

Hypothalamic Non-AgRP, Non-POMC GABAergic Neurons Are Required for Postweaning Feeding and NPY Hyperphagia

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The hypothalamus is critical for feeding and body weight regulation. Prevailing studies focus on hypothalamic neurons that are defined by selectively expressing transcription factors or neuropeptides including those expressing proopiomelanocortin (POMC) and agouti-related peptides (AgRP). The Cre expression driven by the pancreas-duodenum homeobox 1 promoter is abundant in several hypothalamic nuclei but not in AgRP or POMC neurons. Using this line, we generated mice with disruption of GABA release from a major subset of non-POMC, non-AgRP GABAergic neurons in the hypothalamus. These mice exhibited a reduction in postweaning feeding and growth, and disrupted hyperphagic responses to NPY. Disruption of GABA release severely diminished GABAergic input to the paraventricular hypothalamic nucleus (PVH). Furthermore, disruption of GABA-A receptor function in the PVH also reduced postweaning feeding and blunted NPY-induced hyperphagia. Given the limited knowledge on postweaning feeding, our results are significant in identifying GABA release from a major subset of less appreciated hypothalamic neurons as a key mediator for postweaning feeding and NPY hyperphagia, and the PVH as one major downstream site that contributes significantly to the GABA action.

Key words: AgRP; feeding; GABA; NPY; Pdx1-Cre; PVH

Significance Statement

Prevalent studies on feeding in the hypothalamus focus on well characterized, selective groups neurons [e.g., proopiomelanocortin (POMC) and agouti-related peptide (AgRP) neurons], and as a result, the role of the majority of other hypothalamic neurons is largely neglected. Here, we demonstrated an important role for GABAergic projections from non-POMC non-AgRP neurons to the paraventricular hypothalamic nucleus in promoting postweaning (mainly nocturnal) feeding and mediating NPY-induced hyperphagia. Thus, these results signify an importance to study those yet to be defined hypothalamic neurons in the regulation of energy balance and reveal a neural basis for postweaning (nocturnal) feeding and NPY-mediated hyperphagia.

Introduction

Prevailing studies on brain control of feeding focus on neurons that are defined by a selective expression of either transcription factors or

neuropeptides (Liu et al., 2012; Morton et al., 2014). As a result, the function of those neurons that cannot be defined by known transcription factors or neuropeptides is much less appreciated. Accumulating data suggest that these yet to be defined neurons are important for feeding and body weight regulation. Deletion of leptin receptors (LepRs) in GABAergic neurons leads to obesity similar to that of *db/db* mice (Vong et al., 2011). In contrast, deletion of LepR in each of the known, well studied neuronal groups, including POMC and AgRP neurons, only leads to mild obesity (Balthasar et al., 2004; van de Wall et al., 2008). Although deletion of LepRs in GABAergic neurons occurs in regions beyond the hypothalamus, these results suggest an importance for non-POMC, non-AgRP neurons in mediating leptin action on body weight regulation. Thus, to fully appreciate the role of hypothalamic neural pathways in energy metabolism, it is imperative to examine the role of yet to be defined hypothalamic neurons.

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NPY is a well established orexigenic neuropeptide. Despite its broad expression in the brain (Leibowitz, 1991; Thorsell and Heilig, 2002), its expression in the hypothalamus is particularly abundant (Kalra and Kalra, 2004), and the hypothalamus is the predominant site that mediates its orexigenic action (Leibowitz, 1991; Pu et al., 1999). Specifically, site-specific injections of NPY to the paraventricular hypothalamic nucleus (PVH) or lateral hypothalamus (LH) induce robust feeding (Lee and Stanley, 2005). It appears that the activity of Arc NPY/AgRP neurons correlates with feeding levels (Aponte et al., 2011; Krashes et al., 2011). Importantly, NPY neuronal activity is increased after fasting and before the dark cycle in rodents (Takahashi and Cone, 2005; Krashes et al., 2013), suggesting a role for NPY in fasting-induced hyperphagia and nocturnal feeding. In addition, the development of NPY/AgRP neurons, an orexigenic group of neurons in the brain, is not complete until early postnatal age (Xu et al., 2005), suggesting a role for NPY/AgRP neurons in postnatal feeding. Interestingly, NPY expression peaks around the postweaning age, during which its expression is highly sensitive to nutritional changes (Grove and Smith, 2003; Leibowitz et al., 2005; Rocha et al., 2014), supporting an importance of NPY in postweaning feeding. However, the neural pathways that mediate the NPY action and regulate feeding in the postweaning period remain poorly understood.

The hypothalamus contains abundant GABAergic neurons, especially the arcuate (Arc), dorsomedial (DMH), and lateral hypothalamus (Xu and Tong, 2011). Notably, AgRP/NPY neurons represent one of the best studied GABAergic groups of neurons. GABA release from AgRP/NPY neurons promotes feeding through projections to the PVH and a few other extrahypothalamic sites (Tong et al., 2008; Wu et al., 2009; Atasoy et al., 2012). Interestingly, GABA release from these neurons to the parabrachial nucleus is not sufficient to drive feeding, but is required to prevent anorexia (Wu et al., 2009). These observations suggest the importance for GABA release in feeding regulation. Consistent with this, delivery of GABA or its analogs to the LH and PVH induces feeding (Stanley et al., 1993b; Pu et al., 1999; Lee and Stanley, 2005). However, the role of GABA release from the vast majority of non-POMC, non-AgRP hypothalamic neurons is not clear.

Cre recombinase driven by the pancreas duodenum homeobox 1 promoter (*Pdx1-Cre*) expresses abundantly in the DMH, LH, Arc, and pre-optic area (POA) of the hypothalamus (Honig et al., 2010; Song et al., 2010; Wicksteed et al., 2010). Importantly, outside the hypothalamus, *Pdx1-Cre* is only expressed in serotonergic neurons of the dorsal raphe and glutamatergic neurons of the inferior olivary nucleus (ION) of the brain (Song et al., 2010). Since *Vgat* is not expressed in the mouse pancreas (Kong et al., 2012), *Pdx1-Cre* could be used to examine specific *Vgat* function in the hypothalamus. Toward this, this study aimed to explore the function of hypothalamic non-POMC, non-AgRP GABAergic neurons in feeding regulation.

Materials and Methods

Animal care. Mice were housed at 22–24°C with a 12 h light/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. Breeding pairs (male *Pdx1-Cre:Vgat^{fllox/fllox}* mice and female *Vgat^{fllox/fllox}* mice and male *Sim1-Cre:γ2^{fllox/fllox}* mice and female *γ2^{fllox/fllox}* mice) were maintained to generate the study subjects. To visualize *Pdx1-Cre* expression, *Pdx1-Cre* mice were crossed with B6.Cg-Gt(*ROSA*)26Sortm9(*CAG-tdTomato*)Hze/J mice (*Ai9* mice, purchased from The Jackson Laboratory) to generate *Pdx1-Cre:Ai9* mice, in which Cre expression can be directly visualized by

tdTomato, a variant RFP, under fluorescent microscopes (Madisen et al., 2010).

Studies with intracerebroventricular injections. The protocol for the implantation of intracerebroventricular cannulas to the right lateral ventricle was the same as previously described (Tong et al., 2007). After 1 week recovery, mice were used for food intake studies after 2 h fasting in early morning with intracerebroventricular bolus injections of saline (2 μ l), NPY (4 μ g/2 μ l), or Muscimol (4 μ g/2 μ l). Overnight fasting was used to induce fasting-induced hyperphagia.

In situ hybridization. RNAscope Multiple Fluorescent Assay, a novel ISH technique designed to visualize multiple cellular RNA targets in fresh frozen tissues (Wang et al., 2012), was used for detecting the colocalization of *Vgat* and *Vglut2* in the brain (Advanced Cell Diagnostics).

