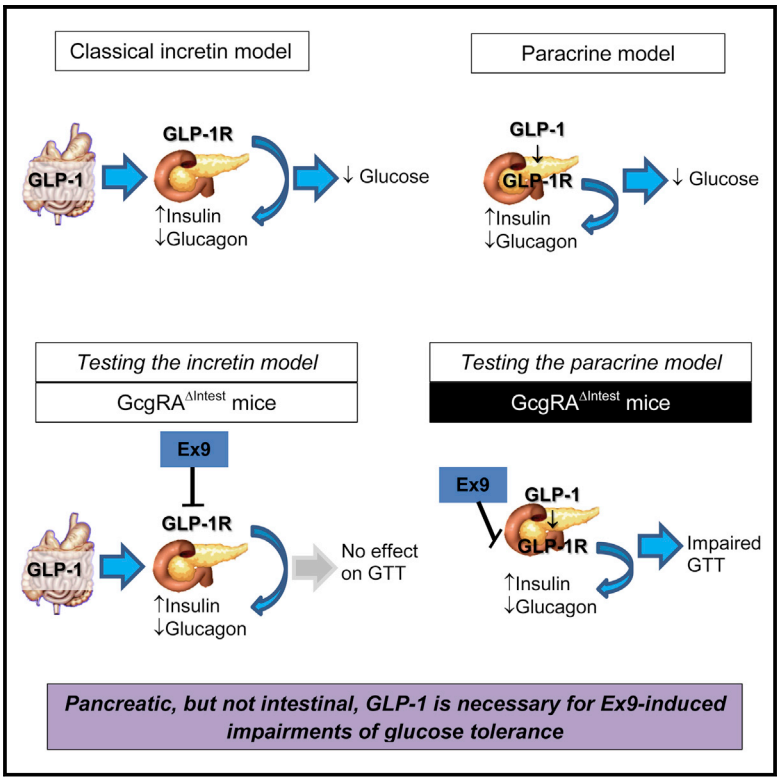
Short Article

Cell Metabolism

The Role of Pancreatic Preproglucagon in Glucose Homeostasis in Mice

Graphical Abstract



Highlights

- Intestinally secreted GLP-1 is presumed to regulate glucose via incretin action
- Exendin-9 does not alter glucose in mice that only produce GLP-1 in the intestine
- Exendin-9 does impair glucose in mice that only produce GLP-1 in the pancreas
- Alternative to the incretin model, islet GLP-1 is crucial for gluco-regulation

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In Brief

GLP-1 is necessary for normal gluco-regulation, and it has been widely presumed that this function is the action of peptide released from enteroendocrine L cells. The data from Chambers et al. challenge this dogma and find that intestinally produced GLP-1 is dispensable, while pancreatic production of GLP-1 is necessary for gluco-regulation.

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The Role of Pancreatic Preproglucagon in Glucose Homeostasis in Mice

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SUMMARY

Glucagon-like peptide 1 (GLP-1) is necessary for normal gluco-regulation, and it has been widely presumed that this function reflects the actions of GLP-1 released from enteroendocrine L cells. To test the relative importance of intestinal versus pancreatic sources of GLP-1 for physiological regulation of glucose, we administered a GLP-1R antagonist, exendin-[9-39] (Ex9), to mice with tissue-specific reactivation of the preproglucagon gene (*Gcg*). Ex9 impaired glucose tolerance in wild-type mice but had no impact on *Gcg*-null or GLP-1R KO mice, suggesting that Ex9 is a true and specific GLP-1R antagonist. Unexpectedly, Ex-9 had no effect on blood glucose in mice with restoration of intestinal *Gcg*. In contrast, pancreatic reactivation of *Gcg* fully restored the effect of Ex9 to impair both oral and i.p. glucose tolerance. These findings suggest an alternative model whereby islet GLP-1 also plays an important role in regulating glucose homeostasis.

INTRODUCTION

The continued pharmaceutical investment in development of glucagon-like peptide 1 (GLP-1) secretagogues as potential therapies for metabolic disease is based on the generally accepted physiological model that GLP-1 is an incretin, that is, a factor secreted from the intestine that acts in an endocrine fashion on β cell GLP-1 receptors (GLP-1Rs) to stimulate insulin secretion. Despite widespread belief in this model, there are many observations that raise questions about the metabolic importance of gut-derived GLP-1. GLP-1 is rapidly metabolized by dipeptidylpeptidase-4 (DPP4), a serine protease widely distributed in the vasculature and circulation that limits the circulatory half-life of GLP-1 to 1–2 min (Meier et al., 2004). Indeed, data demonstrate that 67% of GLP-1 secreted from the pig intestine is already degraded to inactive GLP-1 by DPP4 within the intestinal mucosal capillaries (Hansen et al., 1999). These data raise doubts about whether intestinally derived GLP-1 circulates at concentrations sufficient to activate receptors on target tissues.

We have recently demonstrated that β cell-specific GLP-1R deletion impairs glycemic control in response to intraperitoneal (i.p.) glucose, a setting in which the gut does not secrete GLP-1 (Smith et al., 2014), which begs the question of the source of ligand for β cell GLP-1R in these studies. However, these data are consistent with data from humans and in isolated islets in which the GLP-1R antagonist exendin-[9-39] (Ex9) impairs basal and glucose-stimulated insulin secretion at low or absent plasma GLP-1 concentrations (Masur et al., 2005; Salehi et al., 2010; Schirra et al., 1998). Although similar findings have been used to suggest that Ex9 is an inverse agonist (Serre et al., 1998), this finding has been reported in only a single cell line.

There is now evidence that bioactive GLP-1 is produced in the islet and that α cell production is increased in settings of β cell damage, metabolic demand, and inflammation (Donath and Burcelin, 2013; Whalley et al., 2011). However, it is not clear whether islet GLP-1 has a physiological role in regulating glucose under normal feeding or fasting conditions.

Despite these caveats, the concept that intestinal GLP-1 acts as a hormone to regulate insulin secretion persists. Part of this persistence has been the limitation of the tools available to probe the GLP-1 system, specifically with regard to assessing the relative roles of the various pools of GLP-1. Herein, we describe a new mouse model that allows manipulation of the preproglucagon gene (*Gcg*), and hence GLP-1 production, in a tissue-specific fashion. GLP-1R blockade in these animals permitted determination of relative glucose regulation by GLP-1 produced within the intestine or pancreas.

RESULTS

Gcg Loss of Function

We inserted a constitutive stop signal flanked by loxP sites in the proximal portion of *Gcg* to prevent gene transcription

