表　　題　光遺伝学による視床下部背内側核 GABA 作動性ニューロン の 摂食亢進作用の検証
The role of GABAergic neurons in dorsomedial hypothalamus in feeding regulation

論文の区分　博士課程

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Contents

1 Abstract of thesis

Background and aim

Obesity is a major risk factor for metabolic diseases including diabetes, hypertension and cardiovascular diseases, and for certain types of cancer. To date, no effective treatment for obesity has been developed. Obesity results from hyperphagia as well as excessive energy accumulation in the body. To solve the problems of obesity, it is essential to elucidate the mechanisms for regulation of feeding. In the central feeding regulatory circuit, several anorexigenic neurons have been identified in several regions of brain including hypothalamus and hind brain. As orexigenic neurons, in contrast, only neuropeptide Y/Agouti related protein (NPY/AgRP) neuron in the arcuate nucleus of hypothalamus (ARC) has been established by now. However, several lines of knockout mice studies have suggested that ɣ-aminobutyric acid (GABA)ergic neuron may also serve as an orexigenic neuron. GABA is a classical inhibitory neurotransmitter which inactivate postsynaptic neurons. The dorsomedial hypothalamus (DMH) has been considered an orexigenic center since its lesion decreases food intake and body weight. However, the mechanisms by which DMH promotes food intake, particularly the neuron that regulates food intake, are unclear. In DMH, GABAergic and glutamatergic neurons are localized. The present study aimed to clarify the role of DMH GABAergic neurons in regulation of food intake and to elucidate the downstream neural pathway.

Methods

Channelrhodopsin fast receiver (ChRFR)-C167A, one of bistable variants of chimeric channelrhodopsin, has been used to activate neurons. ChRFR-C167A

was fused with Venus, yellow fluorescent protein variant, and cloned into adeno-associate virus (AAV)2-GAD1 promoter-WPRE-BGH-polyA vector. GAD1 promoter is specifically expressed in GABAergic neuron. Blue light (473 nm) stimulation opens this channel with longer duration than ChR, and yellow light (589) stimulation closes the channel. The vector was injected to DMH bilaterally in wild type male mice (C57BL/6J) to express ChRFR-C167A-Venus in GABAergic neurons. Food intake was measured with or without blue (473 nm) and yellow (586 nm) laser light exposure for 3 h via optical fiber inserted above DMH. To study the regulation of DMH-GABAergic neurons, their responses to systemic hunger and satiety factors, low glucose and leptin respectively, were examined. For this, membrane potential of ChRFR-C167A-expressing neurons was recorded by slice patch clamp. In another study to elucidate the downstream neural pathway, fluorescence protein Venus in axonal fibers and terminals of DMH GABAergic neurons were acquired with a confocal laser-scanning microscope. In response to light exposure via optical fiber inserted above paraventricular nucleus of hypothalamus (PVN), inhibitory synaptic transmission was measured by patch clamp and food intake was measured.

Results

ChRFR-C167A-Venus fluorescence was observed in DMH GABAergic neurons. It was shown by using slice patch clamp with current clamp mode, that the ChRFR-C167A-Venus expressing neurons were long-lastingly depolarized by blue LED light and repolarized by yellow LED light exposure. The optogenetic selective activation of GABAergic neurons in DMH, significantly increased cumulative food intake at 2 and 3 h of light exposure, compared to corresponding

values in mice without light exposure. ChRFR-C167A-expressing neurons in DMH were depolarized by lowering glucose from 2.5 to 0.5 mM and hyperpolarized by administration of leptin at 10^{-7} M. Venus fluorescence in axonal terminal of ChRFR-C167A-expressing neurons was detected in the PVN, ventromedial hypothalamus (VMH), ARC, and dorsal motor nucleus of vagus (DMNV), the areas known as satiety centers. LED light exposure to PVN increased inhibitory postsynaptic current onto PVN neurons *in vitro*. The light exposure for 3 h to PVN via optical fiber increased cumulative food intake for 3 h and 6 h significantly as well as a trend for increase of cumulative food intake for 0.5, 1 and 2 h. These data indicated the DMH GABAergic neuron's inhibitory synaptic transmission to PVN promotes food intake.

Discussion

Although orexigenic function of DMH has long been postulated, actual role of DMH in the feeding regulation has remained unclear. I have demonstrated that selective activation of DMH GABAergic neurons by optogenetics promoted food intake. Furthermore, DMH GABAergic neurons substantially projected and exerted inhibitory synaptic transmission to the neurons of PVN, the integrative center of satiety. Moreover, DMH GABAergic neurons were directly inhibited by leptin and activated by lowering glucose, the representative factors reflecting systemic energy states and implicated in physiological regulation of feeding. These results suggest that the DMH-GABAergic neuron serves as an orexigenic neuron, which is activated by peripheral hunger signal and inhibited by satiety signal, and hence promote food intake.

Conclusion

The DMH GABAergic neuron is activated by orexigenic signal, lowering glucose, and inactivated by anorexigenic signal, leptin. The DMH GABAergic neuron projects to and inhibits satiety center PVN, and thereby promotes food intake.

2 Introduction

2.1 Obesity

Obesity is becoming a global health problem since it is one of major risk factors for diseases such as hypertension, cardiovascular diseases, diabetes, and certain forms of cancer. The study of World Health Organization (WHO) estimated that in 2014 more than 1.9 billion adults were overweight and over 600 million were obese. The development of obesity is linked with increase in high caloric intake on one hand and decrease in general physical activity on other hand in modern life (1).

Obesity, the state of increased body weight, results from long-term energy imbalance, hyperphagia and excessive energy accumulation in the form of fat (2, 3). We know, for more than six decades, that body weight is regulated via homeostatic systems involving the central nervous system (4). However, the effective treatment for obesity has not been established yet. Thus, understanding the mechanism of central regulation of food intake is crucial in the development of therapeutic strategies for solving the problems of obesity.

2.2 Feeding control by the dorsomedial hypothalamus (DMH) of hypothalamus

Increasing evidences based on studies utilizing conventional techniques suggest that the hypothalamus plays an essential role in feeding control (5, 6).

