Original Article

MOLECULAR METABOLISM

Acute activation of GLP-1-expressing neurons promotes glucose homeostasis and insulin sensitivity

Xuemei Shi [1,3,*], Shaji Chacko [3], Feng Li [5], Depei Li [6], Douglas Burrin [3], Lawrence Chan [4], Xinfu Guan [2,3,4,**]

ABSTRACT

Objective: Glucagon-like peptides are co-released from enteroendocrine L cells in the gut and preproglucagon (PPG) neurons in the brainstem. PPG-derived GLP-1/2 are probably key neuroendocrine signals for the control of energy balance and glucose homeostasis. The objective of this study was to determine whether activation of PPG neurons *per se* modulates glucose homeostasis and insulin sensitivity *in vivo*.

Methods: We generated glucagon (*Gcg*) promoter-driven Cre transgenic mice and injected excitatory hM3Dq-mCherry AAV into their brainstem NTS. We characterized the metabolic impact of PPG neuron activation on glucose homeostasis and insulin sensitivity using stable isotopic tracers coupled with hyperinsulinemic euglycemic clamp.

Results: We showed that after ip injection of clozapine N-oxide, *Gcg*-Cre lean mice transduced with hM3Dq in the brainstem NTS downregulated basal endogenous glucose production and enhanced glucose tolerance following ip glucose tolerance test. Moreover, acute activation of PPG neurons^{NTS} enhanced whole-body insulin sensitivity as indicated by increased glucose infusion rate as well as augmented insulin-suppression of endogenous glucose production and gluconeogenesis. In contrast, insulin-stimulation of glucose disposal was not altered significantly.

Conclusions: We conclude that acute activation of PPG neurons in the brainstem reduces basal glucose production, enhances intraperitoneal glucose tolerance, and augments hepatic insulin sensitivity, suggesting an important physiological role of PPG neurons-mediated circuitry in promoting glycemic control and insulin sensitivity.

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Keywords Preproglucagon neurons; Glucagon-like peptides; Glucagon-Cre mice; Insulin sensitivity; Endogenous glucose production; Gluconeogenesis

1. INTRODUCTION

In response to food intake, glucagon-like peptides (GLP-1/2) are co-released from enteroendocrine L cells in the gut and preproglucagon (**PPG**) neurons in the nucleus of the solitary tract (NTS) of the brainstem, which together constitute the key nutritional signals for the control of energy balance and glucose homeostasis. Notably, GLP-1 receptor (GLP-1R) and GLP-2 receptor (GLP-2R) agonists are approved by the FDA for the treatment of type 2 diabetes and short bowel syndrome, respectively. PPG neurons widely project to central autonomic regions where Glp1r/2r are expressed [1—3]. PPG neurons are depolarized by leptin and may play a role in energy homeostasis and peripheral metabolism [4—7]. Intracerebroventricular (icv) infusion of exogenous GLP-1 or GLP-2 enhances glucose tolerance and insulin sensitivity [8—10]. However, it is unknown if PPG neurons play a physiological role in peripheral glucose metabolism and insulin sensitivity, though the physiological significance of endocrine GLP-1/2 is highlighted in maintaining glucose homeostasis.

Increased gluconeogenesis is a primary feature of fasting hyperglycemia and type 2 diabetes (up to 40% of diabetic patients) [11]. Thus,

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Abbreviations: aCSF, artificial cerebrospinal fluid; AP, area postrema; ARC, the arcuate nucleus; CC, central canal; ChAT, choline acetyltransferase; CNO, clozapine N-oxide; DMH, the dorsomedial hypothalamic nuclei; DMV, dorsal motor vagal nucleus; DREADD, Designer Receptors Exclusively Activated by Designer Drugs; DVC, the brainstem dorsal vagal complex; EGP, endogenous glucose production; Gcg, glucagon; GIR, glucose infusion rate; GNG, gluconeogenesis; GLP-1/2, glucagon-like peptides; GLP-1R (or *Glp1r*), GLP-1 receptor; GLP-2R (or *Glp2r*), GLP-2 receptor; HGP, hepatic glucose production; icv, intracerebroventricular; ipGTT, intraperitoneal glucose tolerance test; NTS, the nucleus of the solitary tract; PPG, preproglucagon; PVH, the paraventricular hypothalamic nuclei.

Received July 25, 2017 • Revision received August 20, 2017 • Accepted August 23, 2017 • Available online 1 September 2017

http://dx.doi.org/10.1016/j.molmet.2017.08.009

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it is important to quantify *in vivo* gluconeogenesis [12]. Pyruvate tolerance challenge has been used as an indirect measurement for gluconeogenesis in mouse models. Except for glucose concentration, it does not actually quantify any metabolic flux of *de novo* glucose production. Stable isotopic tracers enable *in vivo* quantification of fractional gluconeogenesis in humans (e.g., by measuring the incorporation of deuterium from the body water into newly formed glucose) [12]. In order to define the physiological impact of PPG neurons that express the glucagon (Gcg) gene (also called Gcg neurons) *in vivo*, we wanted to quantify glucose kinetics and insulin sensitivity using dual stable isotopic tracers in conjunction with hyperinsulinemic euglycemic clamp.

The designer receptors exclusively activated by designer drugs (DREADD) approach has been developed for remote control of targeted neurons in the mouse brain for mapping feeding circuitry [13–19], and has been used to dissect the acute, neural control of periphery metabolism [20–22]. Increasing evidence indicates that acute activation of distinct populations of neurons in the brain influences feeding behavior, food intake and body weight. To elucidate if PPG neurons regulate peripheral glucose metabolism, we wanted to create a genetic mouse model to enable their remote activation in a Gcg-dependent manner. Our objective was to define if acute activation of PPG neurons enhances peripheral glycemic control and insulin sensitivity in lean mice.

In the present study, we established a pharmacogenetics mouse model for the remote control of activation of PPG neurons *in vivo*. We first generated glucagon (Gcg) promoter-driven Cre transgenic mice and used them to create a mouse model for remote control of activation of PPG neurons using the DREADD approach. Moreover, we characterized the physiological significance of acute activation of PPG neurons on glucose metabolism and insulin sensitivity using stable isotopic tracers (6,6-$^2H_2$-D-glucose and ^2H_2O). We showed in Gcg-Cre lean mice infected with excitatory hM3Dq virus in the brainstem NTS that acute activation of Gcg neurons enhances glucose tolerance, suppresses basal endogenous glucose production, and augments hepatic insulin sensitivity. We conclude that acute activation of PPG neurons in the brainstem NTS promotes glucose homeostasis and insulin sensitivity, suggesting a physiological role of PPG neurons-mediated circuitry in glycemic control.