Body weight, body length, and food intake. Weekly body weight was monitored on all genotypes of mice 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 1- to 20-week-old mice. Weekly growth rate was expressed as net increase in body weight during the week. Body length was measured as the distance between the tip of nose and the base of tail in mice under anesthesia. Weekly food intake was measured from individually housed mice from 3- to 15-week old mice. Normalized food intake was calculated by dividing weekly food intake by the average of body weights measured at the beginning and at the end of the week.

Immunohistochemistry assays. The immunohistochemistry studies used the previously described protocol (Xu et al., 2012). Primary antibodies against β -endorphin (1:1000; Phoenix Pharmaceuticals), orexin (1:2000; Phoenix Pharmaceuticals), melanin-concentrating hormone (MCH; 1:200; Phoenix Pharmaceuticals), pSTAT3 (1:400; Cell Signaling Technology), and tryptophan hydroxylase 2 (TPH2; 1:1000; Millipore) were used. Sections were visualized and photographed with a TCS SP5 confocal microscope (Leica). For the pSTAT3 experiment, *Pdx1-Cre:Ai9* mice were administered either saline or leptin (2 mg/kg body weight, i.p.) and perfused 45 min later.

Brain slice electrophysiological recordings. Brain slices were prepared from 4- to 6-week-old mice. Coronal brain slices (350 μ m) were cut in ice-cold ACSF containing the following (in mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 1.3 MgSO₄, 2 CaCl₂, 26 NaHCO₃, and 11 glucose and adjusted to pH 7.4 by bubbling with 95% O₂/5% CO₂. Slices containing the PVH were immediately transferred to a holding chamber and submerged in oxygenated ACSF. Slices were recovered for at least 1 h at 32°C before transferring to a recording chamber. ACSF at 32°C was perfused into slice chambers with ~2 ml/min flow rate regulated by Minipuls 3 peristaltic pump (Gilson).

To minimize potential background difference in electric properties of diverse PVH neurons, we focused on PVH neurons located in the dorso-medial part of PVH for recording. For voltage-clamp recordings, glass electrodes (4–6 M Ω) were filled with internal solution containing the following (in mM): 145 K gluconate, 10 HEPES, 0.2 EGTA, 1 MgCl₂, 4 MgATP, 0.3 Na₂GTP, and 10 Na₂-phosphocreatine and adjusted to pH 7.3 with KOH. For voltage-clamp recordings, spontaneous IPSCs were recorded at the holding potential of 0 mV. Recording pipettes were filled with cesium-based intracellular solution containing the following (in mM): 120 CsCH₃SO₃, 20 HPEPs, 0.4 EGTA, 5 TEA-Cl, 2 MgCl₂, 2.5 MgATP, 0.3 GTP, 1 QX-314, and 10 Na₂-phosphocreatine, pH 7.3.

Throughout the recording experiments, 1 mM kynurenic acid was used to block glutamate-mediated EPSCs. At the end of experiments, 20 μ M bicuculline was applied to the bath to confirm the nature of GABA-A receptor-mediated currents. All recordings were made using a Multi-clamp 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 and Mini Analysis software (Synaptosoft).

Comprehensive Lab Animal Monitoring System measurements. Energy expenditure was measured by oxygen consumption by indirect calorimetry. Individual housed mice were maintained on chow diet at 4–5 weeks of age. Mice were placed at room temperature (22–24°C) in chambers of a Comprehensive Lab Animal Monitoring System (CLAMS; Columbus Instruments). Food and water were provided *ad libitum*. Mice were ac-

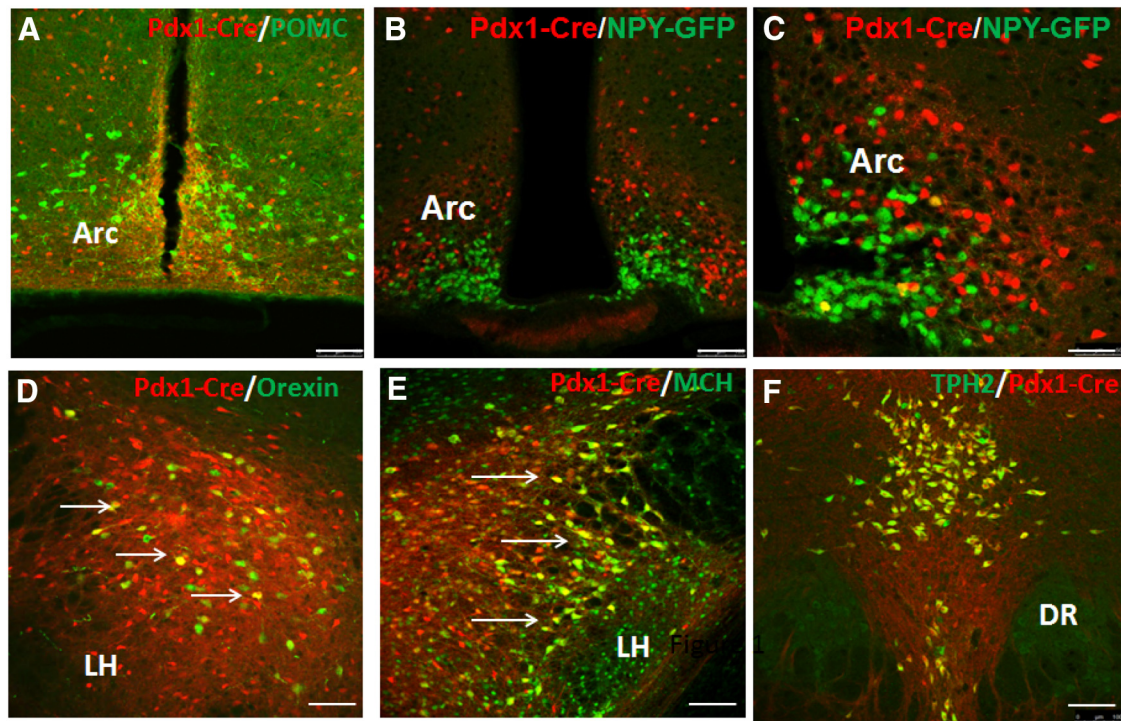


Figure 1. Pdx1-Cre neurons are non-POMC, non-AgRP neurons in the hypothalamus. Immunostainings of the brain were performed for the indicated markers (green) and merged pictures were presented for both green and red (dsRed-positive Pdx1-Cre neurons). **A**, Immunostaining for β -endorphin, a cleaved product of POMC neurons of Pdx1-Cre: Ai9 reporter mice. **B**, GFP immunostaining for NPY/AgRP neurons in Pdx1-Cre: Ai9: NPY-GFP mice. **C**, The same in **B** but shown in a higher magnification. **D**, **E**, Immunostaining for orexin (**D**) and MCH (**E**) in the lateral hypothalamus. **F**, Immunostaining for TPH2, a marker for serotonergic neurons in the dorsal raphe. DR, dorsal raphe. Scale bars: **A**, **B**, **D–F**, 100 μ m; **C**, 50 μ m.

climatized in the chambers for at least 48 h before data collection. Food intake, O₂ consumption, and beam break data were collected.

Locomotion and body temperature measurements using the E-mitter system. As previously reported (Xu et al., 2014), precalibrated sensitive transmitters (PDT-4000 G2 E-Mitter sensors; Respironics) were used for performing telemetric measurements. For measuring body temperature and locomotor activity, mice were anesthetized with ketamine/xylazine and then E-Mitters were implanted in the space under the skin between the scapulae. For core body temperature monitoring, E-Mitters were placed into the peritoneal cavity. Mice were allowed 1 week recovery before all data were collected. Signals emitted by the E-Mitter transponders were sensed by a receiver positioned underneath the animal's housing cage and analyzed using VitalView software (Respironics). Locomotor activity counts are recorded as a gross motor activity indicator. For all experiments, activity counts and temperature measurements were taken every 1 min. All mice were acclimated for at least 3 d and then data were collected for 24 h. Multiple series of data at the same collected time point and from the same genotype mice were summed and then averaged to get their mean temperature and movement.