During the last three decades, progression of researches clarified that neural projections of orexigenic agouti-related protein (AgRP) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons from the arcuate nucleus

(ARC) to paraventricular nucleus (PVN) play a critical role in feeding control (2, 4, 7). In addition, PVN neurons are also projected form several nuclei in the hypothalamus and brainstem, including the dorsomedial hypothalamus (DMH). Classical studies suggested that DMH has been considered an orexigenic nucleus, since its lesion reduces food intake and body weight (8) and induces resistance against diet-induced obesity (9, 10). The DMH expresses feeding regulatory neuropeptides including NPY (11), CART (12), and prolactin-releasing peptide (PrRP) (13). It also expresses various receptors, including leptin receptor (14), melanocortin 3/4 receptors (MC3/4) (15, 16), Y1 receptor, Y5 receptor (17), and CCK receptor (18, 19). Region specific knock down and overexpression studies demonstrated that NPY neurons in DMH, which are GABAergic and leptin insensitive (11, 20), play a role to promote food intake in rats (21-23), being consistent with the DMH-lesion studies. However, it was reported that the level of NPY expression is very low in mice fed with a normal chow, questioning its physiological role, while it is increased in diet-induced obesity (24). Hence, the principal orexigenic neuron in DMH under physiological conditions remains to be identified.

2.3 Hypothalamic GABAergic neuron in feeding regulation

ɣ-Aminobutyric acid (GABA) is an inhibitory neurotransmitter vital in brain function (4). In situ hybridization and transgenic animal model experiments showed that GABA is abundantly expressed in the hypothalamic ARC, LH, and DMH (25, 26). Optogenetic activation of GABAergic neuron in LH or ARC increased feeding (27-29). Also, $GABA_A$ and $GABA_B$ receptors are localized in the hypothalamic region that regulate feeding. (4, 30). Pharmacological

activation of GABAa receptors in the PVN increased feeding (31). However, GABA release from DMH remains to be studied.

It was reported that the mice deficient in leptin receptor specifically in GABAergic neurons develop greater increases in food intake and body weight compared to the mice deficient in leptin receptor specifically in AgRP, POMC or steroidogenic factor 1 (SF1) neurons (26). These results suggested that GABAergic neurons including those in DMH could be a principal orexigenic neuron targeted by leptin.

2.4 Objective

The principal orexigenic neuron in the DMH remains to be defined. In this study, I aimed to clarify the role of the DMH GABAergic neuron in feeding regulation by using optogenetics and electrophysiological techniques. I found that optogenetic activation of DMH GABAergic neurons promotes food intake and elicits inhibitory synaptic transmission to the paraventricular nucleus of hypothalamus (PVN) where anorexigenic neurons are localized. Furthermore, DMH GABAergic neurons are depolarized by lowering glucose and hyperpolarized by leptin.

3 Materials and Methods

3.1 Animals

Male mice (20-25g; C57BL/6J) aged about 8 weeks were obtained from Japan SLC (Hamamatsu, Japan). Mice were housed in individually in specific pathogen-free environment, maintained on a 12 h light/dark cycle (lights on at 07:30 a.m.) and provided with libitum access to water and standard diet (Rodent Diet CE-2, Japan CLEA, Inc., Tokyo, Japan) containing 24.9% protein, 4.6 % fat, and 4.1% fiber. All study protocols involving mice were approved by the Animal Care and Use Committees at Jichi Medical University Institute of Animal Care and Use Committee and in accordance with the institutional Regulation of Animal Experiments and Fundamental Guideline for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the Jurisdiction of the Ministry of Education, Culture, Sports and Technology.

3.2 Adeno-associated virus (AAV) 2 production

Mouse GAD1 promoter gene and cDNA of ChRFR-C167A, one of bistable variants of chimeric channelrhodopsins (32), Venus cDNA and terminator sequences were subcloned to pAAV1-WPRE- vector. The AAV2 virus coded ChRFR-C167A-Venus was generated using GD1001-RV (Genedetect.com Ltd. Auckland, New Zealand). The titer of the virus was 1.1×10^{12} genomic particles/ml. The vector construct was kindly provided by Prof. Hiroshi Onodera (Tokyo University, Japan).

3.3 Stereotaxic surgery

The AAV2-GAD1-ChRFR-C167A vector (50 nl/injection site) was injected stereotaxically to bilateral DMH under anesthesia with tribromoethanol (200 mg/kg). The coordinates for the injections into the DMH were determined as anteroposterior (AP) = -1.4 mm, mediolateral (ML) = ±0.2 mm and dorsoventral (DV) = -5.4 mm with respect to bregma. The optical fiber (Edmund Optics, Japan) with 250 or 500 μm diameter was stereotaxically placed above DMH or PVN. The stereotaxic coordinates used were AP = -1.4 mm, ML = ±0.25 mm and DV = -4.8 mm for DMH and AP = -0.6 mm, ML = ±0.25 mm and DV = -4.4 mm for PVN.

3.4 Feeding experiments

Mice were allowed to recover from surgery by handling at least for 2 weeks before being subjected to tests. On the day of experiments, mice were fasted for 4 h before the onset of the dark phase (start at 15:30) and refed immediately at the beginning of the dark phase (at 19:30) in order ensure the similar feeding onset the of animals. Furthermore, food intake was measured manually using a digital balancer (A and D GX2000, Japan) at 0.5, 1, 2, 3 and 6 h. Exposure to blue laser (473 nm) followed by yellow (589 nm) laser (LUCIR, Tsukuba, Japan) was performed via optical fibers with 10 ms pulses, 50 Hz for 2 s, repeated every 5 s to the DMH or 3 s yellow pulse following 2 s blue pulse repeated every 10 s to the PVN for 3 h from 19:30 to 22:30.

3.5 To verify positions of virus infection and optical fiber and detection of axonal fibers and terminals

3 weeks after vector injection (detection of axonal fibers and terminals) or at the end of food intake experiments (to verify positions of virus infection and optical fiber), mice were perfused transcardially with heparinized saline (heparin 20 unit/ml), followed by 4% paraformaldehyde (PFA) in phosphate buffer (PB, 0.1M, pH 7.4) for about 7 mins for each solution. The brains were removed and subjected for immunohistochemistry. Coronal sections (40 μm) of the hypothalamus were cut using a freezing microtome (Leica SM2000R, Leica Microsystems, Germany) and collected at 120 μm intervals. Sections were mounted on coated glass slides, dried and covered by Fluorescence Mounting Medium (Dako, USA), to histologically verify the position of the virus infection and optical fiber. Confocal fluorescence images of virus infection, axonal fibers and terminals were acquired with a Confocal laser-scanning microscope (fluoreview FV1000-TO, Olympus, Japan).

3.6 Acute slices preparation

The brains were rapidly removed from C57BL/6J male mice injected AAV2-GAD1-ChRFR-C167A. The isolated brains were placed in ice-cold, carboxygenated (95% O_2 and 5% CO_2) high mannitol solution containing 229 mM mannitol, 3 mM KCl, 6 mM $MgCl_2$, 0.5 mM $CaCl_2$, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 10 mM glucose at pH 7.4 with 0.5 μM tetrodotoxin (osmolarity; 300–305 mOsm). A block of tissue containing the hypothalamus was isolated and 200 μm coronal brain slice containing DMH was prepared using a vibratome.

