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GABAergic Projections from Lateral Hypothalamus to Paraventricular Hypothalamic Nucleus Promote Feeding

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Lesions of the lateral hypothalamus (LH) cause hypophagia. However, activation of glutamatergic neurons in LH inhibits feeding. These results suggest a potential importance for other LH neurons in stimulating feeding. Our current study in mice showed that disruption of GABA release from adult LH GABAergic neurons reduced feeding. LH GABAergic neurons project extensively to the paraventricular hypothalamic nucleus (PVH), and optogenetic stimulation of GABAergic LH → PVH fibers induced monosynaptic IPSCs in PVH neurons, and potently increased feeding, which depended on GABA release. In addition, disruption of GABA-A receptors in the PVH reduced feeding. Thus, we have identified a new feeding pathway in which GABAergic projections from the LH to the PVH promote feeding.

Key words: **feeding; GABA; LH; optogenetics; PVH**

Introduction

Recent studies have identified important, neuropeptide-expressing hypothalamic neurons that regulate feeding (Luquet et al., 2005; Leinninger et al., 2011; Atasoy et al., 2012; Krashes et al., 2014). However, a large number of neurons that do not express known peptides may also have critical roles in feeding (Xu and Tong, 2011). Lesions of the lateral hypothalamus (LH) lead to hypophagia (Bernardis and Bellinger, 1996), suggesting that the net effects of LH neurons are to promote eating. However, it has been recently shown that selective activation of glutamatergic neurons in the LH inhibits feeding (Jennings et al., 2013). These contrasting results suggest a potential importance for other types of LH neurons in promoting feeding.

Increasing evidence supports an importance for GABA as a neurotransmitter, in mediating feeding behavior. However, the vast majority of previous studies regarding GABA action focus on GABAergic AgRP neurons (Wu et al., 2009; Atasoy et al., 2012; Krashes et al., 2013). At the same time, the functions of numerous non-AgRP GABAergic neurons elsewhere in the hypothalamus have not been elucidated. Interestingly, our previous study showed a selective role for GABA release from a subset of Arc GABAergic neurons, which express Cre driven by the rat insulin promoter (Rip-Cre) (Song et al., 2010), in energy expenditure regulation, but not in feeding (Kong et al., 2012), suggesting the importance of nonpeptidergic GABAergic hypothalamic neurons in regulating metabolism. However, the potential role of GABAergic neurons in other hypothalamic sites, including LH, remains largely unknown.

Previous pharmacological studies suggest a role for GABA in the paraventricular nucleus of hypothalamus (PVH) in feeding regulation (Pu et al., 1999; Stanley et al., 2011). However, it is unknown whether the GABA action is mediated directly or indirectly by PVH neurons given the potential widespread diffusion associated with the GABA administration. Further electrophysiological studies suggest that GABA may mediate the effects of NPY on PVH neurons (Melnick et al., 2007), and optogenetic studies demonstrate that GABA release from AgRP neurons onto PVH neurons directly mediates feeding behavior (Atasoy et al., 2012). However, the physiological relevance of GABA release to PVH neurons is not clear. Of note, GABA release from AgRP neurons is surprisingly not required for feeding behavior (Krashes et al., 2013), suggesting that other redundant AgRP neural pathways exist for promoting food intake.

We and others have shown that Cre expression driven by the pancreas duodenum homeobox 1 promoter (Pdx1) exhibits abundant expression in the LH region caudal to the PVH, dorsomedial hypothalamus (DMH), arcuate nucleus, and a few other brain sites (Honig et al., 2010; Song et al., 2010). By taking advantage of this Cre expression, our current study identified a

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potent role of LH GABAergic neurons in promoting feeding through projections to the PVH, in which GABA release is required. Our findings suggest that the physiological function of this projection is to promote nocturnal feeding.

Materials and Methods

Animal care. Mice were housed at 21°C–22°C with a 12 h light/12 h dark cycle with food and water provided *ad libitum*. Animal care and procedures were approved by the University of Texas Health Science Center at Houston Institutional Animal Care and Use Committee. Rip-Cre and Pdx1-Cre mice were described previously (Song et al., 2010). Mice with Cre expression driven by the single-minded 1 gene promoter (Sim1-Cre) were described previously and these mice show Cre expression mainly in the PVH, but also in the other brains sites, including the amygdala and nucleus of lateral olfactory tract (Balthasar et al., 2005). Mice with the conditional allele of vesicular GABA transporter (*Vgat*, also named *Slc32a1*) and mice with the conditional allele of GABA-A receptor γ2 subunit were reported previously (Wulff et al., 2007; Tong et al., 2008). Breeding pairs (male *Pdx1-Cre:Vgat*$^{flox/flox}$ mice and female *Vgat*$^{flox/flox}$ mice; and male *Sim1-Cre:γ2*$^{flox/flox}$ mice and female *γ2*$^{flox/flox}$ mice) were maintained to generate the study subjects. Male and female *Vgat*$^{flox/+}$ mice were interbred to generate wild-type and *Vgat*$^{flox/flox}$ mice for AAV-Cre injection study described below. In addition, *Sim1-Cre* mice were bred to Ai9 reporter mice (Madisen et al., 2010) to generate *Sim1-Cre:Ai9* mice for electrophysiological recording.