2. MATERIAL AND METHODS

2.1. Animals

The protocols of this study were approved by the Animal Care and Use Committee of Baylor College of Medicine and carried out in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Bethesda, MD). Glucagon (Gcg)-Cre mice were generated using the Gcg-Cre cryo-preserved embryos (ID 358-UNC donated by Pedro L. Herrera). Mice were maintained under a 12h:12h light–dark cycle with room temperature (~22 °C) and humidity (~50%) conditions and provided *ad libitum* access to water and a standard chow. To determine the metabolic impact of acute activation of targeted neurons, excitatory AAV-hM3Dq-mCherry viruses (serotype 8) were injected in to the brainstem NTS of Gcg-transgenic mice at the age of ~8 wk. Two weeks later, the mice were fitted with the jugular cannulation for primed-continuous infusion of stable isotopic tracers. After one-week recovery, glucose tolerance test and hyperinsulinemic euglycemic clamp in conjunction with stable isotopic tracers were employed to quantify glucose homeostasis and insulin sensitivity in conscious mice with remote activation of targeted neurons. After mice were euthanized under isoflurane anesthesia, brain samples were dissected for immunohistochemistry and electrophysiology. Experimental Procedures in detail are provided in the Supplementary Material.

2.2. Immunohistochemistry

The whole brain was harvested 0.5 h after ip injection of clozapine N-oxide (CNO, 0.3 mg/kg), fixed and cut at 25 μm for coronal sections. Brain slices were immunostained for c-Fos and mapped for PPG neurons-innervated central autonomic regions [8].

2.3. Glucose tolerance test

Three weeks after viral injection, mice were fasted with free access to water. After 6-h fast, basal blood glucose concentrations were measured. 30 min after ip injection of CNO (0.3 mg/kg) or vehicle (PBS), mice were then challenged (at 1.5 g/kg BW, i.p.), and blood glucose concentrations were measured at the time points of 15, 30, 45, 60, 90, and 120 min.

2.4. Stable isotopic tracers in conjunction with hyperinsulinemic euglycemic clamp

Glucose metabolic fluxes were quantified with stable isotopic tracers in conjunction with hyperinsulinemic euglycemic clamp. Glucose metabolic fluxes and insulin sensitivity were assessed in mice after an overnight fast under ip injection of CNO (0.3 mg/kg) or saline ($n = 8$–10/group). A dual stable isotopic tracer method (^2H_2O and 6,6-$^2H_2$-D-glucose) coupled with hyperinsulinemic euglycemic clamp was employed to quantify glucose metabolism (including gluconeogenesis) and insulin sensitivity at a steady-state in conscious mice. In brief, mice were primed-continuously infused with 6,6-$^2H_2$-D-glucose via a jugular catheter for 3 h during basal period; and then for 3 h during insulin clamp (see Figure 4), whereas 6,6-$^2H_2$-D-glucose plus D-glucose were infused to maintain blood glucose level at ~100 mg/dL (see Fig. S2). Blood samples (5 μL each) were collected at 0, 3, and 6 h post infusion for analyzing isotopic enrichment of ^2H_2O with IR-MS; and spotted at 2:50, 2:55, and 3:00 h for the baseline; and at 5:50, 5:55, and 6:00 h for the clamp to analyze isotopic enrichment of 6,6-$^2H_2$-D-glucose with GC–MS.

2.5. Calculations for glucose kinetics

(1) $Rd = Ra = \mathrm{GIR} \cdot \mathrm{MPE(glucM2)}_{infusate}/\mathrm{MPE(glucM2)}_{blood;}$

(2) $\mathrm{EGP} = Ra - \mathrm{GIR};$

(3) $\text{Fractional GNG} = 100 \cdot \mathrm{MPE(glucM1)}_{blood}/\mathrm{MEP}(^2H_2O)_{blood;}$

(4) $\mathrm{GNG} = \mathrm{EGP} \cdot \text{Fractional GNG};$

Where $\mathrm{MPE(glucM2)}_{infusate}$ is $^2H_2$-glucose enrichment in infusate (in mole % excess, MPE); $\mathrm{MPE(glucM2)}_{blood}$ is $^2H_2$-glucose enrichment in blood; MPE(glucM1) is ^2H-glucose enrichment in blood; MPE(^2H_2O) is ^2H_2O enrichment in blood. Glucose kinetics at the steady state is calculated for GIR (glucose infusion rate), *R*d (rate of glucose disappearance), *R*a (rate of glucose appearance), EGP (endogenous glucose production), and GNG (gluconeogenesis) [8,23,24].

2.6. Electrophysiological recordings

Gcg-Cre mice injected with excitatory AAV8-hM3Dq-mCherry viruses were used to validate acute activation of Gcg neurons upon CNO application. Membrane potential and firing rate of Gcg-mCherry$^+$ neurons in the brainstem NTS were measured by the whole-cell current patch clamp [8].

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2.7. Statistical analysis

Using the mixed procedure (SAS 9.4), we analyzed data with the fixed effects (including treatment, status, and time), the random effect (including individual mouse), and repeated measures (at different time points in the same mouse) [8]. A full model for analysis of covariance includes BW, treatment, status, and their interactions. Note that BW is considered as covariates to remove effects of body size. Least squares mean was reported and difference is considered significant at $p < 0.05$. Error bars were indicated by SEM.

3. RESULTS

3.1. Generation of glucagon (Gcg)-Cre mouse line

To determine the metabolic impact of glucagon (Gcg) neurons in the brainstem, we generated a Gcg promoter-driven Cre transgenic mouse line. a) We first obtained the Gcg-Cre cryo-preserved embryos (ID 358-UNC donated by Pedro L. Herrera at University of Geneva Medical School) from the NIH-supported Mutant Mouse Regional Resource Center at the University of North Carolina at Chapel Hill (UNC). b) We then used C57BL/6J female mice for *in vitro* fertilization at the Genetically Engineered Mouse Core at Baylor College of Medicine. c) We further validated the Gcg-Cre mouse line by Cre-dependent Rosa26-eGFP reporter mice. It has been documented that this Gcg promoter-driven Cre expresses efficiently, reproducibly, and specifically in pancreatic α cells [25,26]. Gcg-Cre-mediated expression of eGFP was revealed authentically in distinct types of enteroendocrine L cells in the gut epithelium, endocrine α cells in the pancreas, and preproglucagon neurons in the brainstem NTS (not shown). As we show in Figure 1, this Gcg-Cre-mediated eGFP reporter was displayed in the brainstem (including the dorsal motor vagal nucleus (DMV), the NTS, and the intermediate reticular nucleus (IRT, **C**)) and projected to the brainstem DMV (**D**) and hypothalamus (including the paraventricular/dorsomedial nuclei and arcuate nucleus, **E-F**) where PPG neurons projected and GLP-1R/2R localize [1]. Thus, Gcg neurons are positioned to fine-tune sympathetic and parasympathetic outflow.