Following recovery for 1–2 h, slices were moved to a recording chamber mounted on BX51WI upright microscope (Olympus) equipped with video-enhanced infrared-differential interference contrast (DIC) and fluorescence. Slices were perfused with a continuous flow of carboxygenated aCSF containing 127 mM NaCl, 2.5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1.23 mM NaH_2PO_4, 26 mM $NaHCO_3$, and 2.5–10 mM glucose at pH 7.4. Neurons were visualized with Olympus Optical 40× water-immersion lens.

3.7 Patch-clamp recording

Whole-cell patch clamp recordings were performed. Pipettes were used with 3–9 MΩ resistance after being filled with pipette solution. Pipettes were made of borosilicate glass (Narishige) using a PP-83 vertical puller (Narishige) or a Sutter micropipette puller (P-1000). The pipettes with 3–9 MΩ resistance after being filled with pipette solution were used. The composition of the pipette solution was : 135 mM K-gluconate (for current clamp recording) or KCl (for inhibitory post synaptic current (IPSC) recording), $MgCl_2$ mM 2, HEPES 10 mM, EGTA 1.1 mM, Mg-ATP 2.5 mM, Na_2-GTP 0.3 mM, and Na_2-phosphocreatine 10 mM at pH 7.3 with KOH (osmolarity; 290–295 mOsm). Membrane potential and action potential were recorded at 0 pA holding current with current clamp mode. IPSC was measured at -60 mV holding potential with voltage clamp mode. Axopatch 200B amplifier and Clampex 10 software (Axon Instruments) were used for data acquisition. Pclamp 10 (Axon Instruments) software was used for analysis. Liquid junction potential correction was performed off-line. Access resistance was continuously monitored during the experiments. Only those cells

in which access resistance was stable (changes ~30%) were included in the analysis. The data was analyzed by Clamp fit 10 (Axon instruments) software and GraphPad Prism6 software. If a change of membrane potential was at least twice the standard deviation of membrane potential for 2 min before addition of agents, it was considered the response.

Irradiation was carried out using power LEDs (each from Lumileds, San Jose, CA) emitting either blue light (peak, 460–490 nm, LXHL-NB98) or yellow light (peak, 587–597 nm, LXHL-NL98) controlled by a regulator (SLA-1000-2, Mightex, Toronto, Canada). If the cumulative distribution of IPSC amplitude for 20 s after light exposure was significantly larger than that before light exposure by kolomogrv-semirnov test.

3.8 Statistical analysis

Data are expressed as means ± s.e.m. Two-way ANOVA followed by Sidak multiple range tests was used for food intake experiments and one-way ANOVA followed by Dunnet multiple range tests for membrane potential experiments.

4 Results

4.1 The selective activation of GABAergic neurons in DMH using optogenetic technique

As depicted in Figure 1A, to selectively activate GABAergic neurons in DMH**,** AAV2 encoding ChRFR-C167A-Venus under GAD1 promoter was injected to DMH. ChRFR-C167A, a bistable variant of chimeric channelrhodopsin, provides bimodal regulation: exposure to blue light (470 nm) induces long lasting opening, which is subsequently terminated by exposure to yellow light (592 nm)(32). The mice expressing ChRFR-C167A-Venus in DMH GABAergic neurons were studied. Venus fluorescence was observed in DMH at 2 weeks after virus infection (Figure 1B). In acute slices including DMH under current clamp mode, the ChRFR-C167A-Venus expressing neurons were long-lastingly depolarized by blue LED light and repolarized by yellow LED light exposure (Figure 1C). These *in vitro* data indicated that blue light depolarized ChRFR-C167a expressing neuron DMH neuron.