Studies with stereotaxic injections. At the start of surgical procedures, mice were anesthetized with ketamine and xylazine and placed on a stereotaxic frame (David Kopf Instruments). AAV-Cre-GFP vectors were purchased from the viral core facility of the University of Pennsylvania and were stereotaxically injected into bilateral LH (50 nl) with the following coordinates: bregma −1.6 mm; midline ±1.0 mm; dorsal surface −5.1 mm, of wild-type or *Vgat*$^{flox/flox}$ mice using a 0.5 μl Hamilton syringe controlled by a nano-injector (Stoelting). The injection speed was 0.5 nl/min, and the syringe was withdrawn 15 min after the final injection. Mice were used for experiments after a 2 week period recovery. We aimed to delete *Vgat* in the LH region with Pdx1-Cre expression. Because Pdx1-Cre is only expressed in the LH region located caudal to the PVH (Song et al., 2010), we targeted AAV-Cre-GFP to this specific LH region. To avoid the concern that *Vgat* deletion in nearby non-LH regions affects feeding, we excluded all mice with obvious AAV-Cre-GFP expression in non-LH regions as defined by Paxinos and Franklin (2004). We scored those mice with AAV-Cre-GFP expression limited to bilateral LH posterior to the PVH but across at least 1 mm in the rostral–caudal dimension of LH as hit.

In situ *hybridization.* Then RNAscope Multiple Fluorescent Assay, a novel *in situ* hybridization technique with a unique "double Z" probe design, which greatly increases signal-to-noise ratio and can visualize RNA transcripts at a single molecular level (Wang et al., 2012; Xu et al., 2013), was used to detect transcripts in the brain (Advanced Cell Diagnostics). Matched LH section from *Vgat*$^{flox/flox}$ and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice ($n = 3$ each) were used to estimate percentage of LH neurons with *Vgat* deletion by Pdx1-Cre.

Food intake measurements. Weekly body weight was monitored in all genotypes fed standard mouse chow (Teklad F6 Rodent Diet 8664, 4.05 kcal/g, 3.3 kcal/g metabolizable energy, 12.5% kcal from fat, Harlan Teklad) from 4- to 5- week-old mice. For 24 h feeding pattern measurements, food intake was determined every hour for 24 h and averaged for comparison between genotypes.

Immunohistochemistry assays. For immunohistochemistry studies, we used a previously described protocol (Xu et al., 2012). Primary antibody against γ2 (NB300-190, Novus Biologicals) was used. Sections were visualized and photographed with a TCS SP5 confocal microscope (Leica). The images from brain sections incubated with the γ2 antibody were obtained using an Axioimager fluorescence microscope with Axiocam digital camera (Zeiss) and then exported in grayscale.

Brain slice electrophysiological recordings. Brain slices were prepared from mice (4–6 weeks of age) anesthetized with isoflurane. Coronal brain slices (350 μm) were cut in ice-cold artificial CSF (aCSF) containing the following (in mM): 124 NaCl, 5 KCl, 1.25 NaH_2PO_4, 1.3 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, and 11 glucose and adjusted to pH 7.4 by bubbling with 95% O_2/5% CO_2. Slices containing the PVH and/or LH were immediately transferred to a holding chamber and submerged in oxygenated aCSF. Slices were maintained for recovery for at least 1 h at 34°C before transferring to a recording chamber. aCSF at 32°C was perfused into slice chambers with ~2 ml/min flow rate regulated by miniplus3 peristaltic pump (Gilson).

Sim1-Cre-expressing neurons in brain slices from reporter mice were identified by ds-Red fluorescence emission using a custom filter set and then visually targeted with infrared differential interference optics. Evoked IPSCs in Sim1 neurons were also recorded in voltage-clamp using the same internal solution. Stimuli (0.1 ms duration) were applied through bipolar stimulating electrodes (FHC) placed into the surrounding area of the PVH. The stimulus intensity was adjusted to the level (ranging from 100 μA to 1 mA) at which an evoked current was ~90% of the amplitude of the maximal response. Kynurenic acid (1 mM) was used to block glutamate-mediated EPSCs. At the end of experiments, 20 μM bicuculline was applied to the bath to confirm the nature of GABA-A receptor-mediated currents. All recordings were made using a Multiclamp 700B amplifier (Molecular Devices), and data were digitized at 10 kHz and filtered at 2 kHz using pClamp 10.3 (Molecular Devices). Data were analyzed off-line with Clampfit (Molecular Devices) and Mini-Analysis software (Snaptosoft).

Channelrhodopsin 2 (ChR2)-assisted circuitry mapping and feeding. The ChR2-assisted circuitry mapping was performed similarly as previously described (Kong et al., 2012). Adeno-associated vectors with Cre-dependent expression of ChR2 and yellow fluorescent protein (YFP), AAV-FLEX-ChR2-YFP (generated by Dr. Ben Arenkiel) (Herman et al., 2014) were delivered to bilateral LH of 6- to 7-week-old male *Pdx1-Cre* and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice, and the recordings were performed 2–3 weeks after viral delivery. The same coordinates as described above for AAV-Cre-GFP delivery were used for the viral delivery. IPSCs were recorded from neurons in the posterior PVH with high Cl^- internal solution (in mM: 140 CsCl, 1 BAPTA, 10 HEPES, 5 $MgCl_2$, 5 Mg-ATP, 0.3 Na2GTP, and 10 QX-314, pH 7.35). To photostimulate the ChR2-expressing fibers, a laser source (473 nm; Opto Engine) was used with 1 Hz frequency stimulation. A previous estimate shows that all PVH neurons are Sim1-Cre positive (Kublaoui et al., 2008); thus, recording experiments on field stimulation-evoked IPSCs in Sim1 neurons and photostimulation-evoked IPSCs in PVH neurons target the same group of neurons.