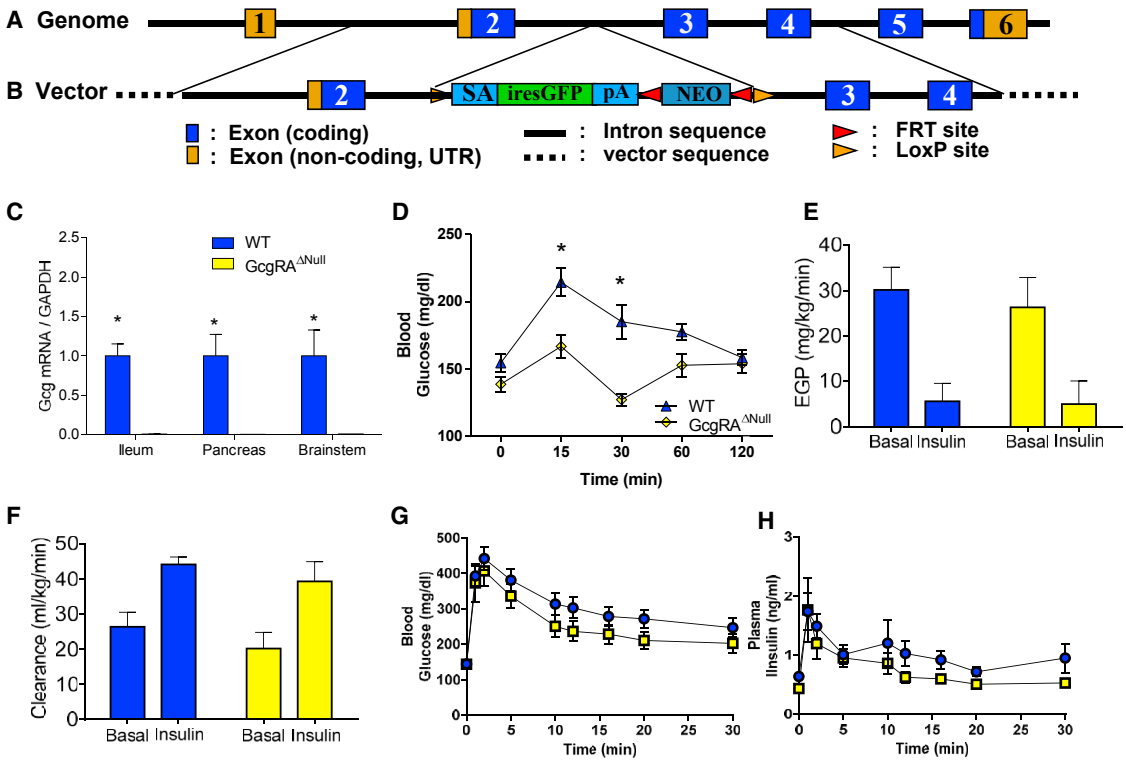

Figure 1. Targeting Construct and Glucose Phenotype of GcgRA$^{\Delta Null}$ Mice

(A) Cartoon of the *Gcg* gene.

(B) A Lox P flanked transcriptional blocking cassette consisting of a splice acceptor (SA), an internal ribosomal entry site (IRES), and a GFP with a poly(A) signal (pA) was inserted into the intron between exons 2 and 3 of the *Gcg* gene. In the absence of Cre recombinase, expression of the targeted allele is suppressed generating a null allele.

(C) *Gcg* gene expression relative to GAPDH was nearly undetectable in GcgRA$^{\Delta Null}$ versus WT mice (n = 4 male WT and n = 6 male GcgRA$^{\Delta Null}$).

(D) Glucose response to Ensure (200 μL) was significantly lower at 15 and 30 min after the gavage in GcgRA$^{\Delta Null}$ versus WT mice (n = 4 female and 6 male per genotype).

(E) Endogenous glucose production (EGP) during baseline and during the final 30 min of a hyperinsulinemic-euglycemic clamp (n = 6 male per genotype) was similar in WT and GcgRA$^{\Delta Null}$ mice (main effect of time, $p < 0.05$).

(F) Glucose clearance was similar in WT and GcgRA$^{\Delta Null}$ mice at baseline and during the final 30 min of the hyperinsulinemic-euglycemic clamp (main effect of time, $p < 0.05$).

(G and H) Glucose (G) and insulin (H) response to an i.v. glucose load (0.5 g/kg) were similar between WT and GcgRA$^{\Delta Null}$ mice (n = 8 male WT, n = 7 male GcgRA$^{\Delta Null}$).

*$p < 0.01$ versus GcgRA$^{\Delta Null}$. Significance in (C) was determined using one-way ANOVA. Significance in (D) to (H) was determined using two-way ANOVA for genotype and time. Data are represented as mean ± SEM.

(GcgRA$^{\Delta Null}$; Figures 1A and 1B). Intestinal, pancreatic, and brainstem *Gcg* expression was nearly undetectable in GcgRA$^{\Delta Null}$ versus wild-type (WT) littermates (Figure 1C). GcgRA$^{\Delta Null}$ mice had similar body mass but lower proportions of fat and higher proportions of lean mass compared with WT mice (Figures S1A and S1B). Consistent with other models in which glucagon signaling is abolished (Gelling et al., 2009; Hayashi et al., 2009), the glucose response to a mixed meal gavage (200 μL Ensure nutrient shake) was lower in chow-fed GcgRA$^{\Delta Null}$ versus WT mice even when animals were weight-matched between genotypes (Figure 1D). However, the improved glucose response was not due to differences in insulin sensitivity, as clamped glycemia, glucose infusion rates (Figures S1C and S1D), basal and insulin-regulated hepatic glucose production, and glucose clearance were all similar between chow-fed GcgRA$^{\Delta Null}$ and WT mice during a hyperinsulinemic-euglycemic clamp (Figures 1E and 1F). In addition, plasma glucose and insulin increased to a similar extent in response to an intravenous (i.v.) glucose load in GcgRA$^{\Delta Null}$ versus WT mice (Figures 1G and 1H). GcgRA$^{\Delta Null}$ mice also had increased pancreatic mass and α cell hyperplasia, similar to what has been previously reported in glucagon receptor knockout (KO) mice (Gelling et al., 2003) (Figures S1D and S1E). Together these data demonstrate that our newly generated GcgRA$^{\Delta Null}$ exhibits features previously reported in models that lack glucagon signaling with lower than normal glucose responses to a mixed nutrient load and increases in pancreatic mass, but differs in not having enhanced insulin secretion.

Gut-Specific Reactivation of the Gcg Gene

We next assessed the effect of intestinal *Gcg* reactivation, and thus gut production of GLP-1, on glucose tolerance. GcgRA$^{\Delta Null}$ mice were crossed with mice that express Cre recombinase in villus and crypt epithelial cells of the small and large intestine (Villin-1 Cre) (el Marjou et al., 2004). Figure 2A demonstrates that