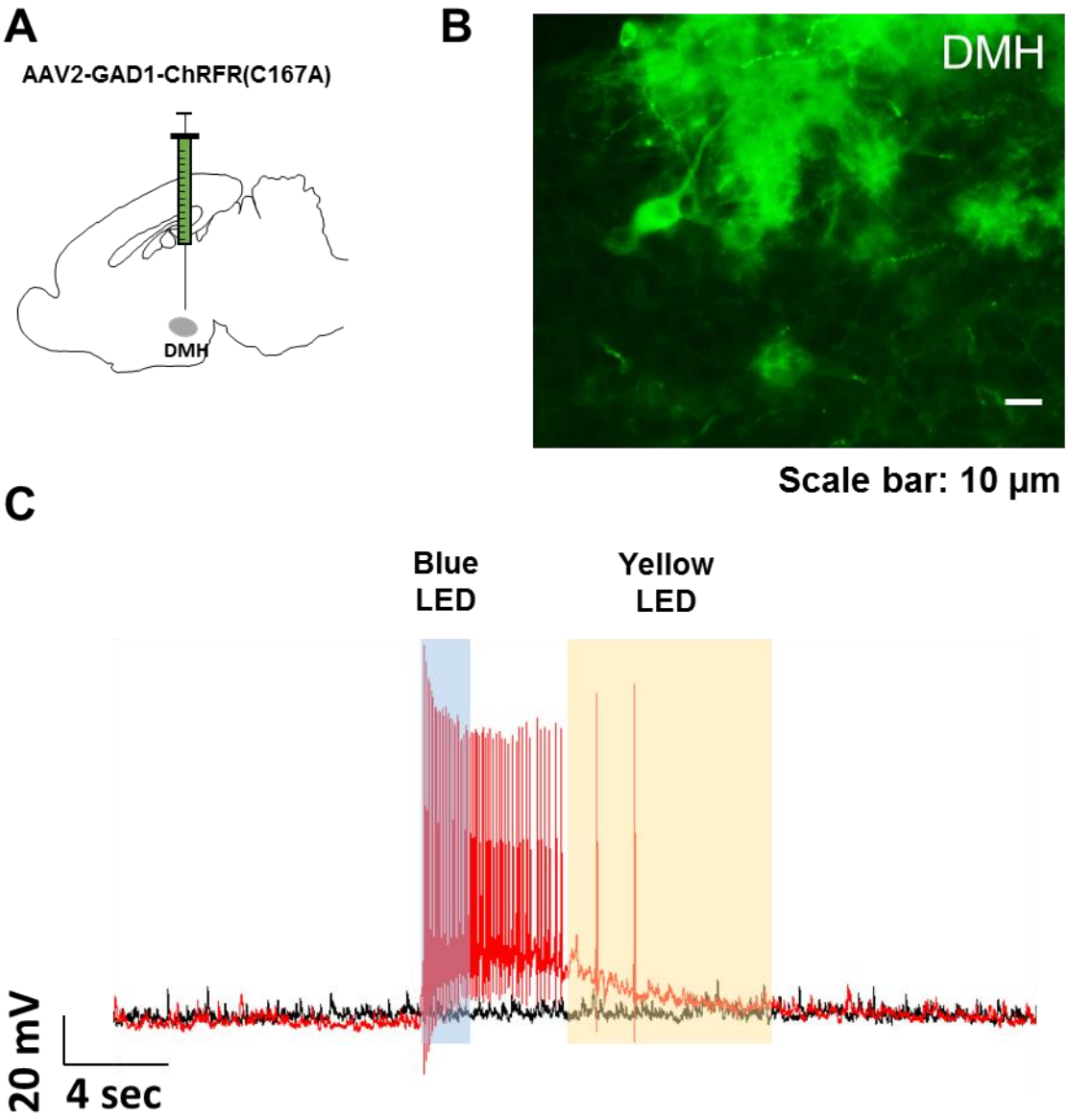

Figure 1. Blue light depolarizes ChRFR-C167A-Venus expressing neurons in DMH. A, Cartoon of a sagittal mouse brain depicting vector injection into DMH B, Representative expression of ChRFR-C167A-Venus in DMH coronal section. Scale bar, 10 μm. C, In vitro experiment. In current clump recording of acute slices prepared from vector injected mice, blue LED stimulation depolarized and Yellow LED stimulation repolarized ChRFR-C167A-Venus expressing neurons in DMH (red trace), while membrane potential was unchanged without light exposure (black trace).

4.2 Selective activation of GABAergic neurons in DMH promotes food intake

To selectively activate GABAergic neurons in DMH, AAV2-ChRFR-C167A-Venus under GAD1 promoter was infected to DMH in mice, food intake was measured with or without blue (473 nm) and yellow (589 nm) laser light (Figure 2A) exposure for 3 h via optical fiber inserted above DMH (Figure 2B). Cumulative food intake at 2 and 3 h of light exposure was significantly greater than the corresponding values in mice without light exposure (Figure 2C). These *in vivo* data indicated that activation of DMH GABAergic neurons promoted food intake.

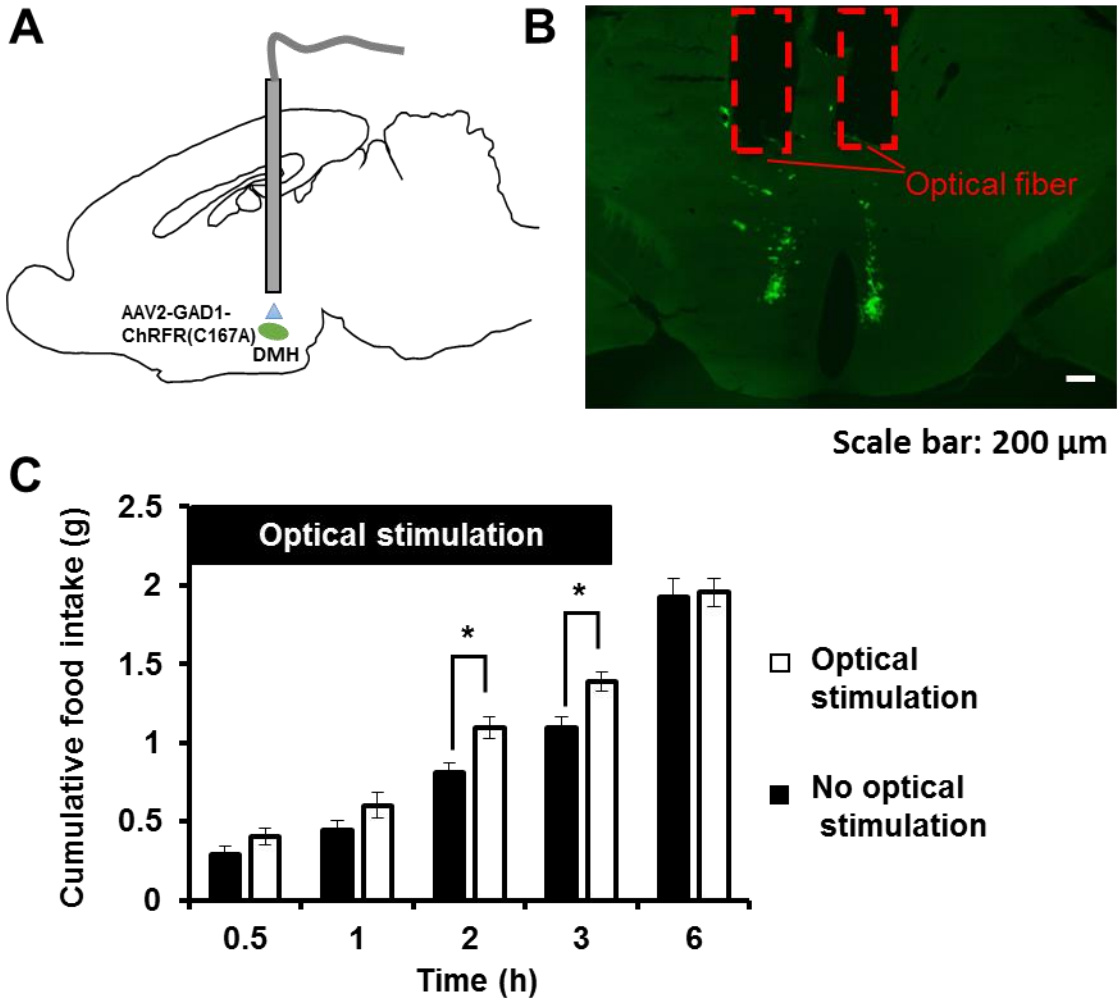

Figure 2. Optogentic activation of GABAergic neurons in DMH promotes food intake. A, Strategy for activation of GABAergic neurons in DMH B, Local infection of AAV2-GAD1-ChRFR-C167A virus expressing ChRFR-C167A in DMH and placement of optical fiber. Scale bar, 200 µm. C, In vivo optogenetic stimulation of DMH GABAergic neurons for 3 h. Activation of DMH GABAergic neuron significantly increased cumulative food intake at 2 and 3 h. Optical stimulation started at the onset of dark phase (7:30 PM). Both group, n=5. * $p < 0.05$, by two-way ANOVA followed by Sidak multiple range test.

4.3 Leptin hyperpolarizes and lowering glucose depolarizes DMH GABAergic neurons

To study the regulation of DMH-GABAergic neurons, their responses to systemic hunger and satiety factors, low glucose and leptin respectively, were examined. For this, the membrane potential of DMH ChRFR-C167A-expressing neurons was measured by patch clump experiments under current clamp mode.