For optogenetic feeding studies, an optic fiber cannula was implanted immediately after the completion of AAV-FLEX-ChR2-YFP delivery. The optic fiber cannula (200 μm in diameter and 0.39 NA, Thorlabs) was implanted ~0.4 mm above the posterior PVH with the following coordinates: bregma: 1.0 mm; midline: 0 mm; dorsal surface: 4.8 mm. Targeted expression of AAV-FLEX-ChR2-YFP in LH Pdx1-Cre neurons and correct implantation of optic fibers were verified by *post hoc* examination. Light power was measured by a PM 100D meter with an S121C sensor (Thorlabs). An online program provided by Dr. Deisseroth laboratory at Stanford University (http://web.stanford.edu/group/dlab/cgi-bin/graph/chart.php) was used for estimating the actual light power on local fibers. After a 2 week recovery from surgery, mice were individually housed and regular chow diet was provided in a Petri dish. Fiber optic cables (1.5 m long, 200 μm diameter; Thorlabs) were firmly attached to the implanted fiber optic cannula with an opaque mating sleeve. Mice were allowed at least 3–4 d to acclimate before experimental sessions. Blue laser stimulation (473 nm, Opto Engine, light power exiting the fiber tip 3 mW) at 20 Hz with 40 ms duration for 10 min generated by Master 8 (AMPI) was used to test feeding levels in early morning (9:00 to 11:00 A.M.), which were compared with the feeding levels during periods of 10 min before and that after laser stimulation.

Statistical analysis. Data are mean ± SEM, and comparison between means was performed with an appropriate statistical analysis specified in figure legends, using Prism 6.0 with $p < 0.05$ indicating statistical significance. Tukey multiple comparison was used for two-way ANOVA tests.

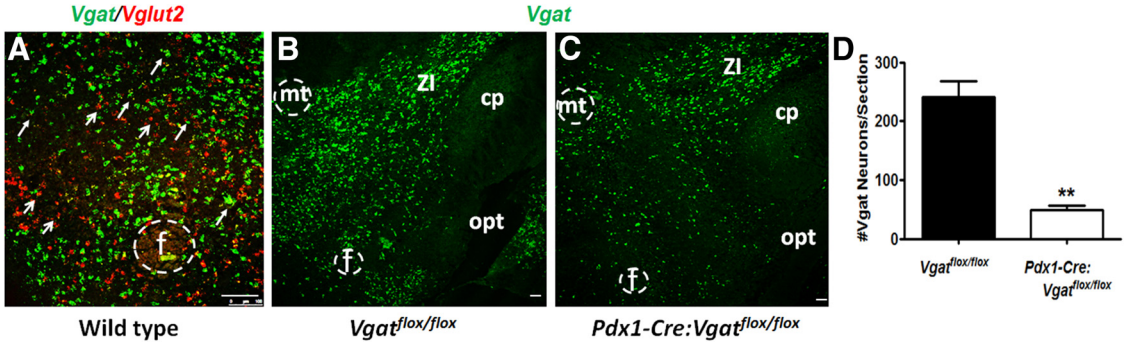

Figure 1. *Vgat* and *Vglut2* expression in the LH. ***A***, *Vgat* (green) and *Vglut2* (red) mRNA expression by dual fluorescent *in situ* hybridization in the LH. Open arrows indicate *Vglut2*-expressing neurons. Solid arrows indicate *Vgat*-expressing neurons. ***B***, ***C***, *Vgat in situ* hybridization in the LH of *Vgat*$^{flox/flox}$ (***B***) and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice (***C***). ***D***, Average number of *Vgat*-expressing neurons in *Vgat*$^{flox/flox}$ and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice ($n = 3$ each). cp, Cerebral peduncle; f, fornix; mt, mammilothalamic tract; opt, optic tract; ZI, zona incerta. $^{**}p < 0.01$, Student's *t* test. Scale bars, 100 μm.

Results

To examine the expression pattern of both *Vgat* and *Vglut2* (also known as *Slc32a1* and *Slc17a6*, respectively, required for presynaptic respective GABA and glutamate release) in the LH, we performed dual-color *in situ* hybridization for both genes. We showed that both mRNAs were abundantly expressed in the LH (Fig. 1*A*). Importantly, none to few LH neurons coexpressed *Vgat* and *Vglut2*, indicating that GABAergic neurons and glutamatergic neurons are largely segregated in the LH (Fig. 1*A*). Although the role of glutamatergic LH neurons has been illustrated (Jennings et al., 2013), the function of GABAergic LH neurons in feeding regulation is, however, unclear.

