatic ganglia, release acetylcholine to the synapse with the acetylcholine, which in turn causes the release of acetylcholine from their terminals within the islet [29,30]. Our results support previous studies that the parasympathetic nervous system regulates blood glucose levels and pancreatic insulin secretion independently. We provided the evidence for these functional connectivity among the parasympathetic nervous system, hypothalamic neurons, and peripheral tissues. Chemogenetic modulation of parasympathetic nervous system on feeding behavior and energy metabolism had changed feeding and glucose and energy expenditure in ChAT-Cre mice (Table 1). However, we were not able to define which neural pathway interplay to control parasympathetic nervous system. Therefore, more detailed working mechanism of the neurons and the researches for functional neural connections are still required. The present study suggest that chemogenetic acute activation or inhibition of the parasympathetic nervous system regulates feeding behavior and energy expenditure.

Fig. 4. Expression of dorsal vagal complex activation/inhibition on hypothalamic AGRP and POMC in ChAT-Cre mice. (A–F) Injection of CNO in ChAT-Cre induce POMC, AGRP, FOS immunoreactivity in the ARC (arcuate nucleus). (A, B) Analysis of average POMC neuron number increased compare with Saline group but did not change both (C) DMV(hM4Di) groups. (F) Analysis of average AGRP neuron number significantly increased compare with Saline group. (F) Co-expression of AGRP and c-fos increased in chemogenetic inhibition of DMV (hM4Di) group. Brain were obtained for analysis 60 min following injection Saline or CNO (1 mg/kg of body weight, i.p, N = 4)(Scale bars:50 μm).
Table 1
Parasympathetic regulates of feeding and glucose metabolism by chemogenetics.

| Stimulation/Inhibition | Energy input | Energy output | Blood glucose (mg/dL) |
|------------------------|--------------|---------------|----------------------|
|                         | Food intake (FI) | Agouti-related protein (AGRP) | Proopiomelanocortin (POMC) | Oxygen consumption (VO2) | Carbon dioxide production (VCO2) | Respiratory exchange ratio (RER) | Energy expenditure (EE) |                       |
|                        |   ↑            |   ↑            |   ↑                   |   ↑                     |   ↑                         |   ↑                              |   ↑                      |   ↓                   |

Author contributions

Conceived and designed the experiments: CNK, HJC. Performed the experiments: CNK, CYK, DHC, SSH.

Discussed and wrote the paper: CNK, WJS, SJW, HJC.

Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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References

[1] G.M. Alexander, S.C. Rogan, A.I. Abbas, B.N. Armbruster, Y. Pei, J.A. Allen, R.J. Nunneman, J. Hartmann, S.S. Mey, M.A. Nicolosi, J.O. McNamara, B.L. Roth, Remote control of neuronal activity in transgenic mice expressing evolved G protein-coupled receptors, Neuron 63 (2009) 27-39.

[2] K.N. Browning, S. Verheijden, G.E. Boeckxstaens, The vagus nerve in appetite regulation, mood, and intestinal inflammation, Gastroenterology 185 (2017) 730-744.

[3] J.A. Cardin, M. Carlen, K. Meletis, U. Knoblich, F. Zhang, K. Deisseroth, L.H. Tsai, C.J. Moore, Driving fast-spiking cells induces gamma rhythm and controls sensory responses, Nature 459 (2009) 663-667.

[4] T. Hampton, Electric stimulation device approved to treat obesity, JAMA 313 (8) (2015) 785.