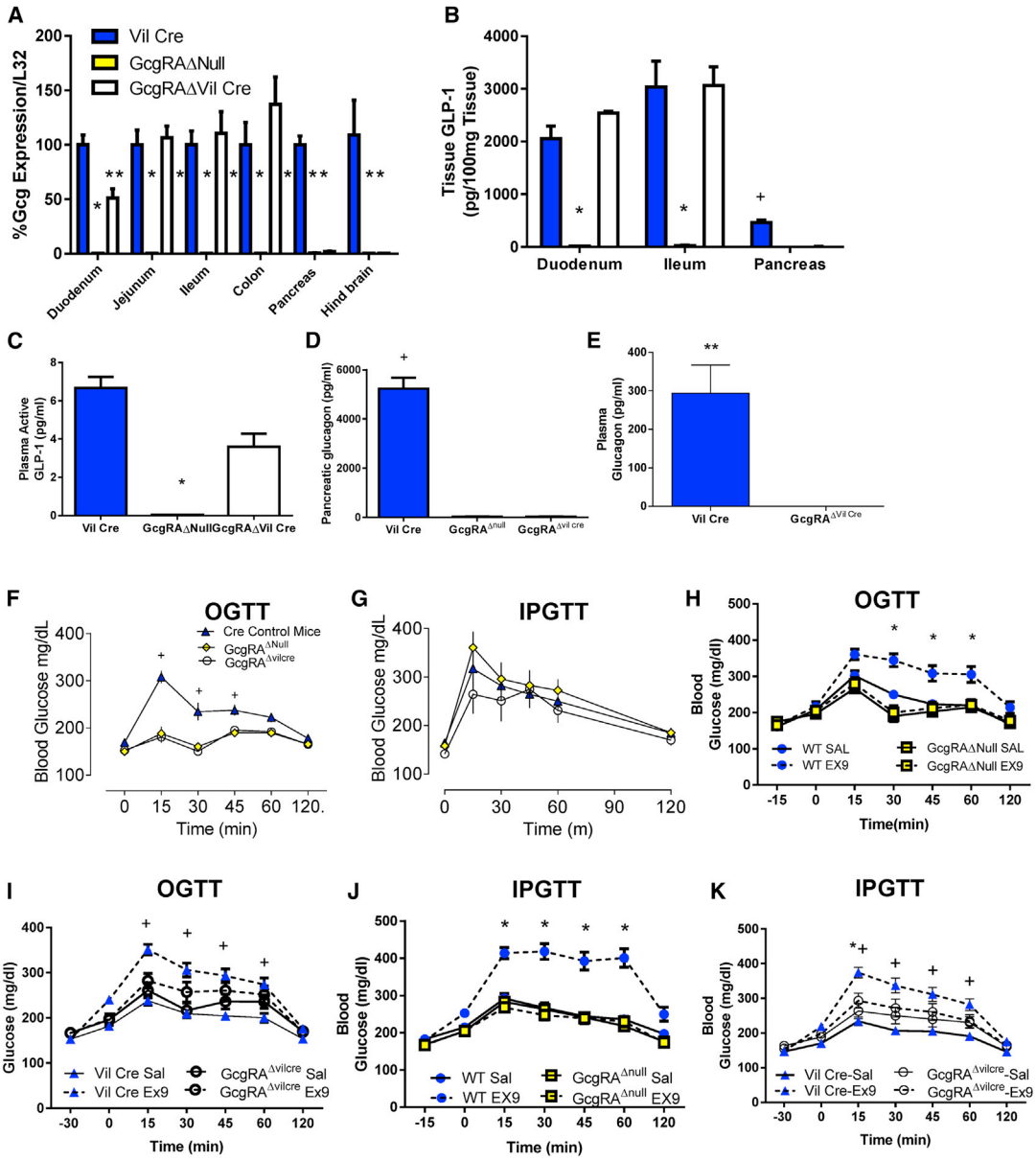

Figure 2. Validation and Glucose Phenotype of Mice with Intestinal Reactivation of Endogenous *Gcg*

(A) *Gcg* expression was significantly greater in Vil Cre versus GcgRA$^{\Delta VilCre}$ mice in the duodenum but similar in the jejunum, ileum, and colon. *Gcg* expression was undetectable in the GcgRA$^{\Delta VilCre}$ mice in the pancreas and hindbrain and in all tissues in the GcgRA$^{\Delta Null}$ mice (n = 7 female and 5 male per genotype).

(B) Tissue GLP-1 levels were not significantly different in the duodenum and ileum in Vil Cre versus GcgRA$^{\Delta VilCre}$ mice and were undetectable in the pancreas of GcgRA$^{\Delta VilCre}$ mice and in all tissues of the GcgRA$^{\Delta Null}$ mice (n = 9 male per genotype).

(C) Circulating levels of active GLP-1 15 min after a glucose (3 g/kg) gavage was similar between Vil Cre and GcgRA$^{\Delta VilCre}$ mice and undetectable in GcgRA$^{\Delta Null}$ mice (n = 7 female and 5 male per genotype).

(D) Pancreatic protein levels of glucagon were undetectable in GcgRA$^{\Delta Null}$ and GcgRA$^{\Delta VilCre}$ mice (n = 10 male Vil Cre and GcgRA$^{\Delta Null}$, n = 9 male GcgRA$^{\Delta VilCre}$).

(E) Plasma glucagon levels 15 min after 1 U/kg of insulin were undetectable in GcgRA$^{\Delta VilCre}$ mice (n = 8 GcgRA$^{\Delta VilCre}$ and n = 10 Vil Cre mice).

(F) Oral glucose tolerance (2 g/kg) was significantly lower at 15, 30, and 45 min in both GcgRA$^{\Delta Null}$ and GcgRA$^{\Delta VilCre}$ mice compared with WT mice (n = 10 male Vil Cre and GcgRA$^{\Delta Null}$, n = 9 male GcgRA$^{\Delta VilCre}$).

(G) i.p. glucose tolerance (2 g/kg) was not significantly different among GcgRA$^{\Delta Null}$, GcgRA$^{\Delta VilCre}$, and WT mice (n = 10 male Vil Cre and GcgRA$^{\Delta Null}$, n = 9 male GcgRA$^{\Delta VilCre}$). *$p < 0.01$, GcgRA$^{\Delta Null}$ versus Vil Cre and GcgRA$^{\Delta VilCre}$ mice; **$p < 0.05$, Vil Cre versus GcgRA$^{\Delta VilCre}$ mice; +$p < 0.01$, Vil Cre versus GcgRA$^{\Delta Null}$ and GcgRA$^{\Delta VilCre}$ mice.

(H–K) Ex9 (50 μg) impaired oral (H and I) and i.p. (J and K) glucose tolerance over saline (SAL or Sal) (100 μl) in WT and Vil Cre versus GcgRA$^{\Delta Null}$ and GcgRA$^{\Delta VilCre}$ mice, respectively (n = 15 male WT and GcgRA$^{\Delta Null}$ per drug treatment, n = 15 male Vil Cre and GcgRA$^{\Delta VilCre}$ per drug treatment). *$p < 0.05$, WT or Vil Cre versus all other groups; +$p < 0.05$, Vil Cre Ex9 versus saline.

Significance in (A) to (E) was determined using one-way ANOVA for genotype. Significance in (F) and (G) was determined using two-way ANOVA for genotype and time. Significance in (H) to (K) was determined using three-way ANOVA for genotype, drug, and time. Data are represented as mean ± SEM.

Gcg expression was not significantly different from controls in the jejunum, ileum, or colon in male and female offspring, hereafter termed GcgRA$^{\Delta VilCre}$ mice. In addition, these mice did not express *Gcg* in the pancreas or brainstem, confirming gut-specific reactivation. Although levels of *Gcg* expression in the duodenum were only 50% of control values, tissue (duodenum and ileum) protein and glucose-stimulated plasma levels of active GLP-1 were not significantly different between Vil Cre and GcgRA$^{\Delta VilCre}$ mice (Figures 2B and 2C). Consistent with our initial observations, *Gcg* gene expression and tissue GLP-1 levels were nearly undetectable in GcgRA$^{\Delta Null}$ mice (Figures 2A–2C), and pancreatic glucagon was undetectable in both GcgRA$^{\Delta Null}$ mice and GcgRA$^{\Delta VilCre}$ mice (Figure 2D). Because intestinal contribution to plasma glucagon has been reported in pancreatectomy patients, we also measured plasma glucagon responses to hypoglycemia in WT versus GcgRA$^{\Delta VilCre}$ mice. We found that plasma glucagon in response to insulin-induced hypoglycemia remained undetectable in GcgRA$^{\Delta VilCre}$ mice (Figure 2E).

Glucagon receptor KO mice have lower glycemic excursions in response to both oral and i.p. glucose, but this occurs in the setting of elevated islet and plasma GLP-1(Gelling et al., 2003). To determine if reactivation of intestinal *Gcg*, which normalized meal-induced increases in plasma GLP-1, would also improve i.p. glucose tolerance, we performed an oral glucose tolerance test and an i.p. glucose tolerance test (IPGTT) with identical doses of glucose over 1 week in a crossover design. In contrast to the results in glucagon receptor KO mice, the blood glucose profiles were lower during the oral glucose tolerance test but not during the IPGTT in GcgRA$^{\Delta Null}$ and GcgRA$^{\Delta VilCre}$ compared with Vil Cre mice (Figures 2F and 2G). These data suggest that lack of glucagon has a potent influence on postprandial glucose regulation, and restoration of intestinal GLP-1 production does not affect glucose homeostasis beyond this.

To directly test the physiologic importance of intestinal GLP-1 on glucose tolerance, we administered the GLP-1R antagonist Ex9 during oral and IPGTTs. Importantly, whereas Ex9 caused glucose intolerance in control mice, there was no effect of Ex9 on blood glucose in the GcgRA$^{\Delta Null}$ mice, regardless of the route of glucose administration (Figures 2H and 2J). These data demonstrate that in vivo, Ex9 lacks inverse agonist properties and instead acts as a pure GLP-1R antagonist. As a demonstration of an absolute requirement for the canonical GLP-1R, Ex9 had no impact on oral or i.p. glucose tolerance in GLP-1R KO mice (Figures S2A and S2B). Given the specificity and pure GLP-1R antagonism of Ex9, it was very surprising that Ex9 failed to impair either oral or i.p. glucose tolerance (Figures 2I and 2K), even with normalized postprandial GLP-1 levels in the GcgRA$^{\Delta VilCre}$ mice.