Ghrelin measurements. Tail blood was obtained at 9:00 A.M. and 7:00 P.M., which was used to measure ghrelin levels using a ghrelin ELISA kit from EMD Millipore (a gift from Dr. Yuxiang Sun of Baylor College of Medicine, Houston, TX) according to the manufacturer's instructions.

Statistical analysis. All data were presented as mean \pm SEM, and comparison between means was performed with an appropriate statistical analysis according to group numbers and treatments using Prism 6.0 with $p < 0.05$ indicating statistical significance. Tukey multiple-comparison was used for two-way ANOVA.

Results

Hypothalamic Pdx1-Cre neurons are neither POMC nor AgRP neurons

To examine Pdx1-Cre expression, we crossed Pdx1-Cre mice with Ai9 reporter mice (Madisen et al., 2010), which express ds-Red in a Cre-dependent manner. Pdx1-Cre was previously

reported to express abundant Cre in the Arc, DMH, LH, and POA, as well as in the dorsal raphe and ION (Honig et al., 2010; Song et al., 2010). To examine whether POMC neurons express Pdx1-Cre, we use the β -endorphin antibody to label POMC neurons in Pdx1-Cre: Ai9 reporter mouse brain sections. POMC neurons were not found to express Pdx1-Cre in the Arc (Fig. 1A). Given the colocalization between AgRP and NPY in AgRP neurons of Arc (Hahn et al., 1998), we crossed NPY-GFP mice with Pdx1-Cre: Ai9 reporter mice to assess whether AgRP neurons express Pdx1-Cre. The vast majority of AgRP neurons were not Pdx1-Cre neurons (Fig. 1B), which can be better appreciated in an amplified picture (Fig. 1C). Thus, Pdx1-Cre targets a major subset of non-POMC, non-AgRP neurons in the hypothalamus. MCH and orexin are two well studied neuropeptides in the LH, in which Pdx1-Cre also showed abundant expression. Pdx1-Cre neurons in the LH partially overlapped with orexin neurons (Fig. 1D) and MCH neurons (Fig. 1E) in the LH. Of note, almost all Pdx1-Cre neurons in the dorsal raphe express TPH2 (Fig. 1F), a marker for serotonergic neurons, suggesting the vast majority of Pdx1-Cre neurons in this region are serotonergic in nature.

Pdx1-Cre neurons partially overlap with LepR neurons

LepR neurons represent a key subset of hypothalamic neurons for body weight regulation. To examine whether Pdx1-Cre neurons significantly overlap with LepR neurons, we colocalized Pdx1-Cre with leptin-induced p-STAT3 expression (Münzberg et al., 2007). Compared with saline treatment, leptin induced a large number of p-STAT3-expressing neurons in the DMH and Arc (Fig. 2A, B, compare A-1 to B-1 and A-2 to B-2), in addition to a few other sites. Interestingly, a subset of Pdx1-Cre neurons in the DMH (Fig. 2B-1) and the vast majority of Pdx1-Cre neurons in the Arc were negative for p-STAT3 (Fig. 2B-2). These results

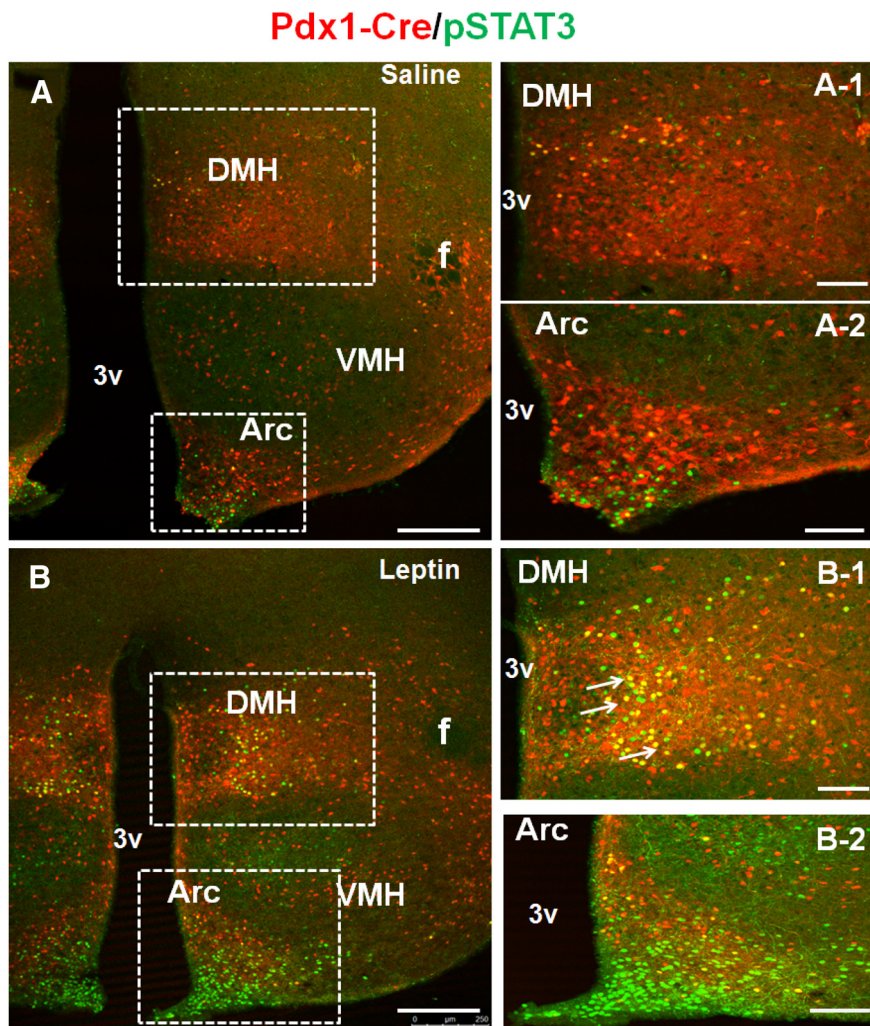


Figure 2. Pdx1-Cre neurons partially overlap with LepR neurons. *Pdx1-Cre: Ai9* mice were intraperitoneally administered saline (**A**) or leptin (**B**) and brain sections were immunostained for p-STAT3 in green. The boxed areas in **A** were enlarged in **A-1** for DMH and in **A-2** for Arc, and those in **B** were enlarged in **B-1** for DMH and in **B-2** for Arc. Arrows point to neurons with coexpression of Pdx1-Cre and p-STAT3. VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; Arc, arcuate nucleus; f, fornix; 3V, the third ventricle. Scale bars: **A, B**, 250 μ m; **A-1, A-2, B-1, B-2**, 100 μ m.

suggest that Pdx1-Cre neurons only partially overlap with LepR neurons in the hypothalamus.

Deletion of *Vgat* by Pdx1-Cre in the hypothalamus

To examine the role of GABA release from Pdx1-Cre neurons, we bred *Pdx1-Cre* mice with mice bearing floxed *Vgat* gene (*Vgat*^{flox/flox} mice). Specific deletion of *Vgat* was examined by the RNA scope technology, which can visualize a single RNA molecule in cells (Xu et al., 2013). We also included ISH for *Vglut2* (also named *Slc17a6*), a marker for glutamatergic neurons, to serve as a control. Our previous results showed that Pdx1-Cre targets ~80% of GABAergic neurons in the LH (Wu et al., 2015). In addition to the LH of control mice, *Vgat* was also found to be abundantly expressed in the Arc (Fig. 3A), DMH (Fig. 3B), and POA (Fig. 3C), in which Pdx1-Cre expression is abundant. In contrast, *Vgat* was not present in ION (Fig. 3D). In *Pdx1-Cre: Vgat*^{flox/flox} mice, *Vgat* was substantially deleted in the Arc (Fig. 3E), DMH (Fig. 3F), and POA (Fig. 3G), and only *Vglut2* and no *Vgat* was present in the ION (Fig. 3H). Combined with our previous data on LH neurons (Wu et al., 2015), these data suggest that

Pdx1-Cre targets a major subset of non-POMC, non-AgRP GABAergic neurons in the hypothalamus.