In 10 recordings from 4 mice, lowering of glucose concentration from 2.5 to 0.5 mM depolarized approximately 60% of ChRFR-C167A-expressing neurons from −46.93 ± 1.9 mV to −42.31 ± 1.6 mV (Figure 3A, B). Anorexigenic leptin hyperpolarized 40% of ChRFR-C167A-expressing neurons from −47.1 ± 1.8 mV to −49.9 ± 1.6 mV (Figure 3C, D). These *in vitro* data indicate that the DMH GABAergic neuron is under reciprocal regulation by leptin and lowering glucose and its activation promotes food intake.

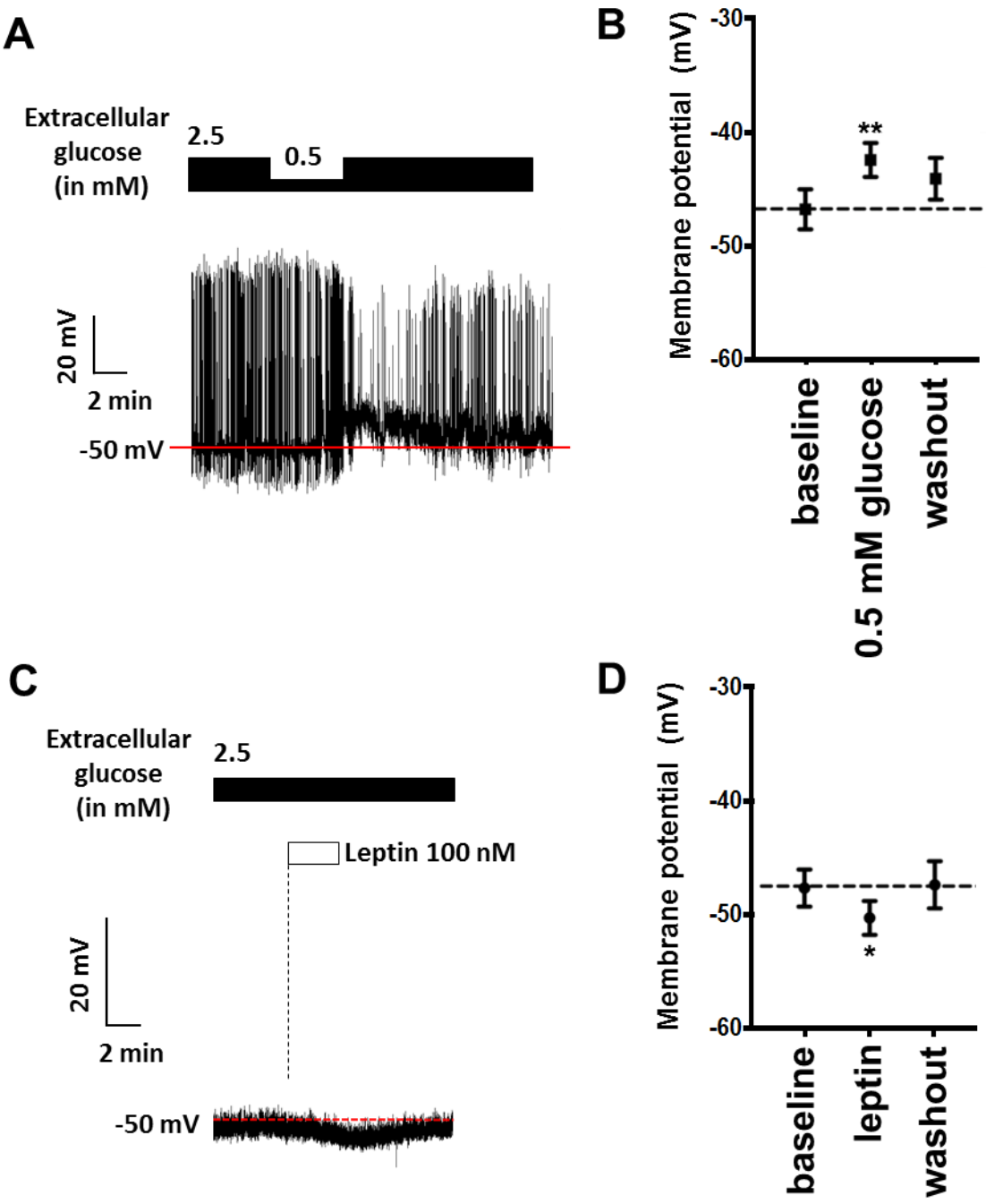

Figure 3. Lowering glucose depolarizes and leptin hyperpolarizes lowering glucose depolarizes DMH GABAergic neurons. In vitro experiment. Representative trace of membrane potential recording on ChRFR-C167A-expressing neuron in DMH. Lowering glucose (from 2.5 to 0.5 mM) depolarized (A), leptin (100 nM) hyperpolarized (C) the neuron, Averaged membrane potential before, during and after application of leptin (B) and lowering glucose (D). *p < 0.05, **p < 0.01. One-way ANOVA followed by Dunnet multiple range test.

4.4 DMH GABAergic neurons project hypothalamus and hind brain

We generated the mice expressing ChRFR-C167A selectively in GABAergic neurons in DMH via local infected AAV2 (Figure 4A). Fluorescence protein Venus in axonal fibers and terminals of DMH GABAergic neurons were acquired with a confocal laser-scanning microscope. Axonal fibers and terminals of DMH ChRFR-C167A-expressing neurons were detected by the Venus fluorescence fused with ChRFR-C167A in hypothalamic VMH, ARC (Figure 4B), PVN (Figure 4C) and DMNV in hind brain (Figure 4D).