Pancreas-Specific Reactivation of the *Gcg* Gene

α cell production of GLP-1 is thought to play a role in pancreatic function only under conditions of high metabolic demand and/or inflammation (Donath and Burcelin, 2013). To determine whether α cell production of GLP-1 plays a physiological role in glucose regulation, and given that Ex9 had no effect on glucose tolerance in mice with restoration of *Gcg* in the intestine, we next restored pancreatic expression of *Gcg* (GcgRA$^{\Delta PDX1Cre}$) by crossing our GcgRA$^{\Delta Null}$ mice with mice that express Cre recombinase under the control of the mouse pancreatic duodenal homeobox promoter (PDX1).

Pancreatic, but not hindbrain or ileal, *Gcg* expression was restored in the GcgRA$^{\Delta PDX1Cre}$ mice (Figure 3A). Because PDX1 is also expressed in the duodenum, we also observed some restoration of *Gcg* expression in the duodenum (Figure 3A). Confirming pancreatic reactivation, plasma glucagon responses to insulin-induced hypoglycemia were restored and, in fact, were significantly greater than in control mice (Figure 3B). In addition, glucose-induced increases in circulating total GLP-1 were approximately 50% of control values in GcgRA$^{\Delta PDX1Cre}$ mice (Figure 3C).

The glycemic responses to oral and i.p. glucose were similar to controls in the GcgRA$^{\Delta PDX1Cre}$ mice, and both of these lines had significantly higher oral glucose excursions than GcgRA$^{\Delta Null}$ mice (Figures 3D and 3E). In contrast to the GcgRA$^{\Delta VilCre}$ mice, treatment with Ex9 impaired both oral and i.p. glucose tolerance in control and GcgRA$^{\Delta PDX1Cre}$ groups relative to saline treatment (Figures 3F and 3G). These data suggest that *Gcg* expression and GLP-1 synthesis within the pancreas is necessary for the normal regulation of glucose. To further confirm these results, we implanted a mini-osmotic pump into these mice and found that 30 day treatment with Ex9, at a dose that had no significant effect on body or fat mass (Figure S3), impaired glucose tolerance in control and GcgRA$^{\Delta PDX1Cre}$ mice but had no effect in GcgRA$^{\Delta Null}$ mice (Figure 3H).

Role of Extra-Pancreatic GLP-1R

Our data suggest that the pancreatic source of GLP-1 is necessary for glucose regulation. Previous work, including our own, has suggested that brain GLP-1Rs are important for gluco-regulation. Because plasma GLP-1 levels were also significantly increased, we tested the hypothesis that this source of GLP-1 could activate CNS GLP-1R and regulate glucose levels. We found that Ex9 impaired both oral and i.p. glucose tolerance equally in GLP-1R f/f controls and both whole-brain (GLP-1RKD$^{\Delta Nestin}$) and hypothalamic (GLP-1RKD$^{\Delta Nkx2.1}$) GLP-1R KO animals (Figures 4A–4D). These findings indicate that the effect of Ex9 to cause glucose intolerance is not mediated in the CNS.

DISCUSSION

The successful targeting of the GLP-1 system for treatment of type 2 diabetes mellitus (T2DM) underscores the critical role GLP-1 plays in regulating glucose homeostasis. GLP-1-focused therapeutics have been conceptualized on the basis of the conventional incretin model whereby peptides made in the gut act as hormones to control insulin secretion and glucose tolerance. In this context, GLP-1R agonists achieve what would be a 10-fold increase in GLP-1 secreted from the gut into the circulation, while DPP-4 inhibitors that protect circulating endogenous GLP-1 and create a 2-fold increase in plasma GLP-1 (Drucker and Nauck, 2006). However, our findings here challenge an independent role for endocrine action of GLP-1 on insulin secretion. Using a novel mouse model, we observed that animals that express *Gcg* only the intestine and have normal amounts of plasma GLP-1 are only minimally affected by GLP-1R antagonism, regardless of how the glucose was administered. In contrast,

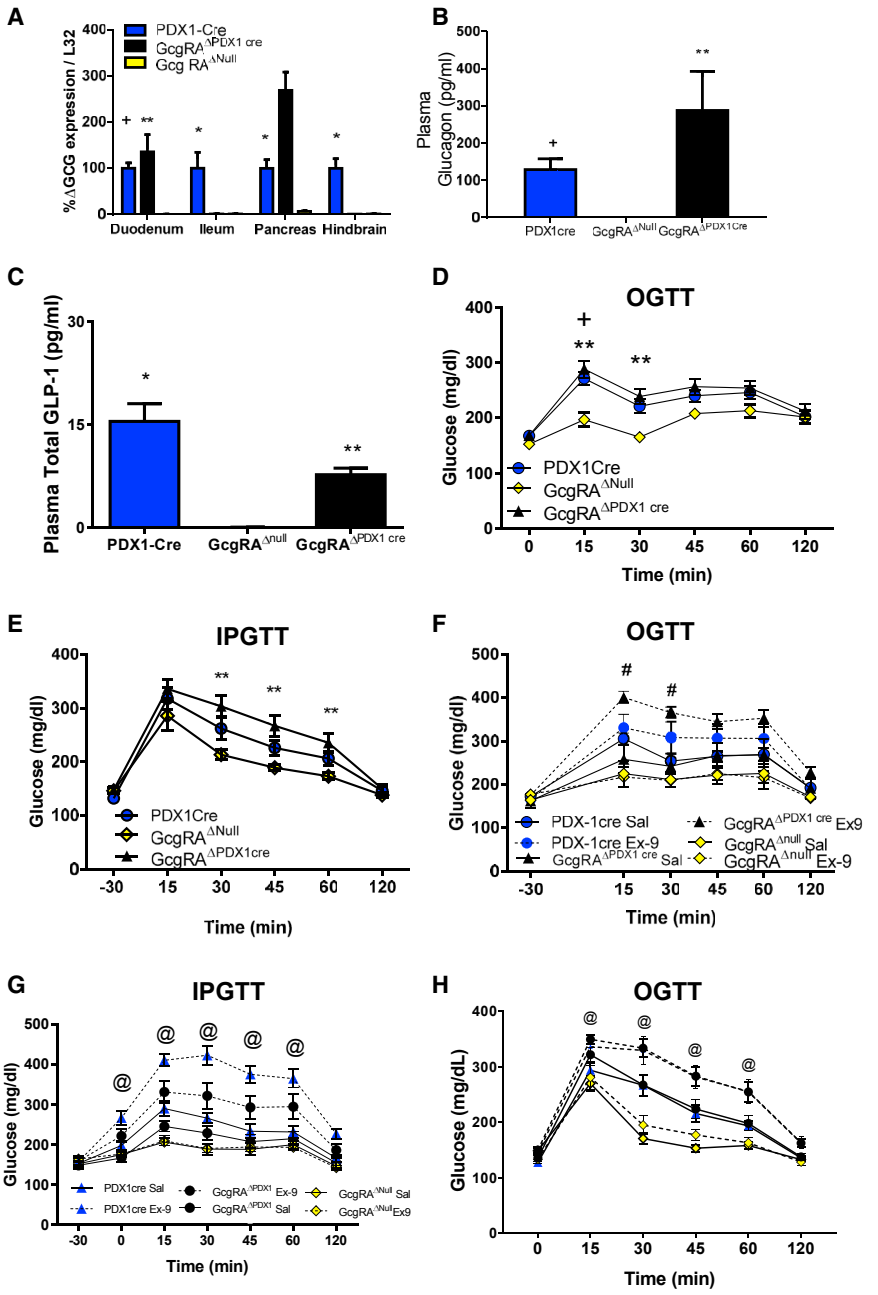

Figure 3. Validation and Glucose Phenotype of Mice with Pancreatic Reactivation of Endogenous *Gcg*

(A) *Gcg* expression was significantly greater in PDXcre control versus GcgRA$^{\Delta PDX1Cre}$ mice in the ileum and hindbrain, was not different in the duodenum, and was significantly lower in the pancreas compared with the GcgRA$^{\Delta PDX1Cre}$ mice. GcgRA$^{\Delta Null}$ mice had undetectable *Gcg* expression across all tissues (n = 10 male per genotype).