Deletion of *Vgat* in Pdx1-Cre neurons impairs GABA release to PVH neurons

In *Pdx1-Cre: Ai9* reporter mice we found abundant ds-Red-positive fibers in the PVH, but not in the surrounding brain regions (Fig. 3I), suggesting that Pdx1-Cre neurons send projections to the PVH. If a significant portion of PVH-projecting fibers are GABAergic, then it is possible that *Vgat* deletion in Pdx1-Cre neurons may cause reduced GABA release to PVH neurons. To examine this possibility, we recorded spontaneous GABA-mediated IPSCs (sIPSCs) in PVH neurons on brain slices of control and *Pdx1-Cre: Vgat*^{flox/flox} mice (Fig. 3J). Whole-cell recordings from brain slices showed that the frequency, but not the amplitude, of sIPSCs of PVH neurons in *Pdx1-Cre: Vgat*^{flox/flox} mice was reduced by two-thirds, compared with *Vgat*^{flox/flox} mice (Fig. 3K,L). These results suggest that *Vgat* deletion in Pdx1-Cre neurons impairs GABA release and that the majority of GABAergic input to the PVH originates from GABAergic Pdx1-Cre neurons within the hypothalamus.

GABA release from hypothalamic neurons is required for postweaning feeding and NPY hyperphagia

To determine the physiological role of GABA release from Pdx1-Cre neurons, we monitored body weight of *Pdx1-Cre: Vgat*^{flox/flox} mice. These mice exhibited reduced body weight from the age of 3 weeks onward in both males (Fig. 4A) and females (data not shown). Of note, the body weight of *Pdx1-Cre: Vgat*^{flox/flox} mice at the ages of 2, 3, and 5 weeks were reported previously (Song et al., 2010). A closer look at their body weight curve revealed that from the age of 5 weeks onward, body weight curves of *Pdx1-Cre: Vgat*^{flox/flox} mice and their controls remained parallel to each other. Interestingly, when growth rate (net body weight growth/each week) was calculated, control mice showed a period of rapid growth rate during the ages of weeks 4–5, a period right after weaning, whereas *Pdx1-Cre: Vgat*^{flox/flox} mice exhibited a dramatic reduction in growth rate in both males (Fig. 4B) and females (data not shown). Notably, growth rates were not different between genotypes at 2 weeks of age and after 6 weeks of age, suggesting that the reduction in growth rate is specific for the postweaning period. To effectively compare food intake between mice of different ages with dramatic body weight difference, we normalized their weekly food intake by their body weight. While control mice exhibited a high level of normalized food intake at the age of 4–5 weeks, which coincided with their rapid growth rate, *Pdx1-Cre: Vgat*^{flox/flox} mice showed significantly blunted feeding (Fig. 4C), suggesting a role for GABAergic Pdx1-Cre neurons

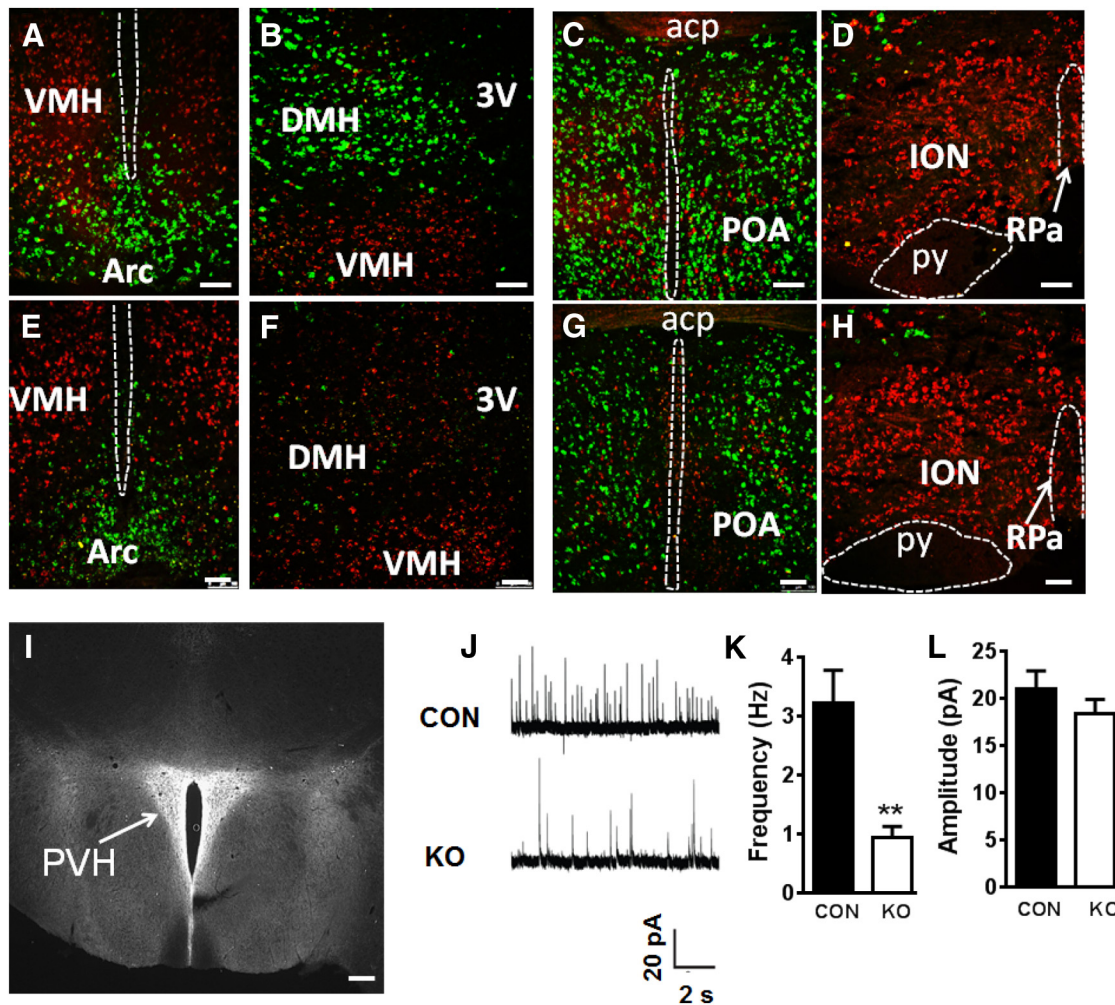


Figure 3. Deletion of *Vgat* in *Pdx1-Cre* neurons impaired GABA release to PVH. **A–H**, *Vgat* (green) and *Vglut2* (red) mRNA expression by ISH in the Arc (**A**, **E**), DMH (**B**, **F**), POA (**C**, **G**), and ION (**D**, **H**). Sections are from *Vgat*^{fllox/fllox} (**A–D**) and from *Pdx1-Cre:Vgat*^{fllox/fllox} mice (**E–H**). In *Pdx1-Cre:Vgat*^{fllox/fllox} mice, *Vglut2* expression is not changed and *Vgat* is largely deleted in the DMH and reduced in the Arc and POA. **I**, Fibers from *Pdx1-Cre* neurons shown by ds-Red fluorescence in the PVH of *Pdx1-Cre: Ai9* reporter mice. **J**, Representative traces showing sIPSCs in a PVH neuron recorded from *Vgat*^{fllox/fllox} mouse (CON, top) and a *Pdx1-Cre:Vgat*^{fllox/fllox} mice (KO, bottom). **K**, **L**, Quantification of the frequency (**K**) and amplitude (**L**) of sIPSCs recorded from PVH neurons in two groups of mice; $n = 19$, ** $p < 0.01$, unpaired Student's *t* tests. Acp, anterior commissure (posterior part); py, pyramid tracts; RPa, raphe pallidus; PVH, paraventricular hypothalamus; CON, *Vgat*^{fllox/fllox} mice; KO, *Pdx1-Cre:Vgat*^{fllox/fllox} mice; POA, preoptic area; ION, inferior olive nucleus; 3V, the third ventricle. Scale bars: **A–H**, 50 μm ; **I**, 100 μm . Data presented as means \pm SEM.

in postweaning feeding. Consistent with the defect in the postweaning period, a key developmental stage, *Pdx1-Cre:Vgat*^{fllox/fllox} mice exhibited reduced body length (Fig. 4D).