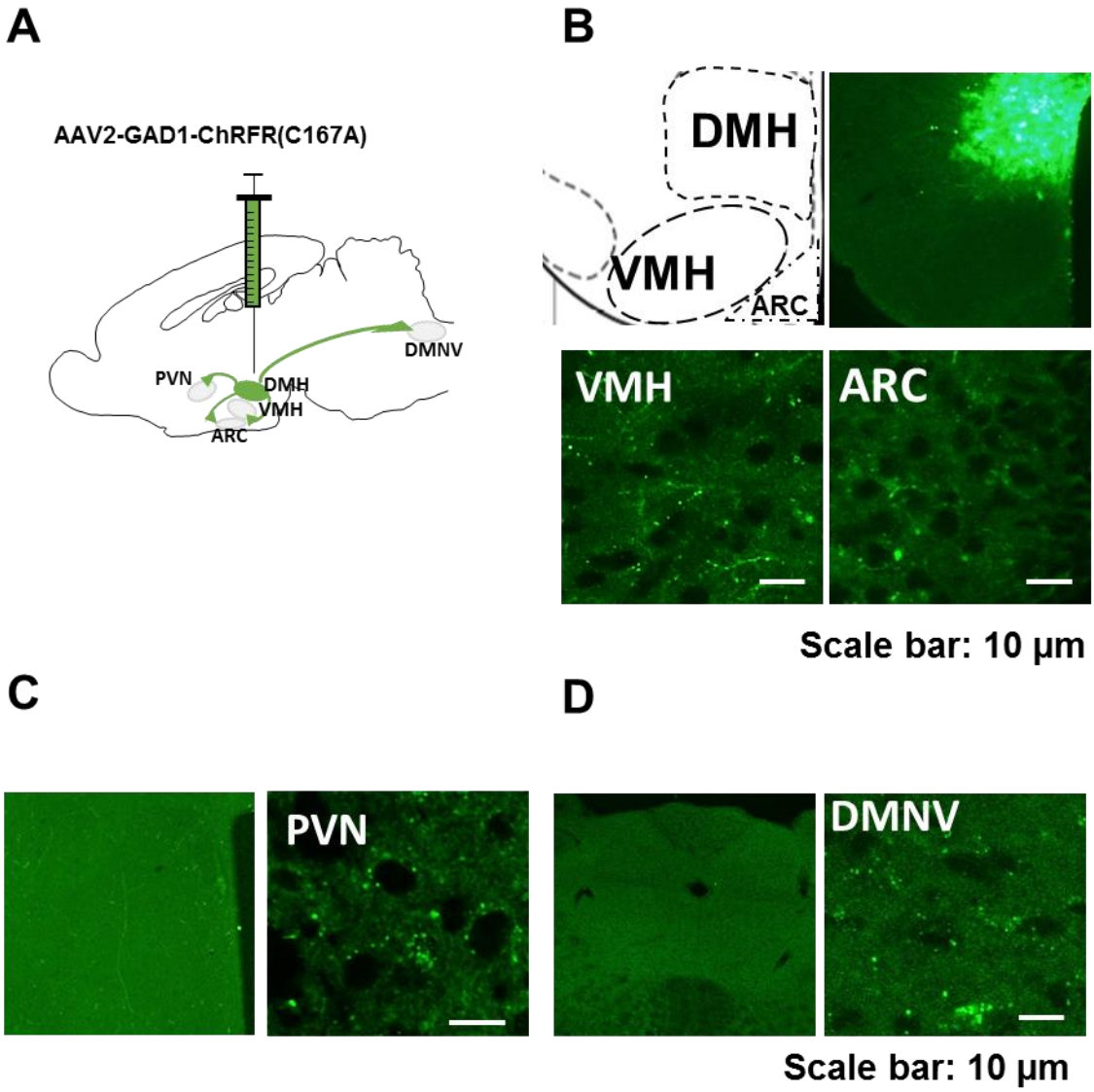

Figure 4. Axonal fibers and terminals of DMH GABAergic neurons projecting to hypothalamus and hind brain. A, Cartoon of a sagittal mouse brain depicting vector injection into DMH and projection of axons. B, Local infection of AAV2-GAD1-ChRFR-C167A virus expressing ChRFR-C167A-Venus in DMH and axon fibers and terminals. Top left, schematic figure of anatomical position of DMH, VMH, ARC nucleus. Top right, Representative low magnification image of expressing ChRFR-C167A-Venus in DMH and axon fibers and terminals in VMH and ARC. Bottom, Axonal terminals of DMH GABAergic neurons projecting to VMH (left) and ARC (right). Scale bar, 10 μm. C,D, Axonal fibers and terminals of DMH GABAergic neurons projecting PVN (C) and hind brain DMNV (D). Left side low magnification and right side high magnification. Scale bar, 10 μm.

4.5 DMH GABAergic neurons inhibit PVN neurons

We next examined the functional connection between DMH ChRFR-C167A expressing neurons and PVN neurons (Figure 5A) *in vitro*, the effect of light exposure on IPSC onto PVN neurons was examined. In 15 recordings from 5 mice, blue light exposure increased amplitude of IPSC in 47% of PVN neurons (Figure 4C, D). This result indicated a functional connection of DMH-ChRFR-C167A expressing neurons to PVN neurons. This electrophysiology result indicated DMH GABAergic neurons inhibit PVN activation.