(B) Plasma glucagon levels 15 min after i.p. insulin (1 U/kg) were similar between control and GcgRA$^{\Delta PDX1Cre}$ mice and were below detection limits in all GcgRA$^{\Delta Null}$ mice (n = 6 male GcgRA$^{\Delta Null}$ and PDX1Cre, n = 8 male GcgRA$^{\Delta PDX1Cre}$).

(C) Plasma GLP-1 levels 15 min after a glucose (3 g/kg) gavage (n = 10 male GcgRA$^{\Delta Null}$ and PDX1Cre, n = 7 male GcgRA$^{\Delta PDX1Cre}$).

(D) Oral (2 g/kg) glucose tolerance in GcgRA$^{\Delta Null}$, PDX1Cre, and GcgRA$^{\Delta PDX1cre}$ mice (n = 13 male per genotype).

(E) i.p. (2 g/kg) glucose tolerance in GcgRA$^{\Delta Null}$ versus PDX1Cre and GcgRA$^{\Delta PDX1cre}$ mice (n = 10 male per genotype).

(F and G) Oral (F) and i.p. (G) glucose tolerance in response to saline (Sal) or Ex9 (50 μg; n = 7 male per genotype).

(H) i.p. glucose tolerance in response to chronic (21 day) infusion of saline or Ex9 (50 μg/day; n = 11 male per genotype). *p < 0.05, PDX1Cre versus GcgRA$^{\Delta Null}$ and GcgRA$^{\Delta PDX1Cre}$ mice; +p < 0.05, PDX1Cre versus GcgRA$^{\Delta Null}$ mice; **p < 0.05, GcgRA$^{\Delta PDX1Cre}$ versus GcgRA$^{\Delta Null}$ mice; #p < 0.05, GcgRA$^{\Delta PDX1Cre}$ mice Ex9 versus saline; @p < 0.05, Ex9 versus Sal in PDX1Cre and GcgRA$^{\Delta PDX1Cre}$ groups.

Significance in (A) to (C) was determined using one-way ANOVA for genotype. Significance in (D) and (E) was determined using two-way ANOVA for genotype and time. Significance in (F) to (H) was determined using three-way ANOVA for genotype, drug, and time. Data are represented as mean ± SEM.

mice with primarily pancreatic *Gcg* expression were subject to the full effect of Ex9 to cause glucose intolerance. These findings supporting a physiological role for islet GLP-1 in glucose regulation and are the first direct demonstration that pancreatic GLP-1 is involved in physiological glucose regulation.

The phenotype of the GcgRA$^{\Delta Null}$ animal is noteworthy and shares some key elements with other murine models of deficient glucagon signaling (Gelling et al., 2003; Hayashi et al., 2009). Similar to another line of *Gcg* null mice (Hayashi et al., 2009) or animals with a glucagon receptor deletion (Ali et al., 2011; Gelling et al., 2003), our mice, devoid of measurable *Gcg*-derived peptides, had improved oral glucose tolerance compared with chow-fed control mice, findings consistent with known glucagon physiology. However, mice with deletion of the glucagon receptor have lower glucose excursions in response to both oral and i.p. glucose challenges, which occur in the setting of a massive increase in pancreatic GLP-1. Instead, the GcgRA$^{\Delta Null}$ and GcgRA$^{\Delta VilCre}$ mice, which were devoid of both glucagon and pancreatic GLP-1, had a nearly identical glucose phenotype to a glucagon receptor/GLP-1R double-KO mouse (Ali et al., 2011), with improved oral but normal i.p. glucose tolerance comparable to WT controls. Thus, the absence of augmented pancreatic GLP-1 signaling, such as occurs in the glucagon receptor KO, is the likely explanation for the absence of supernormal i.p. glucose tolerance in both the GcgRA$^{\Delta Null}$ and GcgRA$^{\Delta VilCre}$ mice.

Our GcgRA$^{\Delta Null}$ mouse does differ from a previous Gcg KO which also had enhanced insulin secretion (Hayashi et al., 2009). Although we cannot readily account for this difference, except perhaps by variation in strain and age of the animals, physiologic

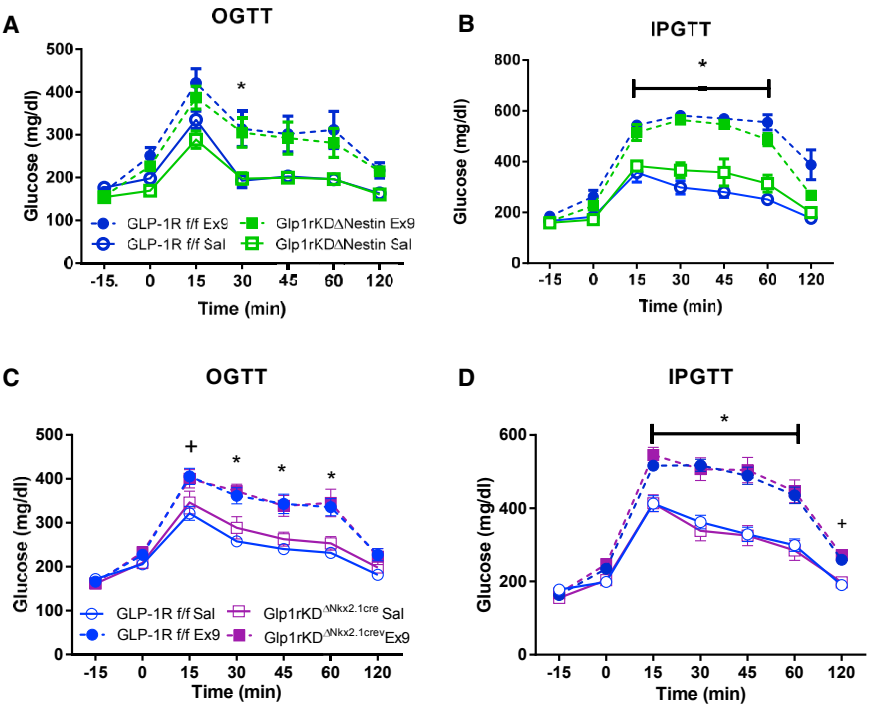

Figure 4. Role of Extra-Pancreatic GLP-1R

(A–D) Ex9 (50 μg) impaired oral (A and C) and i.p. (B and D) glucose tolerance over saline (100 μl) in GLP-1R f/f versus GLP-1RKD$^{\Delta Nestin}$ and GLP-1RKD$^{\Delta Nkx2.1}$, respectively (n = 7 male GLP-1R f/f and GLP-1RKD$^{\Delta Nestin}$ per drug treatment, n = 10 male GLP-1R f/f and GLP-1RKD$^{\Delta Nkx2.1}$ per drug treatment).

*p < 0.05, Ex9 versus Saline within both genotypes; +p < 0.05, Ex9 versus saline within GLP1R f/f only. Significance in (A) to (D) was determined using three-way ANOVA for genotype, drug, and time. Data are represented as mean ± SEM.

actions of glucagon on insulin secretion are not as well established as its cardinal effects on hepatic glucose production. The centrality of this effect is apparent with pancreatic reactivation of *Gcg* where production of islet glucagon causes glucose tolerance to revert to a pattern similar to WT mice.