We previously reported that Cre recombinase driven by the pancreas duodenum homeobox 1 promoter (Pdx1-Cre) is abundant in the LH, the DMH, the arcuate (Arc) and the preoptic area in the hypothalamus (Song et al., 2010). Importantly, in *Pdx1-Cre:Vgat*$^{flox/flox}$ mice, *Vgat* was largely deleted in throughout the LH (Fig. 1*B*,*C*) and was also reduced in other Pdx1-Cre expressing brain sites (data not shown). Based on the comparison of the *Vgat* expression pattern between controls and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice, it appears that the LH regions with higher levels of *Vgat* expression showed more *Vgat* deletion (Fig. 1*B*,*C*). An estimate from matched LH sections from *Vgat*$^{flox/flox}$ and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice ($n = 3$ each) showed that ~80% LH GABAergic neurons expressed Pdx1-Cre (241 ± 27 vs 49 ± 7 GABAergic neurons in the LH of *Vgat*$^{flox/flox}$ and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice, respectively) (Fig. 1*D*). These data indicate that Pdx1-Cre targets a major subset of GABAergic neurons in the LH. Thus, Pdx1-Cre mice, when combined with stereotaxic LH injections, allowed us to specifically target a major subset of GABAergic LH neurons.

To examine the physiological role of GABA release from LH GABAergic neurons, we aimed to disrupt GABA release from LH neurons by stereotaxically injecting adeno-associated viral vectors that express Cre (AAV-Cre-GFP) to the LH of *Vgat*$^{flox/flox}$ mice. To examine the efficiency of AAV-Cre-GFP in deleting the *Vgat* gene, we stereotaxically delivered the Cre vector to one side of LH. Efficient *Vgat* deletion was validated by specific loss of *Vgat* mRNAs in the AAV-Cre-GFP infected area, compared with the other side of LH with intact *Vgat* expression (Fig. 2*A*,*B*). Based on *post hoc* analysis and the scoring criterion (see Materials and Methods), 5 *Vgat*$^{flox/flox}$ mice that received injections were scored as hit (Fig. 2*C–H*). *Vgat* deletion in LH significantly reduced feeding and body weight at 3 weeks after injection (Fig. 2*I*,*J*), indicating that GABA release from LH neurons is required to maintain normal feeding.

To further illustrate the neural circuits that GABAergic LH neurons act upon to regulate feeding, we aimed to optogenetically stimulate these neurons using Pdx1-Cre expression in the LH. To selectively stimulate LH Pdx1-Cre neurons, we delivered Cre-dependent AAV-FLEX-ChR2-YFP vectors to the bilateral LH of Pdx1-Cre mice (Fig. 3*A*). As expected, YFP-expressing neurons were found selectively in the LH (Fig. 3*B–D*). Interestingly, YFP-expressing fibers were not found in the anterior PVH, but abundant in the posterior PVH (Fig. 3*E*,*F*). Upon laser stimulation of YFP-expressing fibers in the PVH, 10 of 12 posterior PVH neurons in control *Vgat*$^{flox/flox}$ mice showed IPSCs (Fig. 3*G*). In contrast, 0 of 12 neurons in *Pdx1-Cre:Vgat*$^{flox/flox}$ mice exhibited IPSCs (Fig. 3*G*). These results support the existence of a GABAergic LH → PVH circuit.

To examine the effect of photostimulation of YFP-expressing fibers in the PVH on feeding behavior, we implanted a fiberoptic directly above the posterior PVH (Fig. 3*H*). Low-intensity blue laser stimulation (3 mW) potently increased feeding during a 10 min photostimulation period in well-fed *Pdx1-Cre* mice (early morning) but had no obvious effect on feeding behavior in *Pdx1-Cre:Vgat*$^{flox/flox}$ mice (Fig. 3*I*). Notably, the distance between the tip of the fiberoptic and the closest LH Pdx1-Cre neurons was ~1 mm, and 3 mW laser intensity used for feeding behavior, according to the estimation (the Deisseroth laboratory at Stanford University), will be reduced to 0.81 mW/mm^2 at LH Pdx1-Cre neuronal soma, which is not sufficient to activate the neurons (Betley et al., 2013). These results, in conjunction with the previously observed hyperphagic GABA action in the PVH (Pu et al., 1999; Atasoy et al., 2012), indicate that activation of the GABAergic LH → PVH circuit promotes feeding behavior.

Despite extensive studies on GABAergic input to the PVH (Cowley et al., 2001; Cole and Sawchenko, 2002; Kalra and Kalra, 2004; Atasoy et al., 2012), its function in feeding, especially in physiological conditions, remains unclear. For example, reduced GABAergic tone to PVH does not contribute significantly to hypophagia following AgRP lesions (Wu et al., 2009). In addition, loss of GABA release from AgRP neurons has no significant impact on the feeding behavior induced by AgRP neuron activation (Krashes et al., 2013), suggesting that GABA release from AgRP neurons is not the sole transmitter that mediates feeding behavior by AgRP neuron activation. To more precisely examine GABAergic input to the PVH, we disrupted GABAergic input to