Since previous results suggest a potential role for NPY in postweaning feeding (Leibowitz et al., 2005), we examined whether *Pdx1-Cre:Vgat*^{fllox/fllox} mice show defects in NPY-induced feeding. In control *Vgat*^{fllox/fllox} mice, intracerebroventricular NPY induced robust feeding during the first 4 h, compared with saline (Fig. 5A). However, in *Pdx1-Cre:Vgat*^{fllox/fllox} mice, the same dose of NPY failed to induce feeding (Fig. 5A). As increased NPY action contributes to refeeding after fasting, we also measured fasting-induced refeeding in these mice. While control mice exhibited robust feeding after fasting (Fig. 5B), *Pdx1-Cre:Vgat*^{fllox/fllox} mice showed a greatly attenuated feeding response to fasting (Fig. 5B). We also tested the effect of Muscimol, a GABA-A receptor agonist known to induce feeding (Pu et al., 1999). Muscimol produced similar hyperphagic responses between control and *Pdx1-Cre:Vgat*^{fllox/fllox} mice (Fig. 5C), suggesting that *Pdx1-Cre:Vgat*^{fllox/fllox} mice have no defects in executing eating behavior. Thus, *Pdx1-Cre:Vgat*^{fllox/fllox} mice exhibited a primary defect in hyperphagic response to NPY, suggesting an im-

portant role for GABA release from *Pdx1-Cre* neurons in mediating NPY action on feeding regulation.

GABA release from hypothalamic neurons is required for nocturnal feeding

Since NPY may promote nocturnal feeding (Sindelar et al., 2005; Krashes et al., 2013), we also monitored hourly food intake for 2 d in 4-week-old *Pdx1-Cre:Vgat*^{fllox/fllox} mice. Control *Vgat*^{fllox/fllox} mice showed typical nocturnal feeding, which was significantly blunted in *Pdx1-Cre:Vgat*^{fllox/fllox} mice (Fig. 6A). This is consistent with the notion that GABA release from *Pdx1-Cre* neurons mediates NPY action on feeding. Of note, blunted nocturnal feeding was persistent when measured in 8-week-old *Pdx1-Cre:Vgat*^{fllox/fllox} mice (data not shown). Interestingly, we noticed that, while ghrelin levels were not different between groups when measured in early morning, they were significantly higher in *Pdx1-Cre:Vgat*^{fllox/fllox} mice in late afternoon, compared with controls (Fig. 6B), which was presumably from a compensatory secretion of ghrelin in response to diminished feeding during the dark period. Nocturnal feeding is normally associated with increased energy expenditure, locomotion, and body temperature. We thus

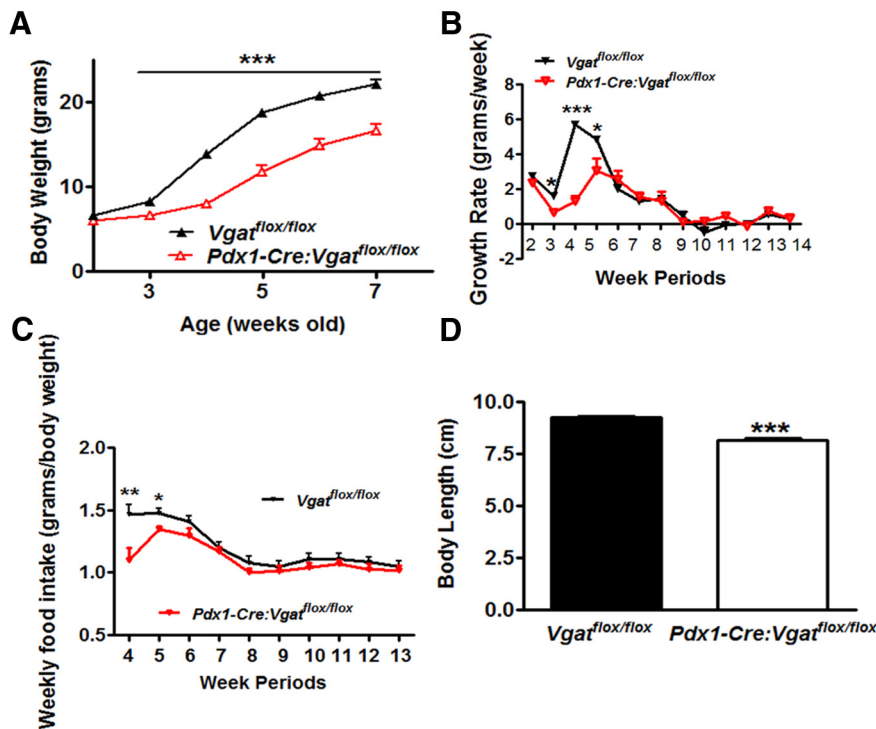


Figure 4. Reduction in postweaning feeding and growth in *Pdx1-Cre:Vgat^{flox/flox}* mice. **A**, Body weight curve of males from the age of 2 weeks onward. **B**, Growth rate expressed as net body weight gain per week in males. **C**, Weekly food intake expressed as net food consumption per body weight in grams. **D**, Body length measured in 8- to 10-week old males. Data presented as mean \pm SEM, $n = 6-8$; * $p < 0.05$ and *** $p < 0.001$ analyzed by Student's *t* tests.

also monitored these parameters in *Pdx1-Cre:Vgat^{flox/flox}* mice and found that, consistent with their reduced nocturnal feeding, these mice showed reduced energy expenditure (Fig. 6C), locomotion (Fig. 6D,E), and body temperature (Fig. 6F). These results suggest that GABA release from a major subset of non-POMC, non-AgRP neurons is required for nocturnal hyperphagia and its associated hyperactivity, increased energy expenditure, and body temperature.

Disrupted GABAergic input to the PVH reduced postweaning growth and attenuated NPY hyperphagia

We previously reported that mice lacking the $\gamma 2$ -subunit in the PVH (*Sim1-Cre:γ2^{flox/flox}* mice) exhibited reduced feeding at 4–5 weeks of age (Wu et al., 2015). The reduced nocturnal feeding supports that the PVH is one of the downstream targets that contribute significantly to the action of GABA released from *Pdx1-Cre* neurons. We next determined whether disrupting GABAergic input to *Sim1* neurons impairs the growth rate of *Sim1-Cre:γ2^{flox/flox}* mice. A reduction in growth rate was observed in *Sim1-Cre:γ2^{flox/flox}* mice ~4 weeks of age, compared with controls (Fig. 7A), which was also observed in females (data not shown). While control $\gamma 2^{\text{flox/flox}}$ mice showed high levels of rapid growth rate (Fig. 7B) during the postweaning period, *Sim1-Cre:γ2^{flox/flox}* mice showed a significantly reduced growth rate (Fig. 7B). *Sim1-Cre:γ2^{flox/flox}* mice exhibited a significant reduction in feeding during week 4 and showed a trend of reduced feeding level in weeks 5 and 6 (Fig. 7C), which remained throughout their adult life (data not shown). Consistent with a reduced feeding during the weaning period, a key period for growth, *Sim1-Cre:γ2^{flox/flox}* mice exhibited reduced body length (Fig. 7D).