One important caveat to our present data is that the PDX-1 Cre promoter we used to reactivate pancreatic *Gcg* is also expressed in the duodenum, and indeed, *Gcg* gene expression was normalized in the duodenum while nutrient-induced increases in GLP-1 were about 50% of control values. Although proximal L cells likely have more direct contact with the nutrient chyme that is thought to stimulate GLP-1 release, the great preponderance of intestinal *Gcg* is expressed in the lower gut. The contribution of the upper gut to postprandial GLP-1 levels has been reported to be significantly less than lower gastrointestinal (GI) sources (Hira et al., 2009). Likewise, we believe that the duodenal activation of *Gcg* in the GcgRA$^{\Delta PDX1Cre}$ mice does not make an important contribution to glucose regulation in these animals. The key example here is the GcgRA$^{\Delta VilCre}$ mice in which *Gcg* was reactivated across the whole intestine, including the L cell-rich jejunum and ileum, yet glucose tolerance did not differ from GcgRA$^{\Delta Null}$ mice, and Ex9 had no effect on these mice. In stark contrast, the pancreatic RA animals had fasting and post-challenge glycemia that did not differ from controls and was significantly different from the *Gcg*-null line. Moreover, Ex9 worsened glucose tolerance when mice expressed GLP-1 in the pancreas and duodenum, whereas it had no effect in mice with only intestinal GLP-1. The contrast between the two reactivation models provides convincing evidence that it is islet GLP-1 that is the key source necessary for regulating glucose homeostasis.

Our previous research demonstrated that β cell GLP-1Rs were necessary for normal glucose clearance during an i.p. challenge, a setting in which plasma GLP-1 concentrations are low and unchanging (Smith et al., 2014). These data, together with our present data that neural or hypothalamic GLP-1R are not necessary for the effect of Ex-9, suggest that the β cell GLP-1R is necessary for glucose homeostasis and is dependent on *Gcg* peptides being produced in the islet. Studies in isolated islets from humans and mice reveal that all the necessary cellular machinery to synthesize and secrete GLP-1 resides within α cells (Whalley et al., 2011). On the basis of these results, we hypothesize a paracrine model whereby GLP-1 secreted from α cells acts on GLP-1R on neighboring cells to regulate insulin secretion, a concept consistent with a number of previous studies (Kahles et al., 2014; Kilimnik et al., 2010; Whalley et al., 2011). The prospect that GLP-1 signaling operates in great part through paracrine fashion at the level of the islet makes intuitive sense. Indeed, because there is evidence that 67% of GLP-1 secreted from the pig intestine is rendered inactive by the time it reaches the portal vein, and greater than 90% by the time it passes the liver (Hansen et al., 1999), it is unclear how much stimulation to β cell GLP-1R is provided through the circulation. Moreover, it seems likely that levels of intact GLP-1 achieved in the islet interstitium are sufficient to potentiate glucose-stimulated insulin secretion (Shigeto et al., 2015). Until now the evidence for a paracrine model has been limited to in vitro systems for which the physiologic relevance is unclear. Our data provide the first in vivo evidence that GLP-1 produced in the islet may have direct paracrine effects on β cells to regulate glucose homeostasis. However, given that plasma GLP-1 was detectable in the pancreatic reactivated animals, this source of plasma GLP-1 could have extra-pancreatic effects as well.

One important limitation of these data is the extent to which they reflect species differences in GLP-1 biology. Critical evidence for the endocrine model of GLP-1 comes from using pharmacological tools in humans (Edwards et al., 1999; Salehi et al., 2012; Schirra et al., 2006). Although our present mouse studies allowed us to very specifically manipulate the GLP-1 system, they cannot account for the possibility that the paracrine action of GLP-1 is more important in mice than it is humans. GLP-1 is found in the pancreas of humans, and the islet architecture in humans (α cells are dispersed throughout rather than around the perimeter in humans versus mice) may even favor a paracrine

role for GLP-1 in the islet (Marchetti et al., 2012; Whalley et al., 2011). Nevertheless, it is the case that there are species differences for the GLP-1 system even between mice and rats (Lachey et al., 2005), and therefore more research is needed to confirm the relative role of pancreatic versus intestinal GLP-1 in regulating glucose homeostasis in humans.

In conclusion, our data demonstrate an underappreciated role for pancreatic GLP-1 in glucose regulation. Using a novel genetic mouse model, our findings demonstrate that GLP-1 produced in the pancreas, almost surely in the α cell, can also be important for insulin secretion and normal glucose tolerance. These findings in mice raise questions about current conceptions of the sole role for entero-insular signaling in regulating glucose homeostasis. Better understanding of the actions of endogenous GLP-1 will be helpful to deploy the current GLP-1-based therapies in a more effective way and in identifying new, more targeted therapeutic strategies that harness the GLP-1 system.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2017.02.008.

AUTHOR CONTRIBUTIONS

A.P.C., R.G.-A., J.E.S., A.H., B.L., K.R., C.R.H., and K.-S.K. were responsible for executing experiments. A.P.C., D.A.D., R.J.S., and D.A.S. were responsible for planning experiments. A.P.C., D.J.D., R.J.S., and D.A.S. were responsible for interpretation of data and drafting of the manuscript. D.A.S. provided final approval of the submitted manuscript.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Guinea pig anti-insulin	Abcam	Ab7842; RRID: AB_306130
Rabbit anti-glucagon	Millipore	AB932; RRID: AB_2107329
Rabbit anti-GFP	Abcam	Ab6556; RRID: AB_305564
Chemical Reagents, Peptides, and Recombinant Proteins		
Exendin-[9-39]	Sigma-Aldrich	133514-43-9
DPP4 inhibitor	EMD Millipore	DPP4-010
[3-3H]-glucose	Perkin Elmer	NET331C250UC
Critical Commercial Assays		
Total GLP-1	Mesoscale Discovery	K150JVC-1
Active GLP-1	Mesoscale Discovery	K150HYC-4
Glucagon	Mesoscale Discovery	K150HCC-1
Pure Link RNA mini kit	Thermo Fisher Scientific	12183025
iScript cDNA synthesis kit	BioRad	170-8891
Taqman University PCR Master Mix	Applied Biosystems	4304437
Taqman gene expression assays	Applied Biosystems	433182
BCA protein assay kit	Thermo Fisher Scientific	23225
Experimental Models: Organisms/Strains		
B6;SJL-Tg(ACTFLPe)9205Dym/J	The Jackson Laboratory	003800
B6.Cg-Tg(Vil1-cre)997Gum/J	The Jackson Laboratory	004586
B6.FVB-Tg(Pdx1-cre)6Tuv/J	The Jackson Laboratory	014647
B6.Cg-Tg(Nes-cre)1Kln/J	The Jackson Laboratory	003771
C57BL/6J-Tg(Nkx2-1-cre)2Sand/J	The Jackson Laboratory	0086611
Oligonucleotides		
Gcg primer	Thermo Fisher Scientific	Mm0129055_m1
RPL32 primer	Thermo Fisher Scientific	Mm 02528467_g1
Recombinant DNA		
Gcg targeting vector	UC Gene Targeting and Mouse Models Core	N/A

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Darleen Sandoval (darleens@med.umich.edu), following an approved MTA between the University of Michigan and the receiving institution.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Male and female mice on a primarily C57BL6/J background were singly housed and maintained on a 12 hr light/dark cycle with ad libitum access to chow diet (Harlan Teklad no. 7012). Studies were conducted in animals 8-20 weeks of age and included age- and sex-matched littermate control mice. All procedures were approved by either the University of Cincinnati or University of Michigan Institutional Animal Care and Use Committee. All animals included in the data analysis did not display any health impairments. The numbers of animals studied per genotype are indicated within each experiment, however the minimal number of animals was 6/genotype in the IVGTT and hyperglycemic-euglycemic clamp studies.