3.2. Remote activation of Gcg^{+} neurons using the DREADD

To define the metabolic impact of activating Gcg^{+} neurons, i.e., preproglucagon (PPG) neurons in the brainstem NTS, we employed the DREADD approach to our generated Gcg-Cre mouse line. To validate if Cre-induced, AAV8-hSyn-DIO-hM3Dq-mCherry virus activated Gcg^{+} neurons, we found in Figure 2 that hM3Dq-mCherry (in red) was expressed in the brainstem NTS-DMV, and upon ip injection of clozapine-N-oxide (CNO, 0.3 mg/kg), hM3Dq-mediated activation of Gcg^{+} neurons induced an abundant expression of the early response gene c-Fos (in green, **C**) in the brainstem NTS-DMV and AP compared to that in **A**, suggesting activation of Gcg^{+} neurons-mediated neural circuitry. To further confirm if hM3Dq-expressing neurons were excited upon CNO application, we recorded the membrane potential and firing rate of Gcg-Cre::hM3Dq-mCherry^{NTS} neurons in brains slices using the whole cell patch clamp. As shown in Figure 3, PPG neurons (in green, **A**) in the brainstem NTS from the Gcg-Cre::Rosa26-eGFP mouse did not respond to CNO application (10 μM, **B**). However, hM3Dq-expressing Gcg^{+} neurons (in red, **C**) in the brainstem NTS-DMV from the Gcg-Cre::hM3Dq-mCherry mouse were acutely excited as their firing rates were increased upon CNO application (10 μM, **D**), suggesting that hM3Dq activation would rapidly excite infected Cre-expressing, Gcg^{+} neurons in the brainstem. Therefore, data obtained using a DREADD approach suggest that hM3Dq^{+}-Gcg neurons can be acutely activated *in vivo* as indicated by enhancing c-Fos expression and acutely excited *ex vivo* by increasing firing rates.

3.3. Remote activation of Gcg neurons in the NTS promotes glucose homeostasis

Gcg-Cre mice fed regular chow were nano-injected in the brainstem NTS with AAV8-hSyn-DIO-hM3Dq-mCherry virus. To characterize the

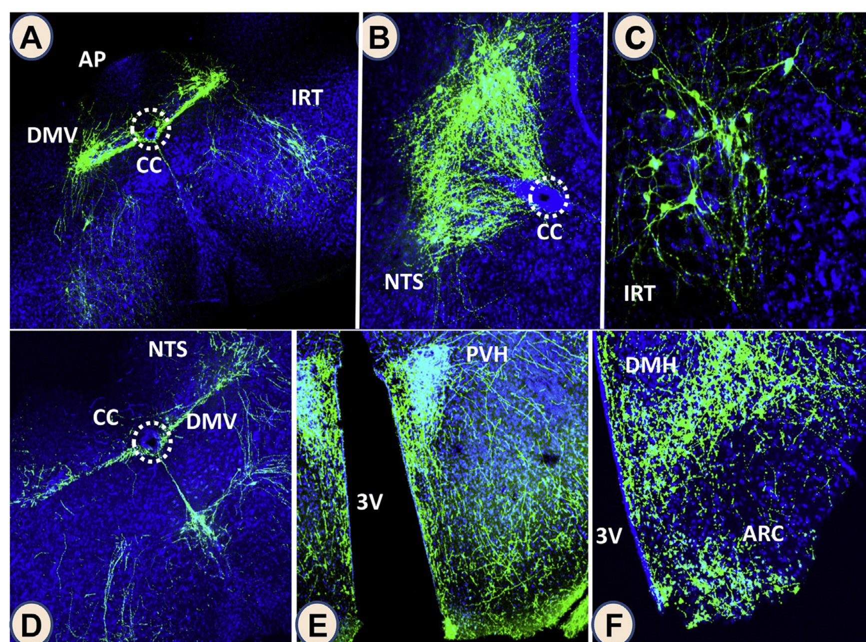

Figure 1: Gcg-Cre transgenic mouse line was validated by Cre-dependent Rosa26-eGFP reporter mouse. Gcg-Cre-mediated eGFP reporter was expressed in distinct neurons in the brainstem the dorsal motor vagal nucleus (DMV, **A**), the nucleus tractus solitarius (NTS, **B**), and the intermediate reticular nucleus (IRT, **C**); and projected to the brainstem dorsal motor vagal nucleus (DMV, **D**) and hypothalamus [the paraventricular/dorsomedial hypothalamic nuclei (PVH/DMH) and arcuate nucleus (ARC), **E**—**F**]. Note that Gcg promoter-driven Cre expression is reported by eGFP (green) from the Gcg-Cre::Rosa26-eGFP mouse brain. CC, central canal; AP, area postrema.