To further examine whether GABAergic input to the PVH mediates the NPY effect on feeding, we also measured food intake

in response to NPY. While control mice exhibited a normal hyperphagic response to NPY, *Sim1-Cre:γ2^{flox/flox}* mice showed significantly blunted food intake response to NPY (Fig. 7E), although the degree in difference was smaller than those observed in *Pdx1-Cre:Vgat^{flox/flox}* mice. We previously reported that *Sim1-Cre:γ2^{flox/flox}* mice exhibited reduced nocturnal feeding (Wu et al., 2015). Associated with this, these mice showed specific attenuation in O_2 consumption during the dark period (Fig. 7F), suggesting an attenuated feeding-induced energy expenditure. These results demonstrate that PVH neurons are one of the important downstream targets of GABAergic input from *Pdx1-Cre* neurons in mediating postweaning feeding and NPY hyperphagia.

Discussion

Prevailing studies on hypothalamic GABAergic action in feeding control primarily focus on AgRP neurons (Wu et al., 2009; Krashes et al., 2011; Atasoy et al., 2012). Here we showed that, within the hypothalamus, *Pdx1-Cre* neurons were distinct from POMC and AgRP neurons. Of note, *Vgat* is not expressed in pancreatic islets (Kong et al., 2012), and *Pdx1-Cre* neurons in the dorsal raphe and ION were shown to be serotonergic and glutamatergic, respectively. Our data showed that disruption of GABA release from hypothalamic non-AgRP, non-POMC neurons impaired postweaning feeding and growth rate. Moreover, these neurons provide direct GABAergic projections to PVH neurons, and disruption of GABAergic input to PVH neurons resulted in similar defects, although to a lesser degree. In both animal models, the growth rate was not altered during the first two postnatal weeks, and the body weight remained parallel to that of controls from the age of 6 weeks onward, suggesting a specific defect in postweaning feeding. The specific defect may indicate that GABAergic input from *Pdx1-Cre* neurons to PVH neurons is most prominent during the weaning period to ensure sufficient feeding that is required for rapid developmental growth. Of note, during the postweaning period, the switch from liquid milk to solid chow food coincides with an increased demand of a high level of feeding during this period of rapid growth (Eisen and Leatherwood, 1978). Thus, this study revealed a key role for hypothalamic non-POMC, non-AgRP neurons GABAergic neurons in postweaning feeding, and will have significant impact on the understanding of early life nutrition and its role in adult metabolism (Lee and Blackshaw, 2014).

Deletion of *LepR* in GABAergic neurons and disruption of GABA release from *LepR* neurons both cause obesity (Vong et al., 2011; Xu et al., 2012). However, disruption of GABA release from *Pdx1-Cre* neurons led to reduced nocturnal feeding and a defective response to NPY-induced hyperphagia associated with blunted growth. These contrasting effects can be explained by the fact that a major subset of *Pdx1-Cre* neurons in the hypothalamus do not overlap with *LepR* neurons. It is likely that the hypothalamic GABAergic neurons that are labeled by *Pdx1-Cre*, but not *LepR*, neurons are required for nocturnal feeding and medi-

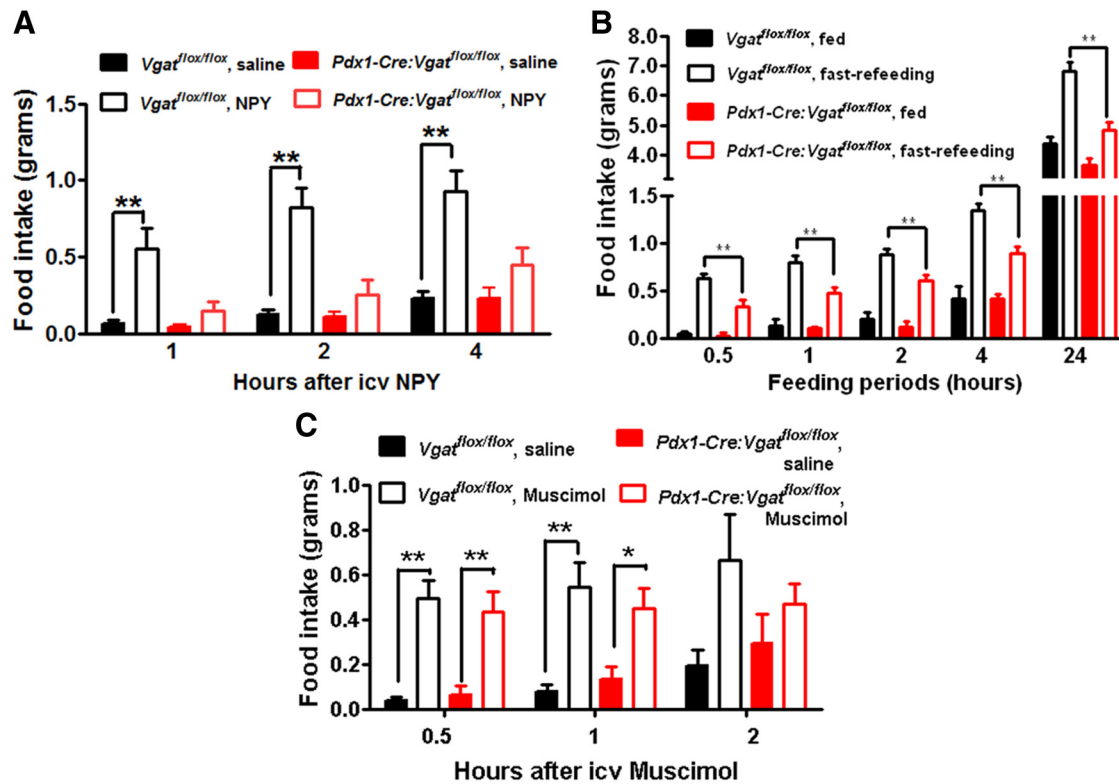


Figure 5. Blunted feeding response to NPY in *Pdx1-Cre:Vgat*^{flox/flox} mice. All mice were implanted with intracerebroventricular (icv) cannulation, which was verified by angiotensin-induced drinking behavior. Food intake response male *Vgat*^{flox/flox} and *Pdx1-Cre:Vgat*^{flox/flox} mice to intracerebroventricular administration of NPY (**A**), overnight fasting (**B**), and Muscimol (**C**). All data presented as mean \pm SEM, $n = 5-8$; * $p < 0.05$ and ** $p < 0.01$, two-way ANOVA tests followed by a Tukey correction.

ate NPY-induced feeding behavior. Thus, our results highlight an important role for a previously uncharacterized group of hypothalamic GABAergic neurons (non-POMC, non-AgRP, and non-LepR) in feeding regulation.

Our findings identified the source of GABA release that is required in feeding regulation. GABA produces robust feeding in the brain (Stanley and Leibowitz, 1984; Stanley et al., 1985; Kalra et al., 1999; Pu et al., 1999; Leininger et al., 2009; Wu et al., 2009). However, the source of GABA release required for feeding regulation remains elusive. Recent studies on AgRP neurons showed that GABA release from these neurons is sufficient (Wu et al., 2009; Atasoy et al., 2012; Krashes et al., 2013), but is not required (Tong et al., 2008; Krashes et al., 2013), to drive feeding. Disruption of GABA release from hypothalamic non-POMC, non-AgRP neurons led to a reduction in postweaning and nocturnal feeding, suggesting that GABA release from these hypothalamic neurons is required to drive feeding. Among different hypothalamic regions with *Pdx1-Cre* expression, LH GABAergic neurons likely contribute significantly to feeding since specific disruption GABA release from LH neurons causes a reduction in feeding and is required for feeding driven by LH GABAergic neurons (Jennings et al., 2015; Wu et al., 2015). Our previous results on mice with almost complete *Vgat* deletion in the Arc show no obvious changes in feeding (Kong et al., 2012), arguing against a significant contribution of Arc non-POMC, non-AgRP neurons to the defect in postweaning feeding observed here. A recent study shows that DMH neurons exhibit an increasing putative inhibitory GABA input to AgRP neurons during periweaning period, arguing against a role for *Pdx1-Cre* GABAergic neurons in the DMH in the postweaning feeding phenotype observed in this study (Baquero et al., 2015). Further studies are required to

specifically address the role of GABAergic neurons in the DMH, as well as in the POA, where *Pdx1-Cre* also shows abundant expression.