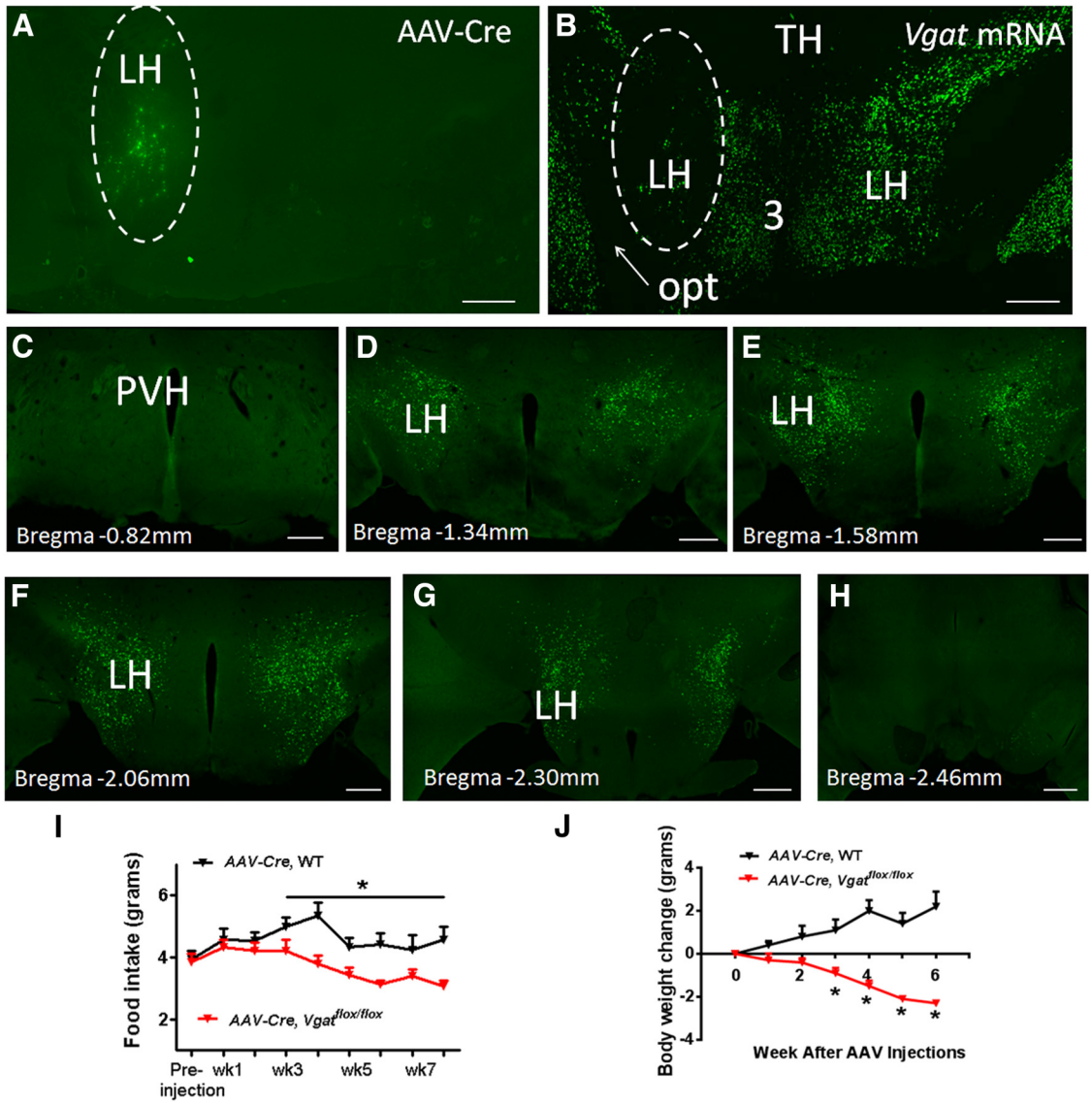

Figure 2. Deletion of *Vgat* in adult LH neurons leads to reduced feeding. ***A***, ***C–H***, GFP expression after AAV-Cre-GFP vector delivery to the brain. ***B***, *In situ* hybridization for *Vgat* in the hypothalamus. ***A***, ***B***, Two adjacent sections from one animal showing delivery of AAV-Cre-GFP vectors only to one side LH of $Vgat^{flox/flox}$ mice (***A***) and expression of *Vgat* mRNA (***B***). ***A***, Micrograph was taken directly from sections freshly cut from frozen brains. White dashed line circle indicates the injected area. ***B***, Micrograph showing sparsely distributed *Vgat* mRNA on the injected side LH (white dashed line circle) with AAV vector delivery, which was in contrast with the abundant *Vgat* mRNA on the opposite side, suggesting effective deletion of *Vgat* by AAV-Cre vectors. ***C–H***, A representative case showing the expression profile of AAV-Cre-GFP vectors, from rostral to caudal levels as indicated by bregma levels, in the LH after stereotaxic delivery of the vectors to bilateral LH of $Vgat^{flox/flox}$ mice. A similar AAV-Cre injection profile was found in 4 other cases that were also scored as hit and peer-reviewed. ***I***, ***J***, Weekly food intake (***I***) and changes in body weight (***J***) measured for 8 weeks after AAV-vector injections. ***I***, ***J***, Data are mean ± SEM; $n = 5–8$. *$p < 0.05$ (unpaired Student's *t* tests). LH, Lateral hypothalamus; Opt, optic tract; PVH, paraventricular hypothalamic nucleus; TH thalamus. Scale bar, 250 μm.