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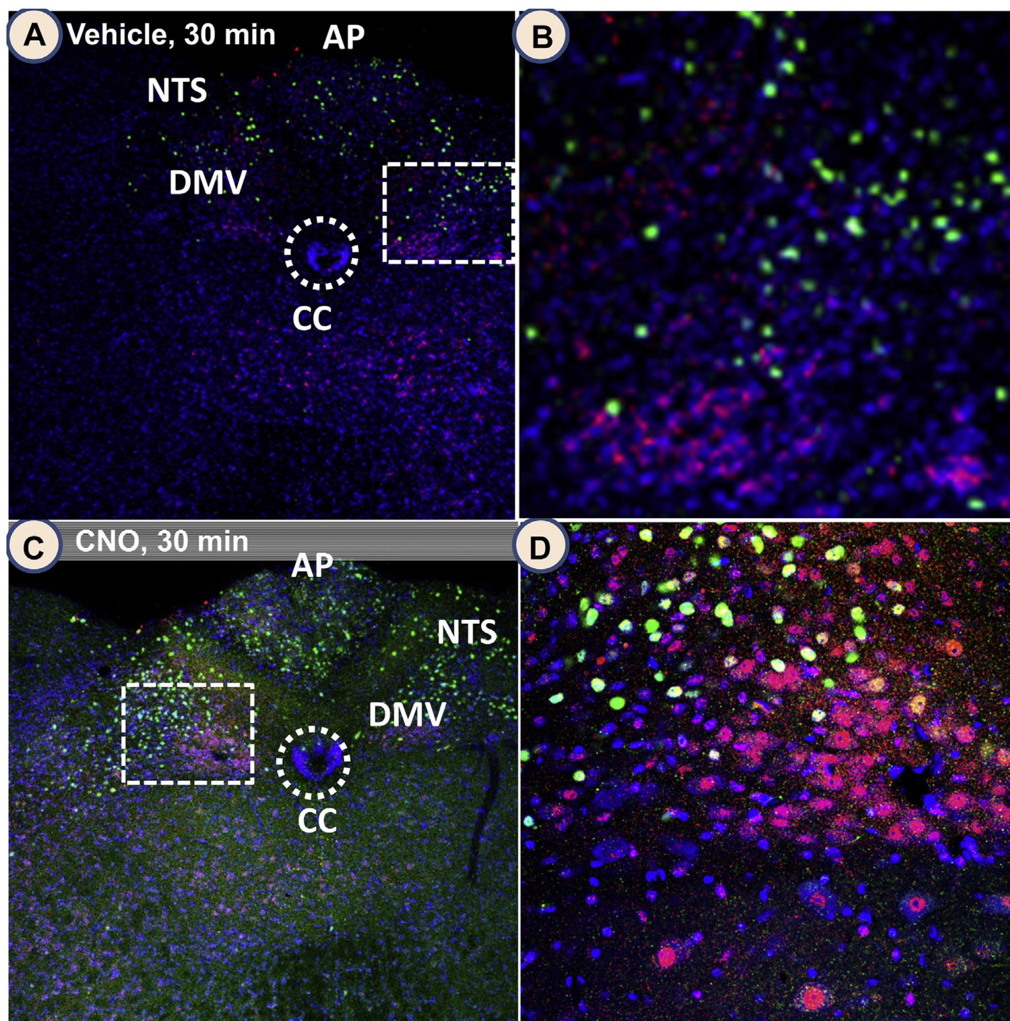

Figure 2: Remote activation of hM3Dq-expressing Gcg neurons induced by CNO. **A**. Weak c-Fos expression in the basal level without CNO injection. **B**. Enlarged image of the squared region in **A**. c-Fos-positive cells (green) were largely segregated from hM3Dq-expressing cells (red). **C**. c-Fos expression was increased at 30 min after ip injection of CNO (0.3 mg/kg). **D**. Enlarged image of the squared region in **C**. c-Fos-positive cells (green) were largely localized to hM3Dq-expressing cells (red). Note that Gcg neurons are indicated by expression of mCherry (red) in the brainstem NTS-DMV from the Gcg-Cre mouse locally infected with AAV8-hM3Dq-mCherry viruses.

metabolic phenotype of acute activation of Gcg^+ neurons in the brainstem NTS, we showed in Figure 5 that blood glucose concentrations were decreased at 3.5 h post ip injection of CNO (0.3 mg/kg) in Gcg-Cre mice after overnight fast (Figure 5, A). It was noted that blood glucose concentrations were temporarily increased at the first 2.5 h post ip injection of CNO (0.3 mg/kg) in as-fed Gcg-Cre mice as shown in supplemental Fig. 1S. Moreover, glucose tolerance was increased in Gcg-Cre mice after a 6-h fast, as revealed by an ip glucose tolerance test (GTT, 1.5 g/kg) 0.5 h post ip injection of CNO (0.3 mg/mL) (Figure 5, C). Furthermore, stable isotopic tracer (6,6-$^2H_2$-D-glucose) was prime-continuously infused for 3.5 h in conscious mice and endogenous glucose production quantified under remote activation of Gcg^+ neurons. Importantly, we found that endogenous glucose production was reduced at 3.5 h post ip injection of CNO (0.3 mg/kg) in Gcg-Cre mice after overnight fast (Figure 5, B). These data suggest that acute activation of PPG neurons in the brainstem NTS suppresses basal endogenous glucose production and enhances glucose tolerance, promoting glucose homeostasis in lean mice.

3.4. Remote activation of Gcg neurons in the NTS enhances hepatic insulin sensitivity

To determine if acute activation of PPG neurons in the brainstem augments insulin sensitivity in a tissue-specific manner, we quantified glucose metabolic fluxes under basal conditions and upon insulin stimulation. To quantify *in vivo* gluconeogenesis in conscious mice after acute activation of PPG neurons, we employed dual stable isotopic tracers (^2H_2O and 6,6-$^2H_2$-D-glucose) in conjunction with hyperinsulinemic euglycemic clamp (Figure 6). This method was used in our laboratory to quantify hepatic insulin sensitivity in mice as reported previously. Glucose metabolic fluxes and insulin sensitivity were determined at steady state in conscious mice after an overnight fast. No difference in clamped levels (approximately at 100 mg/dL) of blood glucose was noted during the last 1-h clamp between two groups (see Fig. S2), indicating that blood glucose levels were truly clamped. This 1-h duration is required for reaching the equilibrium for glucose isotopologues at steady state. Remote activation of Gcg neurons in the brainstem augmented whole-body insulin sensitivity as indicated by higher glucose infusion rate (GIR, Figure 6A), which was largely