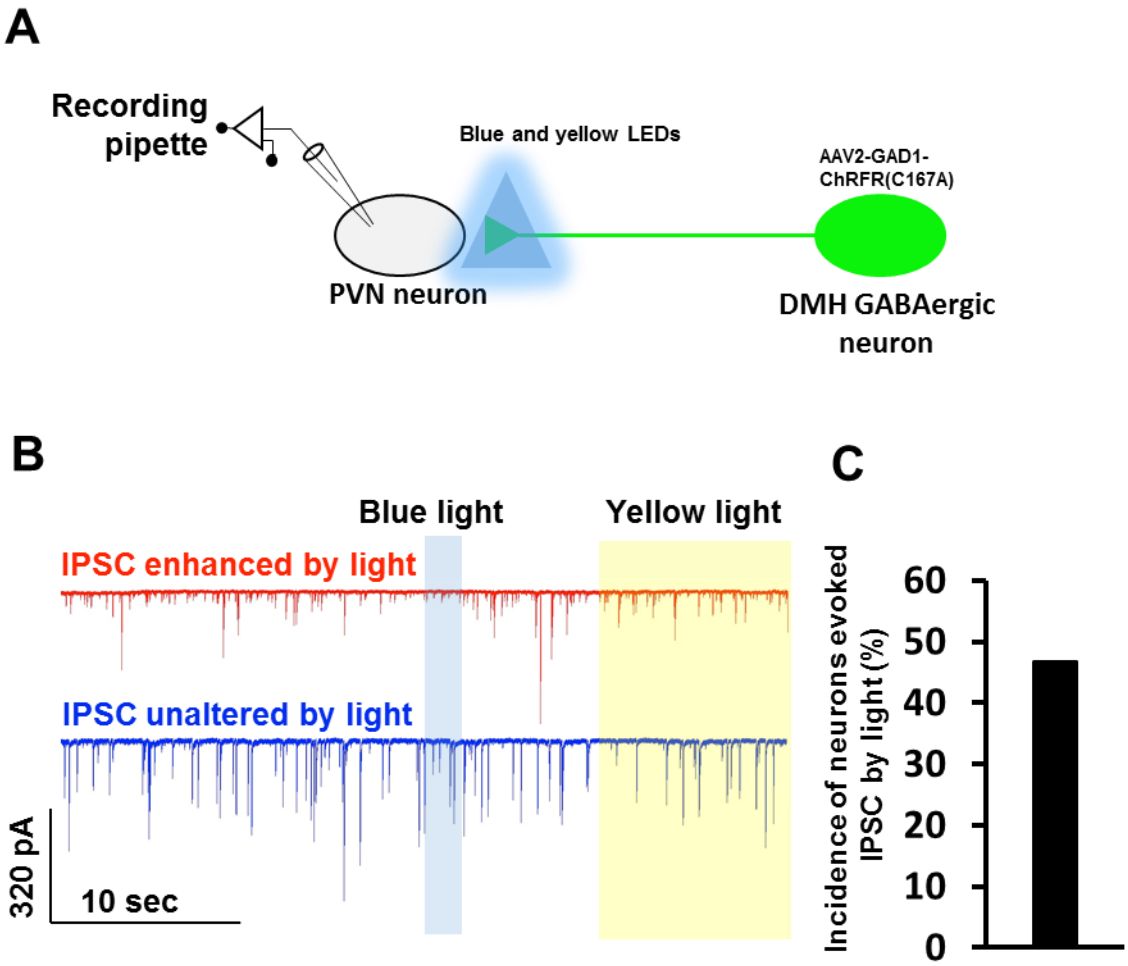

Figure 5. DMH GABAergic neurons inhibit PVN neurons by enhancing IPSC. A, Experimental schematic. B, In vitro experiment. Representative traces showing IPSC that was enhanced (top) or unaltered by light exposure (lower). C, Among 15 PVN neurons, 7 (47%) neurons showed IPSC enhanced by light exposure.

4.6 DMH GABAergic neurons projecting to PVN promote food intake

The anatomical and electrophysiological experiments confirmed the circuit from DMH GABAergic neurons to PVN. Furthermore, the role of this projection in feeding regulation was investigated, AAV2-coded ChRFR-C167A-Venus under GAD1 promoter was infected to DMH and blue (473 nm) and yellow (589 nm) laser light performed for 3 h by optic fiber implanted above PVN (Figure 6A). Food intake is significantly higher in light stimulated group at 3 h and 6 h (Figure 6B). These *in vivo* data indicated the inhibitory synaptic transmission of DMH GABAergic neurons to PVN, which promotes food intake.

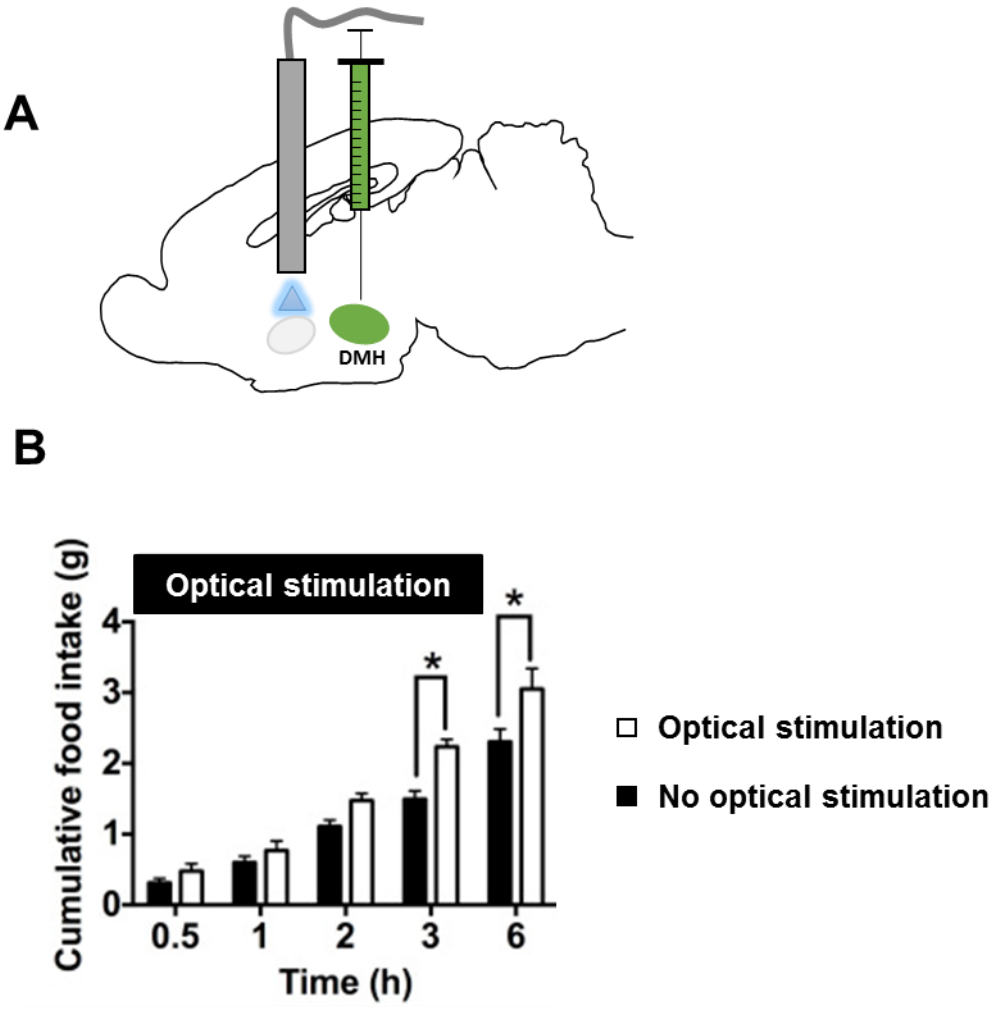

Figure 6. DMH GABAergic neurons projecting to PVN neurons promote food intake. A, Strategy for activation of axon terminal of DMH GABAergic neurons at PVN. B, DMH GABAergic neuron axon terminals at PVN were optogenetically stimulated in vivo. Cumulative food intake was increased at 3 h and 6 h after optical stimulation. Optical stimulation started at the onset of dark phase. Optical stimulation group, n = 3; non-optical stimulation group, n = 4. *$p < 0.05$ by two-way ANOVA followed by Sidak multiple range test.

5 Discussion

Optogenetic technique allows us to investigate not only morphological but also functional neuronal connections including hypothalamic circuit. Using this technique, the present study has demonstrated that DMH GABAergic neurons are activated by lowering glucose, a peripheral orexigenic signal, and inhibited by leptin, a peripheral anorexigenic signal and that they, once activated, promote food intake via inhibitory synaptic transmission to the neurons of PVN.

Mice were infected with AAV2 coded GAD1-ChRFR-C167A specifically in DMH, and at 2 weeks after injection, fluorescence protein Venus was sufficiently expressed in DMH (Figure 1B, 2B). This result was in agreement with previous findings that GABAergic neurons are abundant in DMH (25, 26).