METHOD DETAILS

Genetic mouse generation

A conditional GcgRA$^{\Delta Null}$ mouse was generated by introducing a transcriptional blocking cassette into the intron between exons 2 and 3 of the *Gcg* gene (Figure 1A). Flanking this blocking cassette were *loxP* sites recognized by cre-recombinase. To generate these mice, the *Gcg* targeting vector (University of Cincinnati Gene Targeting and Transgenic Mouse Models Core) which consisted of a splice acceptor (SA), an internal ribosomal entry site (IRES) with green fluorescent protein (GFP), a poly-A signal (pA), the selection marker, neo, and loxP sites flanking this cassette, was inserted into the intron between exons 2 and 3 (Figure 1A). This vector was then electroporated into 129 ES and positive *Gcg* ES clones, identified by Southern blotting, were injected into blastocysts to generate chimera by the ES core at the University of Cincinnati. Germline transmission was verified in agouti mice, and the neomycin resistance cassette was removed by breeding then these mice Flp-recombinase mice (Jackson Laboratory, stock number 003800; C57BL/6). Offspring were then bred to C57BL/6 mice to remove the *Flp* gene and generate breeding pairs for studies. The GLP-1R floxed animals were generated similarly except that *loxP* sites flanking exons 6 and 7 or the *GLP1R* gene were inserted (Smith et al., 2014; Wilson-Pérez et al., 2013). The whole body GLP-1R KO mice were provided by Dr. Dan Drucker and were generated by isolating the mouse *GLP1R* gene and using homologous recombination in mouse embryonic stem cells to generate a null mutation in both alleles (Scrocchi et al., 1996).

Mice with intestinal or pancreatic reactivation of the endogenous *Gcg* gene were generated by crossing GcgRA$^{\Delta Null}$ mice with villin 1-*Cre* (Jax Laboratories, stock number 004586) and PDX1-*Cre* (Jax Laboratories, stock number 014647) mice, respectively, to produce mice with Cre-specific reactivation of the *Gcg* gene, GcgRA$^{\Delta Null}$, and Cre littermate controls. The GLP-1R f/f mice were crossed to nestin Cre (Jax Laboratories, stock number 003771) and Nkx2.1 Cre (Jax Laboratories, stock number 0086611) promoter mice to delete the GLP-1R from the CNS and hypothalamus, respectively. See the Key Resources Table for all mouse lines utilized for this manuscript.

Mouse validation

To validate proper gene targeting, mice were sacrificed by CO_2 asphyxiation and tissues (hindbrain, duodenum, jejunum, ileum, colon, and pancreas) were rapidly removed and frozen. Tissue was homogenized in Trizol reagent and tissue RNA was extracted using a Pure Link RNA mini kit (Invitrogen, Carlsbad, CA). cDNA was isolated (iScript cDNA synthesis kit, BioRad, Hercules, CA), and real-time quantitative PCR (qPCR) was performed using a TaqMan 7900 Sequence Detection System with TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assays (all from Applied Biosystems, Foster City, CA) or with the BioRad CFX 96 Touch RT PCR detection system with Sso Advanced Universal Probes supermix and iTaq Universal probes supermix (BioRad, Hercules, CA). Relative mRNA expression for the *Gcg* (Mm01269055_m1, Thermo Scientific, Waltham, MA) was calculated relative to L32 using the ΔΔCT method.

Tissue and protein levels of GLP-1 and glucagon were also measured to validate appropriate ablation or reactivation of gene function. Ileal and pancreatic tissue was homogenized in T-Per Protein Extraction Reagent (Thermo Scientific, Waltham, MA) with protease/phosphatase inhibitors and a DPP4 inhibitor. Total protein was quantified using a BCA protein assay kit (Thermo Scientific, Waltham, MA).

Plasma GLP-1 was measured 10 min after a gavage of 25% dextrose (3g/kg), CO_2 asphyxiation, and cardiac puncture. Plasma glucagon response was measured 15 min after an IP injection of insulin (1U/kg), CO_2 asphyxiation, and cardiac puncture. In both cases, blood was collected in heparinized syringes and placed into a tube with a mixture of heparin, EDTA, and aprotonin. Plasma levels of total and active GLP-1 and glucagon in protein-extracted tissue were measured using sandwich ELISA kits (Mesoscale Discovery, Rockville, MD, USA).

Glucose tolerance tests

For all glucose tolerance tests, mice were fasted for 4h after the onset of the light phase. For the IP and oral glucose tolerance tests, blood was taken via tail nick. Basal blood glucose was sampled at −15min, and glucose administered as an oral gavage or by IP injection at a dose of 2mg/kg of 25% dextrose or Ensure (200μl) was administered via oral gavage. Blood samples were taken at 15, 30, 45, 60, and 120 min after glucose administration.

On another occasion, oral or IP glucose tolerance tests (100μl of 20% dextrose) were performed 15 min after an IP injection of Ex9 (50μg/100μl) or vehicle control and blood was sampled as described above.

IVGTT

Catheters were placed in the left common carotid artery for sampling and right jugular vein for infusions under isoflurane anesthesia in GcgRA$^{\Delta Null}$ and WT littermate controls (n = 6M/genotype). The catheters were tunneled under the skin to the back of the neck and attached via stainless steel connectors to tubing made of Micro-Renathane (0.033 OD); the tubing was externalized and sealed with stainless steel plugs. Lines were filled with saline containing 200 units/ml heparin and 5 mg/ml ampicillin. Body mass was recorded daily and animals were studied once body mass was within 10% of their presurgery weight (~5d). Animals not reaching this weight were excluded from the study.

One the day of the study, mice were fasted and moved to the procedure room 4h prior to the IVGTT. Approximately 30 min after lines were lengthened for ease of access and patency was verified, all animals received an intravenous bolus of 20% dextrose

(0.5 mg/kg). Blood samples for glucose and insulin were taken at baseline, at 1, 2, 5, 10, 12, 16, 20, and 30 min after glucose administration.

Hyperinsulinemic euglycemic clamp

For this experiment, 8-week old GcgRA$^{\Delta Null}$ and WT (n = 6M/genotype) littermate controls had catheters placed as described for the IVGTT. A hyperglycemic euglycemic clamp with HPLC purified [3-3H]glucose and a continuous infusion of human insulin (2.5 mU/kg/min; Humulin R; Eli Lilly, Indianapolis, IN) was performed as described previously (Ayala et al., 2006). Specific activity for individual time points did not vary by > 15% from the average specific activity during the last 40 min of the clamp, and the slope of specific activity over time was not significantly different from zero.

Mini-Osmotic Pump Study

Body weight-matched mice within a genotype (PDX1 Cre, GcgRA$^{\Delta Null}$, and GcgRA$^{\Delta PDX1Cre}$) had a 30d subcutaneous mini-osmotic pump (Alzet Osmotic Pumps, Cupertino, CA) filled with saline or Ex9 implanted under isoflurane anesthesia. An IPGTT was performed on treatment day 21 as described above.

For all studies above, glucose tolerance tests with and without Ex9 were replicated for data that will be used in publication elsewhere. Animals were placed in groups based on genotype and within each genotype were weight matched between drug and vehicle administrations. Due to the necessity of ensuring the proper drug and glucose dosage administration, no formal blinding was performed in any of these studies. Animals were only excluded from a study if there were apparent and noted experimental mistakes made with drug dosage, injections, with the assay procedures or if the data point in question was greater than 3 standard deviations from the mean of the group.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis

The statistical procedures used are indicated in the figure legends. Briefly, normally distributed data were analyzed utilizing standard parametric statistics including ANOVA's and t tests where applicable. Both male (M) and female (F) mice were used based on availability from the breeding cohorts. For experiments requiring surgery, males were used to avoid the potential surgical complications of doing surgeries in the smaller age-matched females. Statistical analyses were performed using either GraphPad Prism v.6.02 or Statistica v.13 for Windows. Data are expressed as mean ± SEM, and statistical significance was accepted when $p < 0.05$.