Our results have revealed an importance of hypothalamic non-POMC, non-AgRP neurons in mediating NPY hyperphagia. Disruption of GABA release from these neurons greatly attenuated NPY-induced food intake and the hyperphagia induced by fasting, a condition with increased NPY action (Takahashi and Cone, 2005). NPY/AgRP neuronal activity increases in the nocturnal period (Krashes et al., 2013), which is thought to promote nocturnal feeding (Sindelar et al., 2005). Knockdown of NPY in the DMH causes a specific reduction in nocturnal feeding in rats (Yang et al., 2009). In line with this, we found that disruption of GABA release from *Pdx1-Cre* neurons selectively reduced nocturnal feeding, strongly supporting a role for GABA in mediating NPY action and a role for an increased GABA release from these neurons in driving nocturnal feeding. Supporting our observations, NPY action on feeding depends on GABA-A receptors (Jonaidi and Noori, 2012). Furthermore, the LH and Arc express abundant NPY receptors (Lin et al., 2004), and multiple brain sites including the DMH, LH, and Arc have been shown to contribute to NPY-mediated feeding regulation (Stanley and Leibowitz, 1984; Stanley et al., 1993a; b; Tiesjema et al., 2007; Dailey and Bartness, 2009; Yang et al., 2009). One additional key observation supporting that hypothalamic non-POMC, non-AgRP neurons in mediating NPY hyperphagia is the reduction in postweaning feeding. NPY has been suggested to be involved in postweaning feeding. NPY expression peaks and is highly sensitive to undernutrition around weaning time (Grove et al., 2001; Grove and Smith, 2003; Rocha et al., 2014). The action of NPY on GABA release is the strongest in early postnatal life (Melnick et

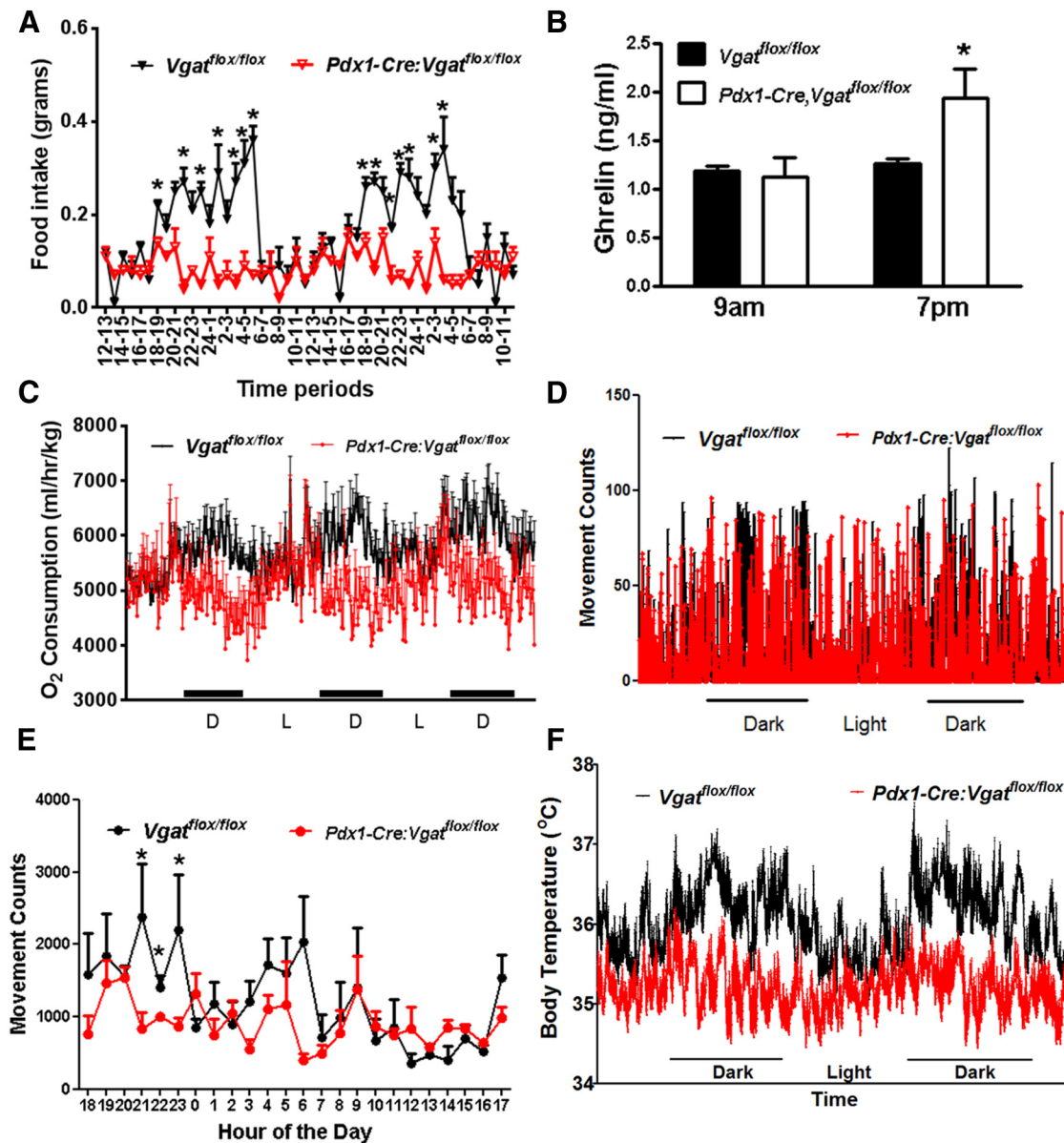


Figure 6. Altered diurnal feeding rhythms in $Pdx1-Cre:Vgat^{flox/flox}$ mice. **A**, Hourly food intake for 2 d measured in 4-week-old $Vgat^{flox/flox}$ and $Pdx1-Cre:Vgat^{flox/flox}$ mice. **B**, Ghrelin levels measured in blood obtained at 9:00 A.M. and 7:00 P.M. of indicated genotypes at the age of 7–8 weeks. **C**, Energy expenditure measured by CLAMS for 2 d in 4-week-old mice. **D**, Movement counts from a representative 8-week-old $Vgat^{flox/flox}$ and $Pdx1-Cre:Vgat^{flox/flox}$ mouse measured from beam breaks of CLAMS. D and L, dark and light periods respectively. **E**, Averaged movement counts measured at the age of 7–8 weeks of age by an E-mitter system. **F**, Real-time body temperature measurement (every minute) at the age of 7–8 weeks of age using an E-mitter system. All data presented as mean \pm SEM; $n = 5–8$ in **A** and **B**, $n = 6$ in **C**, and $n = 5–7$ in **E** and **F**; $*p < 0.05$, Student's *t* tests.

al., 2007), suggesting a role of NPY in controlling GABA neurotransmission during early key developmental periods. Collectively, our study identifies GABA as a key mediator for the NPY hyperphagic action.