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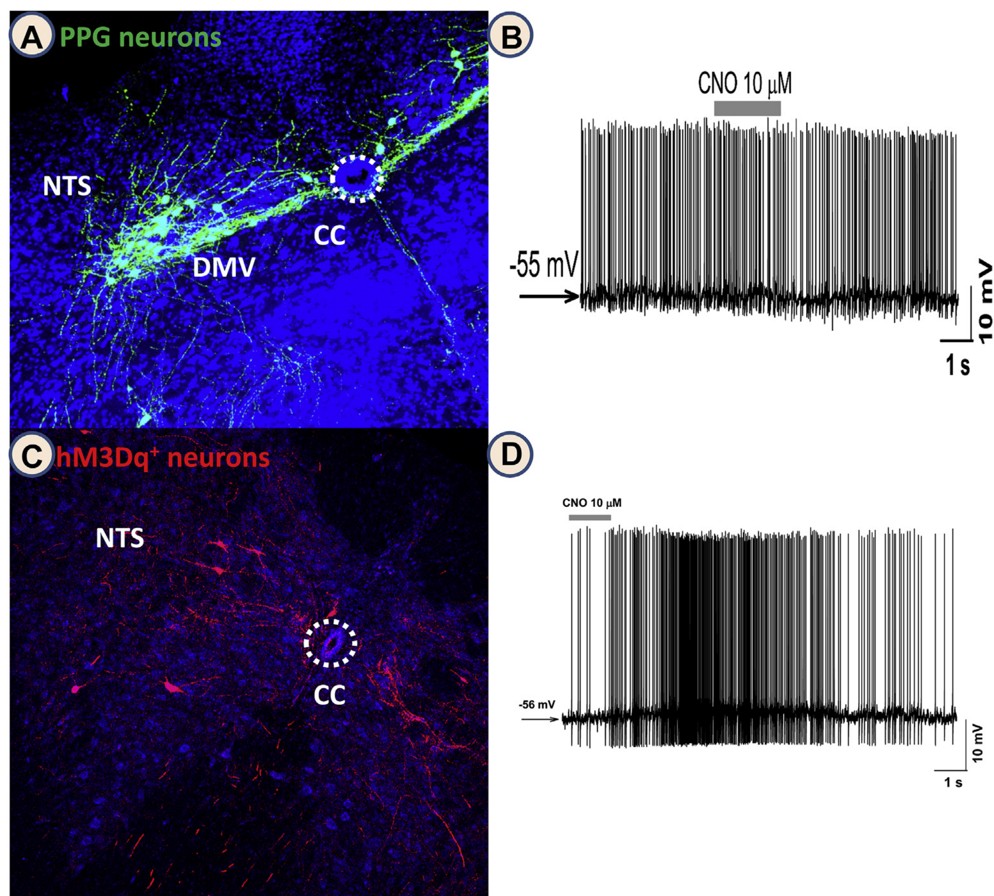

Figure 3: Acute excitation of hM3Dq-expressing Gcg neurons induced by CNO. **A**. Gcg Cre-mediated expression of eGFP (green) in the brainstem NTS—NTS from the Gcg-Cre::Rosa26-eGFP mouse line. **B**. PPG neurons (green) in the brainstem NTS did not respond to CNO (10 μM) application as control. **C**. Gcg neurons indicated by mCherry (red) in the brainstem NTS from the Gcg-Cre mouse locally infected with AAV8- hM3Dq-mCherry viruses. **D**. hM3Dq-expressing Gcg neurons was excited by CNO (10 μM) application as shown representative traces of the whole-cell current patch clamp.

attributed to augmenting insulin-mediated suppression of endogenous glucose production (EGP) and gluconeogenesis (Figure 6B). However, insulin-mediated stimulation of peripheral glucose utilization was not significantly altered as indicated by rate of glucose disappearance. Of note, there was no difference in body weight (Vehicle: 27.2 $\pm$ 0.3 g/n $=$ 8; CNO: 26.7 $\pm$ 0.3 g/n $=$ 6) when hyperinsulinemic euglycemic clamp was performed.

4. DISCUSSION

In addition to enteroendocrine L cells, GLP are produced from **PPG** neurons in the brainstem NTS [28,29]. PPG neurons widely project to central autonomic regions where GLP-1R/2R are expressed [2]. It seems that the $NTS^{PPG} \rightarrow$ PVH circuitry plays a key role in suppressing feeding [7,30,31]. Except for endocrine regulation, glycemic control is achieved by neural regulation [32]. It is challenging to define the physiological relevance of endocrine vs neural GLP to glycemic control. The glycemic impact of PPG neurons-derived neural GLP remains unclear, yet indirect evidence has been reported from both genetic deletion (e.g., *Glp2r* in hypothalamic POMC neurons) and pharmacologic manipulation (e.g., icv administration of GLP-1 or GLP-2). GLP-1 improves glycemic control via its incretin effect while GLP-2 augments hepatic insulin sensitivity via a neural action. As a matter, however, it is unknown if acute activation of PPG neurons in the brainstem alters insulin sensitivity. In order to determine the physiological role of PPG neurons, we thus generated the Gcg-Cre transgenic mouse line. To validate the expression of Gcg-Cre by Cre-mediated eGFP reporter, we show that Cre is expressed authentically in PPG neurons and enteroendocrine L cells.

One major finding in the current study is that acute activation of Gcg^{+}-neurons in the brainstem augmented glucose tolerance and hepatic insulin sensitivity, suggesting the functional importance of the Gcg $neurons^{NTS}$ autonomic circuitry in glycemic control. As an integrative center, the brainstem dorsal vagal complex (**DVC**, including NTS, DMV and AP) integrates neural, hormonal, and nutritional inputs [33,34], particularly integrating hypothalamic inputs and vagal afferent inputs to fine-tune autonomic outputs, thus suppressing **HGP** and food intake [1,27,34—36]. PPG neurons widely project to central autonomic regions. Cholinergic neurons (**ChAT**) regulate HGP [27]. Although largely segregated in peripheral endocrine cells, GLP-1R/2R are co-expressed in central autonomic neurons (including DVC ChAT neurons) [8,37—39]. GLP-1 improves oral glucose tolerance probably via a neural manner [40,41]. Except for the enteroinsular incretin effect, GLP-1-mediated neural action in glycemic control has not been fully defined [42—46], yet brainstem GLP-1R activation suppresses food intake [47,48]. GLP-2 promotes intestinal glucose uptake required for the enteroinsular function. Neural GLP-2 acts as a neurotransmitter linking the hypothalamus and the brainstem [37,49]. GLP-2R activation