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Supplemental Information

The Role of Pancreatic Preproglucagon in Glucose Homeostasis in Mice

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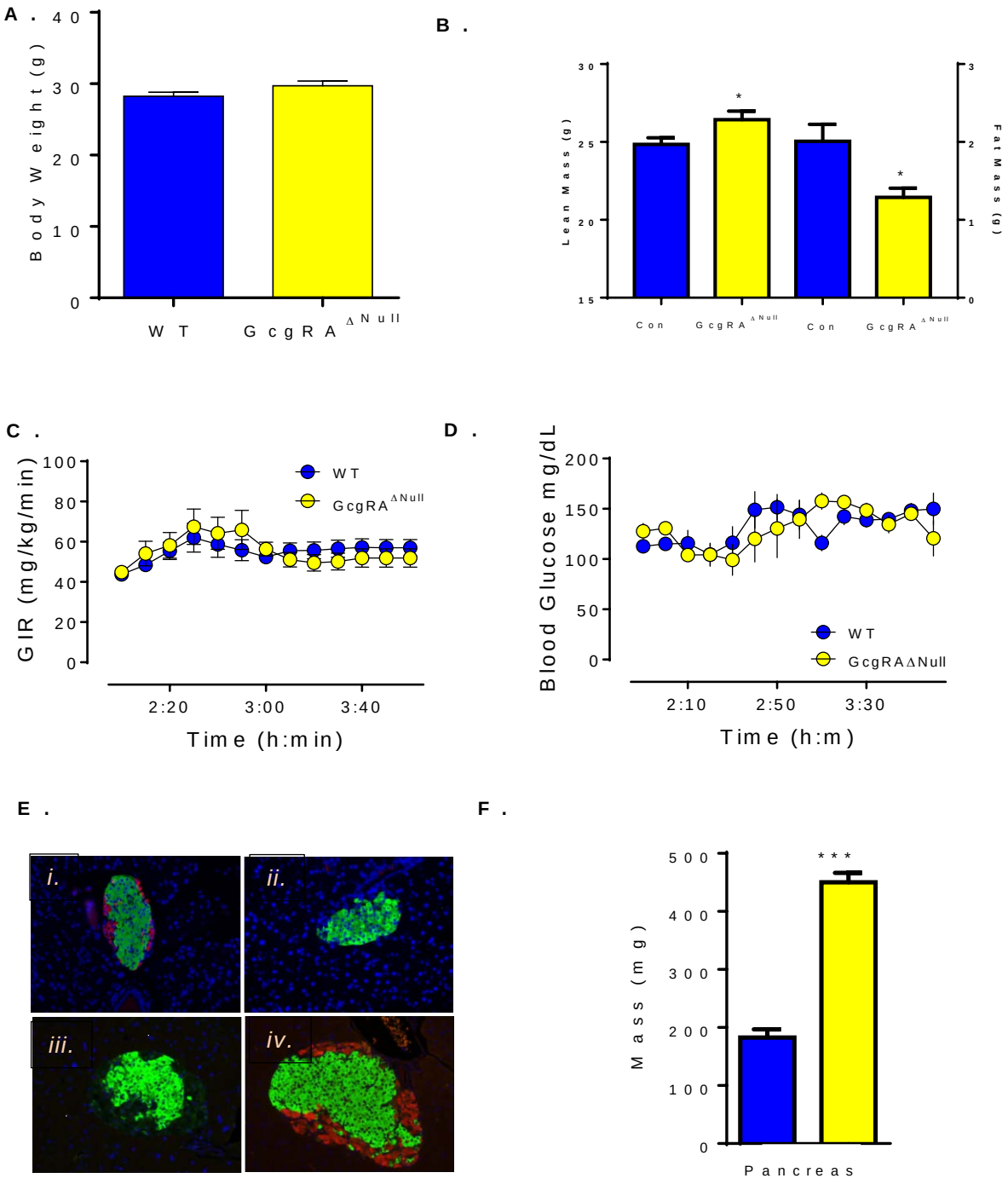
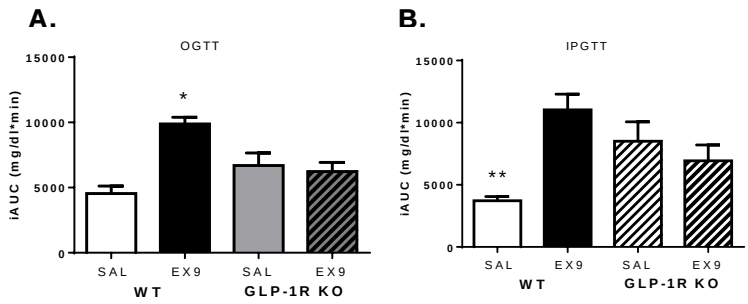
Figure S1 (Related to Figure 1)



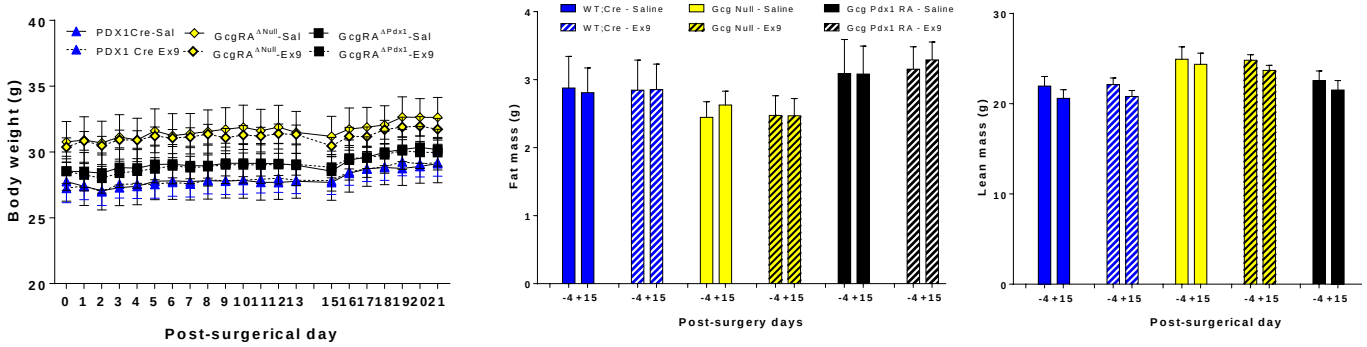
Supplementary Figure 1. A-B. Body weight was similar (A) but lean mass was significantly greater and fat mass was significantly lower in GcgRA$^{\Delta Null}$ (n=18) vs. WT (n=17) male mice at 12 wks of age. *P<0.05 vs. GcgRA$^{\Delta Null}$. **C.** Clamped glucose and **D.** exogenous glucose infusion rates were similar between GcgRA$^{\Delta null}$ (n=18) vs. WT (n=17) male mice (see Methods). **E.** Representative sections of islets from WT mice stained for *i.* insulin (green) and glucagon (red), or, *ii.* insulin (green) and GFP (red; not visible); and representative sections taken from the islets of Gcg$^{RA\Delta Null}$ mice stained for *iii.* insulin (green) and glucagon (red; not visible), or, *iv.* insulin (green) and GFP (red). **F.** Pancreas mass, was significantly lower in WT vs. GcgRA$^{\Delta Null}$ mice (***p<0.001 vs. WT).

Figure S2 (Related to Figure 3)



Supplementary Figure 2. Blood glucose (iAUC) following oral (left) or i.p. glucose challenge (2g/kg) in WT or GLP-1 receptor KO mice given saline (SAL; n=9/genotype) or the GLP-1 recpeptor antagonist Ex9 (n=9/genotype). *p<0.05 vs. all other groups; **p<0.05 vs. WT Ex9 and GLP-1R KO Sal.

Figure S3 (related to Figure 4)



Supplementary Figure 3: Body, fat, and lean mass changes before (-4d) and after mini-osmotic pump infusion of saline or Ex9. There were no significant differences found with Ex9 infusion.