Somatostatin (SST) and growth hormone (GH)-releasing hormone located in the Arc and POA are known to regulate GH secretion. As $Pdx1-Cre$ also expresses abundantly in both regions, it is important to know whether the reduced growth in $Pdx1-Cre:Vgat^{flox/flox}$ mice is from a defective GH action. Indeed, we found that SST neurons significantly overlapped with $Pdx1-Cre$ neurons in the POA, but not in the Arc (data not shown). However, despite the overlap, it is unlikely that defective postweaning growth in $Pdx1-Cre:Vgat^{flox/flox}$ mice is a direct result of a compromised SST neuron function. First, SST-deficient mice do not exhibit reduced growth; instead they show a slightly increased

body weight (Low et al., 2001). Second, the reduced growth in $Pdx1-Cre:Vgat^{flox/flox}$ mice is unlikely because of defective GH action since these mice exhibited a normal level of GH at 3 weeks of age and a trend of increased GH levels at 5 weeks of age (data not shown). Third, $Pdx1-Cre:Vgat^{flox/flox}$ mice exhibited, in addition to a trend of increased GH levels, reduced T3 and T4 levels in blood and reduced *Igf1* expression in liver (data not shown), which are all typical signs of starvation (Boelen et al., 2008; Misra and Klibanski, 2014), suggesting that reduced feeding is the primary defect and that reduced growth is a result of reduced feeding, especially during the weaning period when rapid growth is conceivably most sensitive to nutrient insufficiency. Fourth, specific reduction in nocturnal and NPY-induced feeding suggests an inherent defect in feeding.

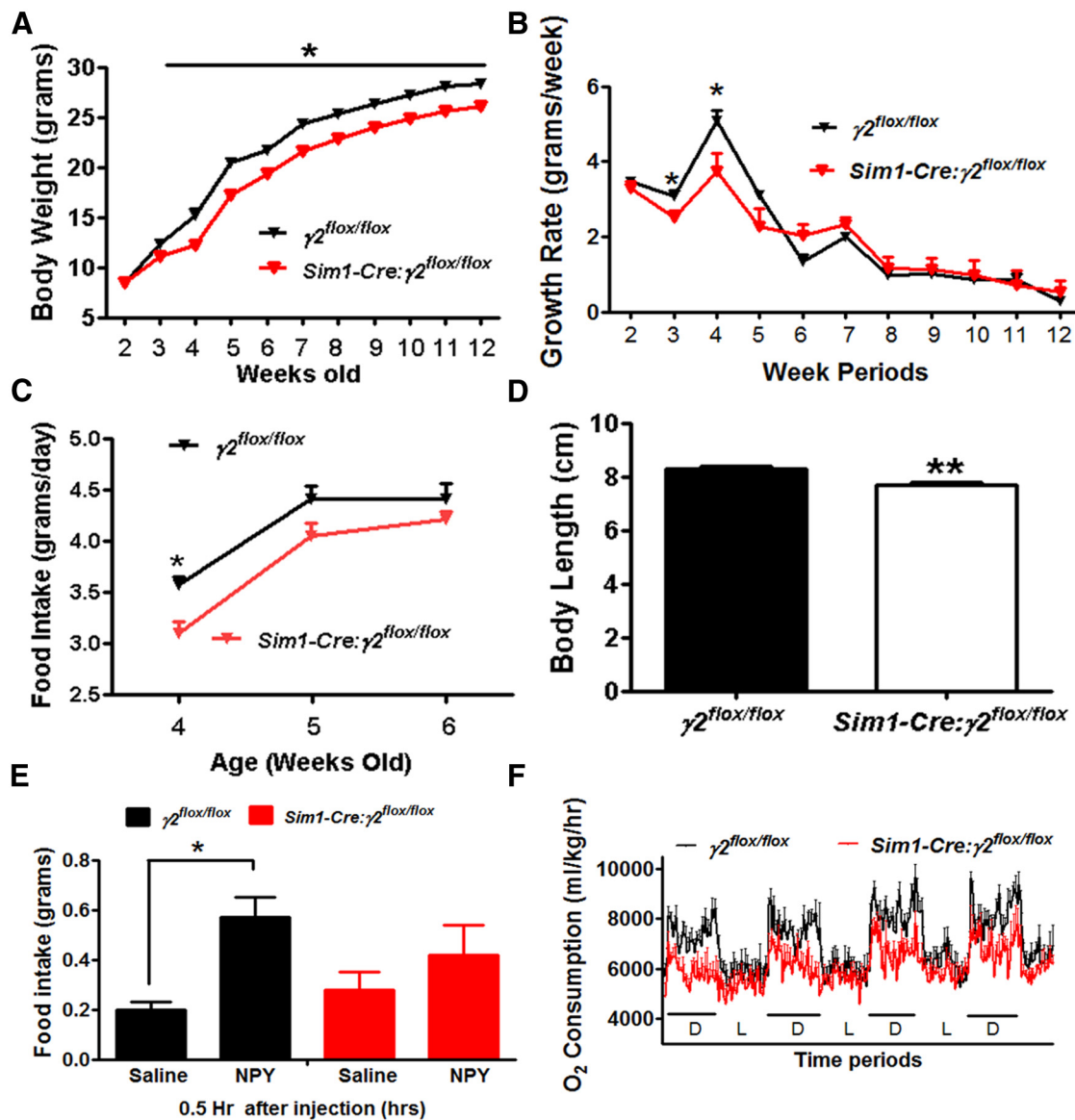


Figure 7. Reduction in postweaning feeding and NPY hyperphagia in *Sim1-Cre:γ2^{flox/flox}* mice. **A**, Body weight curves of male $\gamma_2^{flox/flox}$ and *Sim1-Cre:γ2^{flox/flox}* mice. **B**, Growth rate expressed as net body weight gain each week in males. **C**, Average daily food intake for each week during the age of weeks 4–6 in males. **D**, Body length measured at the age of 5 weeks. **E**, Food intake in $\gamma_2^{flox/flox}$ mice and *Sim1-Cre:γ2^{flox/flox}* mice in response to saline or NPY. **F**, Energy expenditure measured by O₂ consumption at the age of 4 weeks using CLAMS for 4 consecutive days. All data presented as mean \pm SEM; $n = 8–10$ in **A–C**, $n = 5–7$ in **D** and **E** and $n = 6$ in **F**; * $p < 0.05$; ** $p < 0.01$, Student's *t* tests.

GABAergic input to the PVH is involved in the integration of an array of physiologic processes, including modulating the melanocortin input from the Arc (Cowley et al., 2001), energy expenditure and feeding from the Arc (Atasoy et al., 2012; Kong et al., 2012), stress response from the prefrontal cortex (Radley et al., 2009), and circadian metabolic changes from the suprachiasmatic nucleus (Kalsbeek et al., 2008). Despite multiple origins of GABAergic input to PVH, the frequency of sIPSCs of PVH was reduced to one-third by disrupting GABA release from Pdx1-Cre neurons, suggesting a major contribution of these neurons to total GABAergic input to PVH neurons. This notion is consistent with the intense projection of Pdx1-Cre neurons to the PVH and previous observations showing strong GABAergic input to the PVH from both DMH and LH (Boudaba et al., 1996). Importantly, disruption of GABAergic input to PVH neurons led to an array of phenotypes similar to those observed in mice with disruption of GABA release from Pdx1-Cre neurons, including spe-

cific reduction in postweaning feeding and growth, blunted response to NPY-induced hyperphagia, and diminished nocturnal feeding. The striking similarity suggests PVH neurons are one major downstream target of GABA released from Pdx1-Cre neurons in feeding regulation. The reduced food intake by disrupting GABA-A receptors is supported by previous observations that specific GABA injections to the PVH induce feeding (Pu et al., 1999) and that reduced GABA-A receptor levels inhibit feeding (Sheng et al., 2006). A specific reduction in nocturnal feeding highlights an important role for circadian changes in GABA release to the PVH in feeding behavior.

In summary, our study identified GABA released from hypothalamic non-POMC, non-AGRP neurons as a critical downstream mediator for postweaning feeding and NPY hyperphagia, and PVH neurons as one major downstream target for the GABA action. Our results suggest that diurnal changes in hypothalamic GABA action may underline diurnal feeding behaviors.

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