the PVH by deleting γ2 subunit of GABA-A receptors. Toward this end, we crossed Sim1-Cre mice with $\gamma2^{flox/flox}$ mice to generate *Sim1-Cre:*$\gamma2^{flox/flox}$ mice (Wulff et al., 2007; Xu et al., 2013). In these mice, γ2 was largely deleted in the PVH (Fig. 4*A*,*B*), in the supraoptic nucleus and the nucleus of olfactory tract but was not obviously altered in other Sim1-Cre-expressing regions due to undetectable endogenous γ2 expression levels (data not shown). To examine the consequence of γ2 deletion on GABA-A receptors on PVH neurons, we performed whole-cell recording on PVH neurons in brain slices for GABA-A receptor-mediated currents IPSCs. Compared with control mice, evoked IPSCs in PVH neurons of *Sim1-Cre:*$\gamma2^{flox/flox}$ mice were diminished (Fig. 4*C–E*), confirming that the function of GABA-A receptors was disrupted. Interestingly, *Sim1-Cre:*$\gamma2^{flox/flox}$ mice showed reduced feeding (Fig. 4*F*), which was largely due to a specific reduction in nocturnal feeding (Fig. 4*G*). Thus, GABAergic input to PVH neurons promotes feeding, especially during the nocturnal period.

Discussion

Here we identify a novel direct GABAergic projection from the LH to the PVH that promotes feeding. This is the second group of hypothalamic GABAergic neurons, alongside AgRP neurons, that have been identified to elicit feeding (Fig. 4*H*). GABA release is not required for mediating AgRP neurons in promoting feeding (Krashes et al., 2013), whereas it is required for LH GABA neurons. Thus, unlike AgRP neurons, GABA is the sole transmitter that mediates the feeding behavior of the LH → PVH projection.

LH GABAergic neurons promote feeding, whereas glutamatergic ones inhibit feeding (Jennings et al., 2013), suggest-