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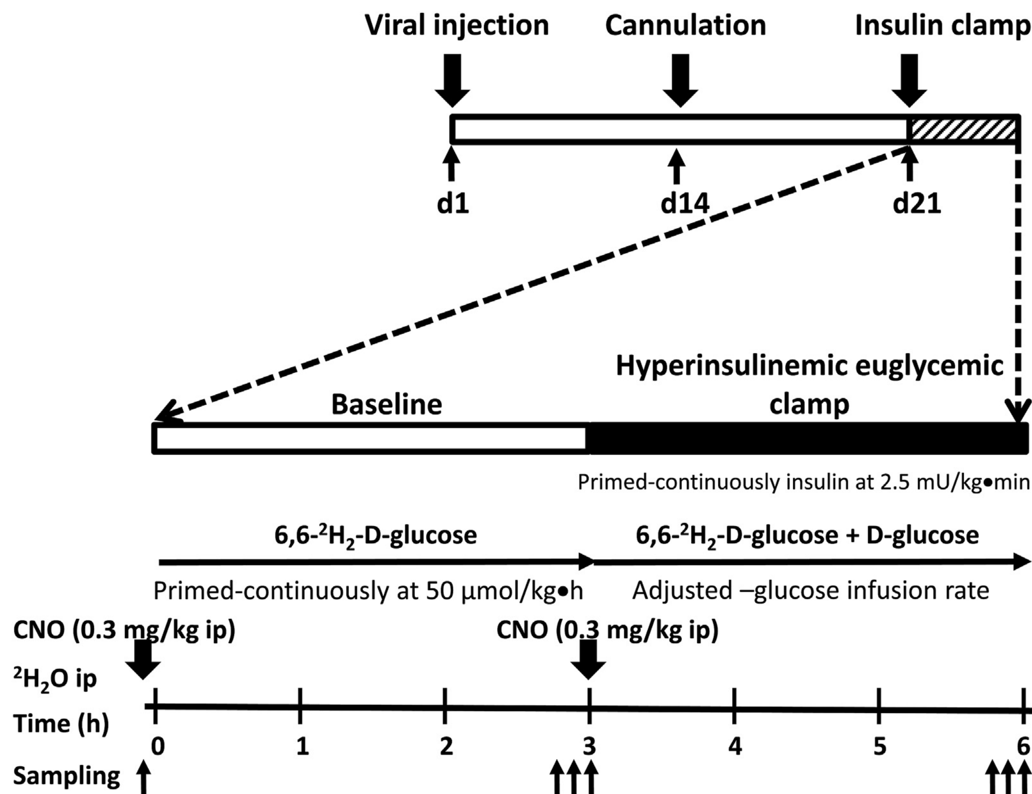

Figure 4: A protocol for quantifying glucose kinetics and insulin sensitivity. A protocol for stable isotopic tracer method in conjunction with hyperinsulinemic euglycemic clamp is pictured [8]. After a 7-d recovery, conscious mice after 12-h fast were primed-continuously infused with stable isotopic tracers (^2H_2O and 6,6-$^2H_2$-D-glucose) for 3 h to quantify glucose kinetics during the basal period; and then for 3 h to assess tissue-specific insulin sensitivity during hyperinsulinemic euglycemic clamp. Two weeks after viral injection, 10-wk-old mice were implanted with jugular vein catheters. Hyperinsulinemic euglycemic clamp was performed in stress-less, conscious mice at one week post cannulation. Note that CNO (0.3 mg/kg) was ip injected at 30 min prior to the primed-continuous infusion of 6,6-$^2H_2$-D-glucose for glucose kinetics and then at the beginning of the insulin clamp. Blood glucose was clamped at ∼100 mg/dL (as shown in Fig. S2) with infusion of glucose during insulin infusion (2.5 mU/kg/h). Isotopic enrichments of blood glucose and water were measured by GC–MS and GC-IR-MS, respectively. Glucose kinetics (including **GIR**, glucose infusion rate; **EGP**, endogenous glucose production; **GNG**, gluconeogenesis; and $\boldsymbol{R_d}$, the rate of glucose disappearance) were quantified at postabsorptive, steady state in conscious mice.

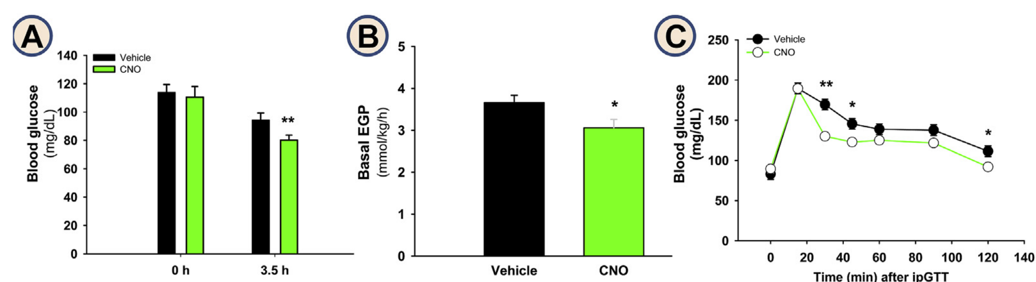

Figure 5: Remote activation of Gcg neurons reduces endogenous glucose production in mice fed regular chow. **A**. 12-h fasting glucose at 0 and 3.5 h post injection of CNO (ip 0.3 mg/kg). Excitatory AAV-hM3Dq-mCherry viruses (150 nL) were injected into the Gcg-Cre mouse brainstem NTS. **B**. Basal endogenous glucose production (**EGP**) in the conscious mice after an overnight fast (at 3 wks post viral injection). To quantify the basal EGP, a stable isotopic tracer (6,6-$^2H_2$-D-glucose) was infused for 3 h to reach an equilibrium for glucose isotopologues. See the detailed stable isotopic tracer methodology in **METHODS**. Mice were ip injected with CNO (0.3 mg/kg) or saline prior to the tracer infusion. n = 6–8/group. *$P < 0.05$. **C**. Blood glucose concentrations (of mice at 3 wks post viral injection) measured in 6-h fast mice after ip glucose challenge test (ipGTT). CNO (0.3 mg/kg) was ip injected 30 min prior to ipGTT.

in POMC neurons enhances glucose tolerance and promotes hepatic insulin sensitivity [8], while brainstem GLP-2 suppresses food intake [48]. The new evidence reported here indicates that acute activation of Gcg neurons *per se* plays an important physiological role in glycemic control and insulin sensitivity. Future studies are warranted to define which neurotransmitters are released from activating Gcg neurons in the brainstem using MS-based metabolomics.