Although orexigenic function of DMH has long been postulated, actual role of DMH in the feeding regulation has remained unclear. I have demonstrated that selective activation of DMH GABAergic neurons by optogenetic promoted food intake. Furthermore, DMH GABAergic neurons substantially projected and exerted inhibitory synaptic transmission to the neurons of PVN, the integrative center of feeding. Moreover, DMH GABAergic neurons were directly inhibited by leptin and activated by lowering glucose, the representative factors reflecting systemic energy states and implicated in physiological regulation of feeding. These results suggest that the projection of DMH GABAergic neurons to the PVN serves as a pathway to promote food intake under physiological conditions. This finding reinforces that DMH plays a role in promoting feeding.

The present study indicated that leptin inhibits orexigenic GABAergic neurons in DMH. This fits with previous report that leptin action on GABAergic neurons prevents obesity, as evidenced by the study on leptin receptor-deficient

GABAergic neurons(26). The leptin activates various brain regions, leptin receptor is expressed largest density in the hypothalamus including DMH. Moreover, it was reported that leptin activates DMH neurons expressing LepR, NPY, galanin or PrRP, and thereby stimulates thermogenesis by brown adipose tissue (13, 24, 33, 34). Additionally, leptin activates PrRP neurons to suppress food intake (13). Thus, it is suggested that in DMH leptin inhibits GABAergic neurons to suppress energy intake and activates another subpopulations of neurons to promote energy expenditure and/or suppress energy intake and that all these actions contribute to reduction of body weight. The opposing effects of leptin on different subpopulations of neurons have been well known in ARC where leptin inhibits NPY/AgRP neurons and activates POMC neurons.

The present study showed that DMH GABAergic neurons project to PVN, being consistent with previous reports that DMH neurons, including GABAergic neurons (35), project to PVN (36, 37) GABA injection to PVN induces feeding. The neurons of the PVN express a various neuropeptides that are critically involved in homeostatic regulation. Although the target neuron in PVN remains to be identified, the neurons expressing oxytocin, CRH, nesfatin-1 and/or MC3/4 (38), potent anorectic neuropeptides and receptor, are the candidates.

The DMH GABAergic projection to PVN may regulate not only feeding but also other functions, in considering diverse functions of DMH and PVN. SCN, the master clock governing circadian rhythm, projects to DMH (39). PVN is part of a multi-synaptic pathway that is responsible for the circadian regulation of several functions including glucocorticoid and melatonin release (40) and feeding behavior (41, 42). These findings suggest a possible functional connection between DMH and PVN for circadian regulation. The DMH GABAergic projection

to PVN, found in the present study, could serve as a neuro-circuit that relays DMH to PVN for circadian regulation.

I found that light activation of the presynaptic terminal of DMH ChRFR-C167A-expressing GABAergic neurons evoked IPSC onto PVN neurons. Open and close time constants of ChRFR-C167A are slower than those of Channelrhodopsin2 under blue light exposure (32). Therefore, we expected that photo-activation of ChRFR-C167A would evoke IPSCs continually and hence increase both amplitude and frequency of total IPSC for a certain period, unlike channelrhodopsin2 that evokes single large IPSC immediately after photo-activation. However, our recording showed that IPSC frequency was not changed, although the cumulative distribution of IPSC amplitude was increased. The unchanged IPSC frequency could be due to possible additional neuronal circuits. DMH GABAergic neurons may project to not only PVN neurons but also GABAergic interneurons around PVN or presynaptic terminals of GABAergic neurons onto PVN neurons. Light-evoked GABA release from presynapses of DMH GABAergic neurons could not only directly suppress PVN neurons but also inhibit GABAergic interneurons and/or presynaptic terminals that secondarily suppress PVN neurons. The net effects of these distinct neuro-circuits could result in little change in IPSC frequency.

Our findings suggested that DMH GABAergic neurons project axonal terminals to 47% of PVN neurons (Figure 4C, 5B), similarly to ARC AgRP neurons that project to 49% of PVN neurons (43). However, activation of DMH GABAergic neurons took more than 2 h to increase food intake, whereas activation of AgRP neurons immediately increased food intake (43). Although the mechanisms underlying the different time course of feeding behavior following DMH

GABAergic and ARC AgRP neuron activation remain to be elucidated, we can speculate a few explanations for the delayed effect of the DMH GABAergic neuron activation on food intake. First, it is still unclear whether DMH GABAergic neurons co-release orexigenic neuropeptide(s). By contrast, AgRP neurons release AgRP and NPY, well-established most potent orexigenic neuropeptides. Secondly, DMH GABAergic neurons were found to project to not only PVN but other feeding regulatory nuclei including ARC, VMH, LH, and DMNV, in consistent with previous reports (21, 44). Some of these projections could inhibit orexigenic neurons and thereby cancel out the effect of the PVN neuron inhibition that increases food intake, which may take place for the initial a few hours. In contrast, AgRP neurons project to the nuclei where anorexigenic neurons dominate (45) (46). It was also observed that the light administration on DMH and that on PVN increased food intake in slightly different time courses. This could be due to that the former activates additional subpopulations of DMH GABAergic neurons.

6 Conclusion

DMH GABAergic neurons are regulated by metabolic signals leptin and glucose and, once activated, promote food intake via inhibitory synaptic transmission to PVN.

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8 List of abbreviations

AAV	-	Adeno-associated Virus
AgRP	-	Agouti-related Protein
ANOVA	-	Analysis of Variance
ARC	-	Arcuate Nucleus of Hypothalamus
CART	-	Cocaine-amphetamine-related transcript
CCK	-	Cholecystokinin
CNS	-	Central Nervous System
CRH	-	Corticotropin-releasing Hormone
CSF	-	Cerebrospinal Fluid
DMH	-	Dorsomedial Hypothalamic Nucleus
DMNV	-	Dorsal motor nucleus of the Vagus
HEPES	-	Hydroxyethyl Piperazine Ethane Sulfonic Acid
IPSC	-	Inhibitory post synaptic current
LED	-	Light emitting diode
LHA	-	Lateral Hypothalamic Area
MC4R	-	Melanocortin-4 Receptor
NPY	-	Neuropeptide Y
NUCB2	-	Nucleobindin-2
OCT	-	Optimal Cutting Temperature
PB	-	Phosphate Buffer
PFA	-	Paraformaldehyde
POMC	-	Pro-opiomelanocortin
PVN	-	Paraventricular Nucleus of Hypothalamus
VMH	-	Ventromedial Hypothalamus

WHO	-	World Health Organization
α-MSH	-	alpha-Melanocyte-stimulating Hormone
GABA	-	ɣ-aminobutyric acid

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