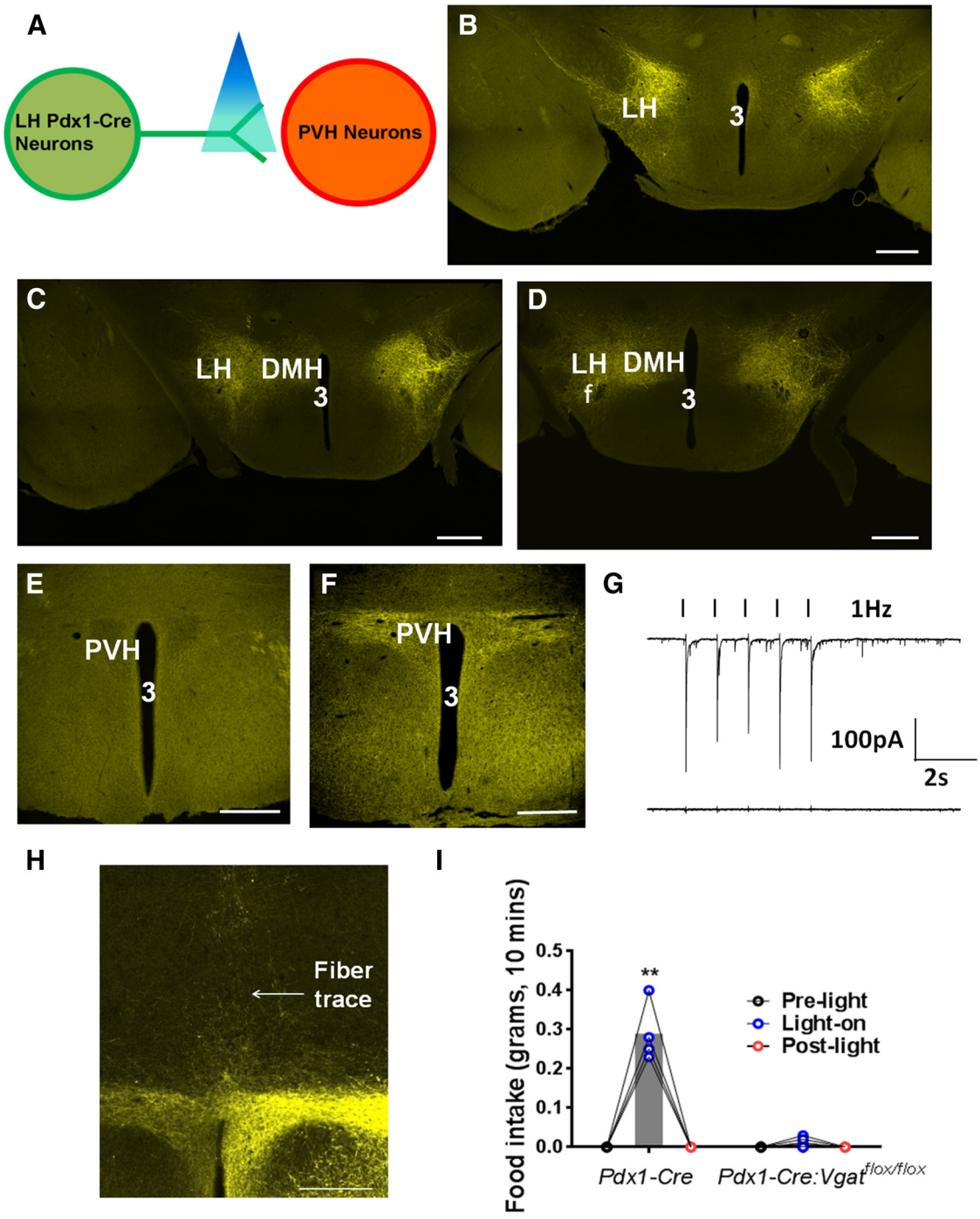

Figure 3. Optogenetic stimulation of GABAergic fibers from LH Pdx1-Cre neurons in the PVH promotes feeding in well-fed animals. ***A***, Diagram showing ChR2-assisted circuitry mapping. ***B–D***, AAV-FLEX-ChR2-YFP expression pattern in bilateral LH of *Pdx1-Cre* mice at 2 weeks after viral delivery at the levels of bregma −1.34 mm (***B***), −1.46 mm (***C***), and −1.58 mm (***D***). ***E***, ***F***, YFP-expressing fibers in the anterior (***E***) and the posterior PVH (***F***). ***G***, IPSCs elicited by blue laser (black ticks) at 1 Hz in *Pdx1-Cre* (top) and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice (bottom). ***H***, A representative implantation of optic fiber cannula above the posterior PVH. ***I***, Feeding response to laser stimulation during 10 min period in well-fed *Pdx1-Cre* and *Pdx1-Cre:Vgat*$^{flox/flox}$ mice. Data are mean ± SEM; $n = 4–6$. $^{**}p < 0.01$ (two-way ANOVA tests). 3, Third ventricle; DMH, ventromedial hypothalamus; LH, lateral hypothalamus; PVH, paraventricular hypothalamic nucleus. Scale bar, 250 μm.

ing divergent roles for LH neurons in feeding regulation. Lesions of LH cause hypophagia (Bernardis and Bellinger, 1996), suggesting that the effect of LH GABAergic neurons on feeding dominates over glutamatergic ones. Neurotensin and melanin-concentrating hormone (MCH) neurons represent two known groups of LH GABAergic neurons. Because LH neurotensin neurons are activated by leptin to reduce food intake (Leinninger et al., 2011), it is unlikely that these neurons contribute significantly to the observed feeding promotion. Lesions of MCH neurons cause hypophagia and reduced body weight (Alon and Friedman, 2006), and MCH partially colocalizes with Pdx1-Cre expression in the LH (data not shown), suggesting that GABA release from MCH neurons might contribute to the feeding behavior. However, two recent studies with optogenetic stimulation of MCH neurons did not report any changes in feeding behavior (Domingos et al., 2013;