4.1. Mechanisms for PPG neurons-mediated metabolic actions

The present study revealed that acute activation of PPG neurons in the brainstem NTS suppresses hepatic glucose production under both basal state and insulin stimulation. PPG neurons (as reported by Gcg Cre-mediated Rosa26-eGFP) are mainly localized to the brainstem NTS-DMV and the intermediate reticular nucleus of the brainstem; and densely projected to the paraventricular, dorsomedial, and arcuate

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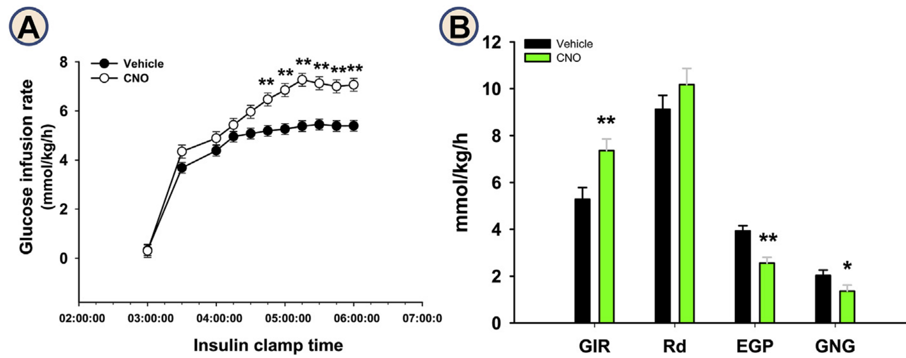

Figure 6: Remote activation of Gcg neurons enhances hepatic insulin sensitivity in mice fed regular chow. **A**. Glucose infusion rate (GIR, mmol/kg/h) during hyperinsulinemic euglycemic clamp. Higher GIR were needed to maintain the same level of blood glucose (clamped at $\sim$100 mg/dL, see Fig. S2) in conscious PPG^{hM3Dq} mice after ip injection of CNO. **B**. Tissue-specific insulin sensitivity of the conscious PPG^{hM3Dq} mice quantified by hyperinsulinemic euglycemic clamp coupled with dual stable isotopic tracers (^2H_2O and 6,6-$^2H_2$-D-glucose as shown in Figure 4). Glucose kinetics was determined at the steady status. Remote activation of Gcg neurons augments hepatic insulin sensitivity largely by further suppression of EGP and GNG. **GIR**, glucose infusion rate; ***Rd***, rate of glucose disappearance; **EGP**, endogenous glucose production; **GNG**, gluconeogenesis. PPG^{hM3Dq} mice (at 3 wks post viral injection) were ip injected with CNO (0.3 mg/kg) or saline prior to the insulin clamp. n = 6–8/group. * or **$P < 0.05$ or 0.01.

nuclei of the hypothalamus. Notably the PPG neurons also project to the dorsal motor nucleus of the vagus (DMV) and the area postrema (AP) in the brainstem. Activation of Gcg neurons induced c-Fos expression mainly localized to central autonomic regions (not shown). Thus, PPG neurons are ideally situated to modulate sympathetic and parasympathetic outflow [3]. PPG neurons receive synaptic inputs from vagal afferents and nutritional signals such as leptin [3,5,33]. These data suggest that acute activation of PPG neurons in the brainstem NTS probably promotes glycemic control and insulin sensitivity. We propose a working model for Gcg neurons-mediated glycemic action as shown in Figure 7. We speculate that activation of PPG neurons may release GLP and unidentified neurotransmitters to the hypothalamic paraventricular/arcuate nuclei and the brainstem DMV, suppressing hepatic glucose production (**HGP**, mainly gluconeogenesis) by fine-tuning hepatic vagal output [3,50] and pancreatic hormonal secretion (of insulin and glucagon). However, studies are warranted to identify which neurotransmitters/neuropeptides are released from activated PPG neurons using MS-based metabolomics and genetically define their contribution to glycemic control.

4.2. The physiological impact on glucose homeostasis

Acute activation of PPG neurons in the brainstem NTS lowered basal glucose production and enhanced glucose tolerance. In hMD3q-infected Gcg-Cre lean mice fasted overnight, blood glucose concentrations were lowered 3.5 h post ip injection of CNO. More importantly, basal glucose production was reduced as assessed with the stable isotopic tracer (6,6-$^2H_2$-D-glucose). Moreover, their glucose tolerance (determined by intraperitoneal GTT) was enhanced in response to ip CNO. We note that oral GTT stimulates GLP release from enteroendocrine L cells, probably compromising the glycemic action of activating Gcg neurons. Interestingly, blood glucose concentrations were increased within the first 2 h post ip injection of CNO in as-fed mice. Notably glucose homeostasis is

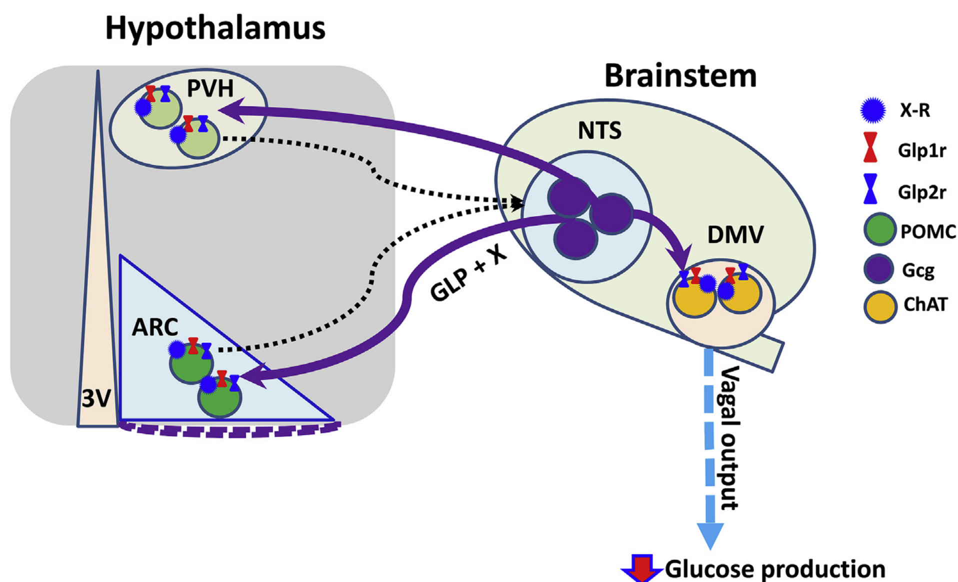

Figure 7: A proposed model for Gcg neurons in glycemic control. Activation of Gcg neurons in the brainstem NTS promotes glycemic control and insulin sensitivity via fine-tuning autonomic outputs to peripheral tissues (liver), suppressing hepatic glucose production (**HGP**, mainly gluconeogenesis), which may involve Glp1r/2r-positive neurons in the hypothalamus (PVH and ARC) and brainstem (DMV). In addition, activation of Gcg neurons may release unidentified neurotransmitter(s) X that modulates postsynaptic neurons in the above nuclei.