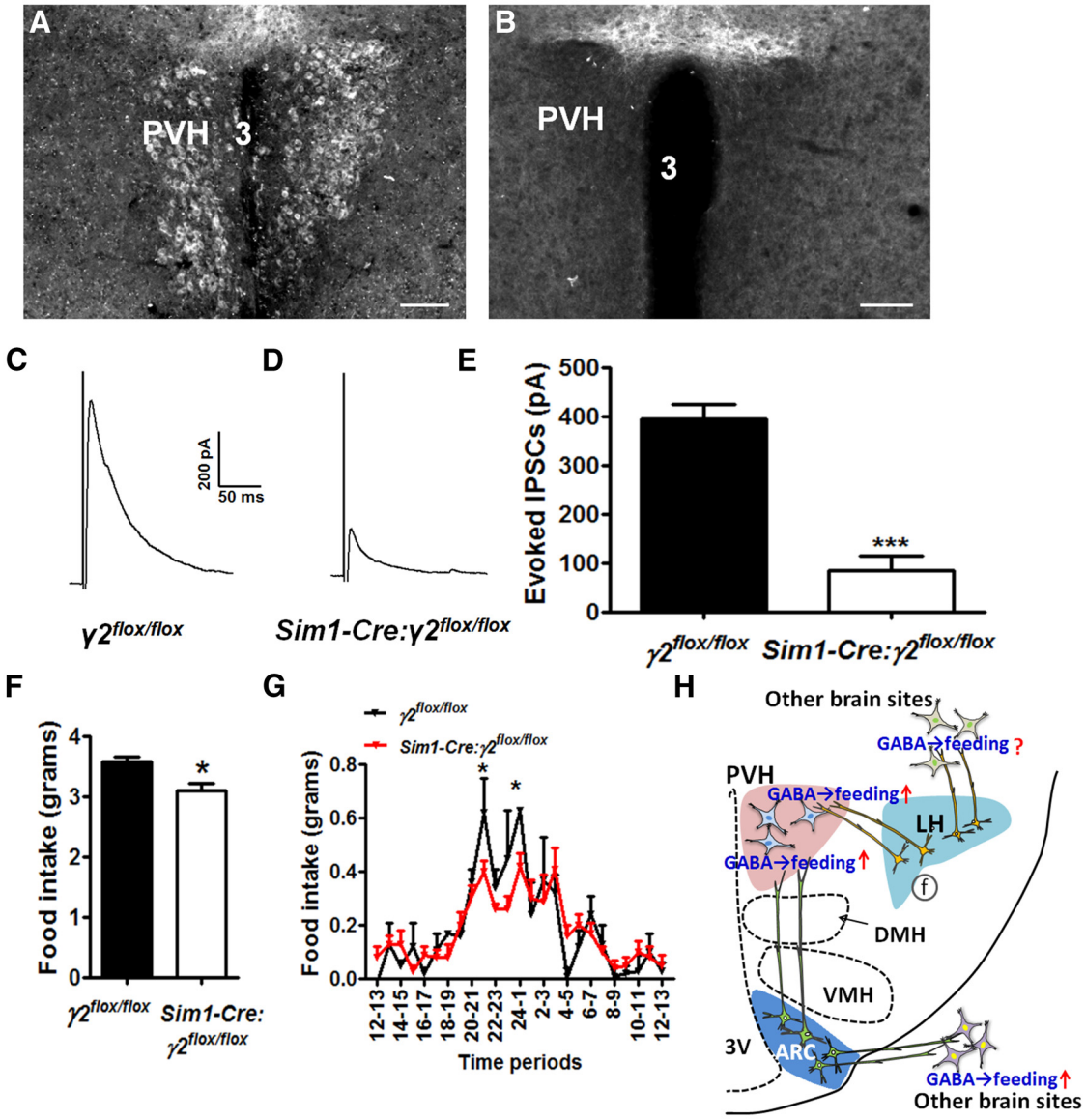

Figure 4. Deletion of GABA-A receptor γ2 subunit in PVH neurons impairs GABAergic input and reduces feeding. ***A***, ***B***, Immunostaining of GABA-A receptor γ2 subunit in $\gamma2^{flox/flox}$ mice (***A***) and *Sim1-Cre:*$\gamma2^{flox/flox}$ mice (***B***). ***C***, ***D***, Representative traces showing evoked IPSCs in PVH neurons of $\gamma2^{flox/flox}$ mice (***C***) and *Sim1-Cre:*$\gamma2^{flox/flox}$ mice (***D***). ***E***, Statistical comparison of evoked IPSC amplitude between $\gamma2^{flox/flox}$ mice and *Sim1-Cre:*$\gamma2^{flox/flox}$ mice. ***F***, Average daily food intake for each week during ages of 4–5 weeks in males. ***G***, Hourly food intake measured for a period of 24 h at the age 6–7 weeks. Data are mean ± SEM; $n = 19$ or 20 (***E***) or $n = 7$ or 8 (***F***, ***G***). $^*p < 0.05$ (unpaired Student's *t* tests). $^{***}p < 0.001$ (unpaired Student's *t* tests). ***H***, A summary diagram showing the role of GABAergic projections from LH to PVH in feeding promotion along with the known GABAergic projections from the arcuate nucleus to the PVH and other brain sites that also promote feeding. Whether LH GABAergic projection to other brain sites also promotes feeding awaits further tests, as indicated by the question mark. 3V, Third ventricle; ↑, increased feeding; ARC, arcuate nucleus; DMH, ventromedial hypothalamus; f, fornix; LH, lateral hypothalamus; PVH, paraventricular hypothalamic nucleus; VMH, ventromedial hypothalamus.

Tsunematsu et al., 2014). Alternatively, a novel group of LH GABAergic neurons that are nonpeptdiergic is responsible for the observed feeding effects.

Disruption of GABA-A receptors in the PVH reduced feeding, which was largely due to reduced nocturnal feeding, suggesting an important role for GABAergic input to PVH in feeding regulation. Among various sources of GABAergic input to PVH neurons that are known to regulate feeding, GABAergic input from the Arc unlikely contributes significantly to the feeding defect because nearly complete deletion of *Vgat* in the Arc by Rip-Cre or *Vgat* deletion by AgRP-Cre causes no obvious feeding defects (Tong et al., 2008; Kong et al., 2012). Notably, GABA release from AgRP neurons is sufficient, but not required for feeding induced by AgRP neuron activation (Krashes et al., 2013). In addition, our preliminary data showed that AAV-Cre mediated deletion of *Vgat* in the DMH led to no obvious changes in feeding (data not shown). Although it could not be ruled out that the GABAergic neurons outside LH contributed to the feeding defect observed in *Sim1-Cre:*$\gamma2^{flox/flox}$ mice, given our results showing that disrupted GABA release from LH reduced feeding, it is likely that LH GABAergic neurons contribute significantly to the reduced feeding. Furthermore, a role for GABAergic LH → PVH pathway in nocturnal feeding is supported by the known role of the LH in arousal (Adamantidis and de Lecea, 2008), a state when mice normally feed. Together, our observations that LH GABAergic neurons provide monosynaptic inputs to PVH neurons, that photostimulation of LH GABAergic fibers in the PVH invokes GABA release-dependent feeding behavior, and that disruption of GABA-A receptors in the PVH reduced feeding strongly support a role for GABAergic LH → PVH projections in promoting feeding. In summary, we have iden-

tified a novel GABAergic projection from the LH to the PVH that is critical for feeding regulation, and our data support a physiologically important role for this circuit in nocturnal feeding.

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