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tightly controlled in a time-relevant, feeding-dependent manner. Paradoxically effects of PPG neurons' activation on blood glucose concentrations may be dependent upon physiological conditions. Considering endocrine secretion of GLP from enteroendocrine L cells, their neural release from PPG neurons might be enhanced postprandially to coordinate glycemic control. These data suggest that acute activation of PPG neurons reduces hepatic glucose production at the basal status and enhances glucose tolerance.

4.3. The physiological impact on insulin sensitivity

The present study was the first time to show that acute activation of Gcg neurons in the brainstem enhanced hepatic insulin sensitivity, as indicated by augmenting insulin-mediated suppression of HGP and gluconeogenesis. The liver plays a major role in maintaining blood glucose levels by regulating *de novo* glucose production (gluconeogenesis) and glycogen breakdown (glycogenolysis). Chronically elevated HGP is caused by increased gluconeogenesis, rather than glycogenolysis in the diabetic state [11]. Thus, inhibition of gluconeogenesis is an important target for developing glucose-lowering drugs. It is well demonstrated that distinct populations of neurons in the hypothalamus and brainstem regulate HGP, and that the brain is responsible for $\sim$50% of oral glucose disposal in the postabsorptive state [51]. The present study suggests that PPG neurons^{NTS} autonomic circuitry plays an important role in glycemic control and hepatic insulin sensitivity. It is plausible that activation of PPG neurons suppresses hepatic gluconeogenesis, which contributes to improving glycemic control and insulin sensitivity via fine-tuning vagal outputs to liver. Further studies are warranted to define if and how PPG neurons regulate postprandial glycemic control and insulin sensitivity and determine if activation of PPG neurons reduces hepatic gluconeogenesis under hyperglycemic condition.

4.4. Innovative tools to define the metabolic impact of targeted neurons in the brain

The designer receptors exclusively activated by designer drugs (DREADD) approach has been developed for rapid activation of targeted neurons in the brain for mapping feeding circuitry, and used extensively for dissecting the acute, neural control of periphery metabolism [20,52,53]. Note that there were no differences in body composition or body weight prior to acute activation of PPG neurons by CNO. Using Gcg-Cre mouse line, we showed that the remote activation of Gcg neurons *in vivo* promotes glucose homeostasis and insulin sensitivity, suggesting that Gcg neurons may release GLP and other neuroendocrine signals to promote glycemic control. However, these results must be interpreted with caution. New evidence indicates that the mechanism of CNO action at DREADDs in the brain is directly mediated by clozapine, a metabolite converted from CNO. Thus, CNO agonistic effects could be confounded by off-target effects of CNO-converted clozapine at endogenous receptors if any. Notably, ip injection of CNO at 1 or 10 mg/kg decreased locomotor activity selectively in Drdla^{hM4Di} or forebrain^{hM4Di} mice, and not in the control [54]. Considering a single, low dosage (0.3 mg/kg) of CNO used in the present study, the metabolic phenotype of PPG^{hM3Dq} mice could be specific. To avoid any off-target effects of locally converted clozapine at endogenous sites, dose—response relationships and more controls are needed in the future studies. To dissect GLP-induced neural function from endocrine action, moreover, it would be interesting to examine the metabolic impact of remote activation of Gcg neurons with mutated Gcg, from which the neurotransmitters other than mutant GLP are released from activation of Gcg neurons in the brainstem NTS.

Stable isotopic tracers have been used to evaluate glucose homeostasis as a powerful approach to quantify metabolic functions and pathways in term of glucose metabolic fluxes. It is the mass balance of glucose metabolic fluxes, such as endogenous glucose production in the liver, exogenous glucose uptake by the gut, and glucose utilization by insulin-sensitive tissues, that determines the concentrations of blood glucose measured. It has been challenging to quantify glucose metabolic fluxes (such as gluconeogenesis, GNG) in conscious mice. Hyperinsulinemic euglycemic clamp has been coupled with radioactive tracers to assess EGP, by which gluconeogenesis and glycogenolysis cannot be quantified separately. To quantify how GNG is regulated in mice, the authors have employed stable isotopic tracers in conjunction with the insulin clamp. Of note, the ^2H_2O method might underestimate GNG. In the present study, rates of glucose metabolism (instead of concentrations of blood glucose) were quantified *in vivo* using stable isotopic tracers, providing insights into glycemic control. One of the important findings is that acute activation of PPG neurons inhibits hepatic gluconeogenesis at the basal status (i.e., after overnight fast) and under the insulin clamp, indicating their activation augments hepatic insulin sensitivity. Thus, PPG neurons may be a therapeutic target for ameliorating hyperglycemia and hepatic insulin resistance.

5. CONCLUSIONS

In summary, we showed that acute activation of Gcg neurons in the brainstem promotes glucose homeostasis and insulin sensitivity in the Gcg-Cre mouse line. Specifically, acute activation of Gcg neurons reduces endogenous glucose production (i.e., hepatic gluconeogenesis at the basal status) and augments hepatic insulin sensitivity as gluconeogenesis is further suppressed by exogenous insulin. However, it is warranted to further define which key neurotransmitters (in addition to glucagon-like peptides) are released from activated Gcg neurons. Acute activation of either Gcg neurons improves glucose tolerance and insulin sensitivity, indicating that Gcg-mediated autonomic circuitry plays an important role in glycemic control. The present study suggests that PPG neurons-mediated neural circuitry is essential for glycemic control and insulin sensitivity. Gcg neurons may be a potential target for improving glycemic control and insulin sensitivity in type 2 diabetes and obesity.

ACKNOWLEDGMENTS

The authors thank Zhuo Yang, Shixiang Wen, Alexander Brown, Qiang Tong, Darryl Hadsell, and Marta Fiorotto at Baylor College of Medicine for scientific and technical support. We also thank Xiaozhi Wang at Binzhou Medical University Hospital for technical support. This work is supported by the USDA CRIS 6250-51000-054-00D-3, NIH DK58338 (DDC 2014 Pilot/Feasibility Award), and LUCTA Research Award (X.G.); the National Natural Science Foundation of China Grant 81570722 (X.S.); and NIH P30DK079638 (L.C.). X.G. and X.S. contributed to the study concept and design, acquisition of data, statistical analysis, interpretation of data, and drafting the manuscript. In addition, X.G. obtained funding, supervised the study, and finalized the manuscript. X.G. and X.S. contributed to the design and generation of the Gcg-Cre mice. S.C. and F.L. assisted with GC—MS analysis. D.L. assisted with electrophysiological recording. D.B. and L.C. provided technical support and critically reviewed the manuscript.

CONFLICTS OF INTEREST

None declared.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.08.009.

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