Original Article

MOLECULAR METABOLISM

Deletion of the glucagon receptor gene before and after experimental diabetes reveals differential protection from hyperglycemia

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ABSTRACT

Objective: Mice with congenital loss of the glucagon receptor gene (*Gcgr*$^{-/-}$ mice) remain normoglycemic in insulinopenic conditions, suggesting that unopposed glucagon action is the driving force for hyperglycemia in Type-1 Diabetes Mellitus (T1DM). However, chronic loss of GCGR results in a neomorphic phenotype that includes hormonal signals with hypoglycemic activity. We combined temporally-controlled GCGR deletion with pharmacological treatments to dissect the direct contribution of GCGR signaling to glucose control in a common mouse model of T1DM.
Methods: We induced experimental T1DM by injecting the beta-cell cytotoxin streptozotocin (STZ) in mice with congenital or temporally-controlled *Gcgr* loss-of-function using tamoxifen (TMX).
Results: Disruption of *Gcgr* expression, using either an inducible approach in adult mice or animals with congenital knockout, abolished the response to a long-acting Gcgr agonist. Mice with either developmental *Gcgr* disruption or inducible deletion several weeks before STZ treatment maintained normoglycemia. However, mice with inducible knockout of the *Gcgr* one week after the onset of STZ diabetes had only partial correction of hyperglycemia, an effect that was reversed by GLP-1 receptor blockade. Mice with *Gcgr* deletion for either 2 or 6 weeks had similar patterns of gene expression, although the changes were generally larger with longer GCGR knockout.
Conclusions: These findings demonstrate that the effects of glucagon to mitigate diabetic hyperglycemia are not through acute signaling but require compensations that take weeks to develop.

Keywords Glucagon; GCGR; GLP-1R; Insulin; Tamoxifen; Diabetes

1. INTRODUCTION

Glucagon receptor (GCGR) signaling helps maintain glucose homeostasis by stimulating hepatic glucose production during periods when both the influx of exogenous glucose and circulating insulin levels are low. Consistent with this, a reduction of endogenous glucagon action lowers fasting blood glucose and reduces glucose excursion during a glucose tolerance test [1,2]. One of the more dramatic demonstrations of the impact of GCGR signaling is in rodent models of type-1 diabetes in which hyperglycemia is almost completely mitigated by deletion of *Gcgr* [3–7]. In fact, while loss of *Gcgr* does not prevent hyperglycemia and death under conditions of complete loss of insulin [8,9], it is sufficient to maintain normoglycemia and promote survival under insulinopenic conditions to a degree that cannot be explained solely by the action the residual insulin [4,7,10].

It is notable that pharmacological or genetic inhibition of GCGR signaling results in the engagement of a number of compensatory mechanisms that potentially impact glucose control. These include alpha-cell hyperplasia [2,11–13] and increased beta-cell proliferation under low insulin conditions [10]. Several mechanisms have been proposed to contribute to the alpha-cell proliferation, including increased ANGPL4 [14], although this has been questioned [15]. More convincingly, the hyperaminoacidemia that follows GCGR interruption leads to activation of the mTOR pathway in the alpha cell [16–18]. Reduced GCGR signaling also leads to altered levels of multiple humoral factors important in the control of glucose. Hence, *Gcgr*$^{-/-}$ mice have increased ghrelin levels even during insulinopenic conditions due to STZ treatment [19], and this may contribute to the prevention of hypoglycemia. On the other hand, loss of *Gcgr* results in supraphysiological increases in Fibroblast Growth factor 21 (FGF21) [6] and GLP-1 [2], hormones that each have glucose-lowering properties. The development of compensatory mechanisms for the loss of GCGR signaling suggests a more complex role of glucagon action in diabetic hyperglycemia than

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simply increased hepatic glucose production. To evaluate the relative impact of the GCGR on blood glucose we crossed *Gcgr* "flox" mice with a line expressing TMX-inducible cre-recombinase under the control of the universally expressed gene *Rosa26*. This allowed for time-controlled disruption of *Gcgr* gene expression following tamoxifen injections and the ability to compare acute and chronic loss of the GCGR during insulinopenic diabetes. Our results demonstrate that engagement of compensatory signals, specifically GLP-1 receptor signaling, rather than loss of GCGR activation per se, attenuates the development of hyperglycemia during insulinopenic conditions.

2. MATERIAL AND METHODS

2.1. Animal studies

These studies were approved by the Institutional Animal Care and Use Committee at the University of Cincinnati in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were housed in a 12-h light, 12-h dark cycle room held at 22 °C with free access to food and water and housed as 3–4 per cage, or single-housed after STZ administration.

Congenital *Gcgr* KO mice: $Gcgr^{flox/flox}$ mice were generated as previously described [20] and crossed with mice expressing Cre-recombinase driven by the cytomegalovirus minimal (CMV) promoter to induce the deletion of LoxP–flanked sequences in all tissues, including germ cells [21]. The CMV-cre transgene was then eliminated by subsequent breeding, and heterozygous mice were mated to generate littermate mice with congenital global disruption ($Gcgr^{-/-}$), or wild-type ($Gcgr^{+/+}$), expression of the *Gcgr* gene.

Inducible *Gcgr* KO mice: $\boldsymbol{Gcgr^{flox/flox}}$ mice were crossed with $\boldsymbol{Rosa26\text{-}^{Cre\text{-}ERT2}}$ (tamoxifen-inducible) mice ($\mathbf{Gt(ROSA)26Sor^{tm1(cre/ERT2)Tyj}}$, The Jackson Laboratory, Stock number #008463) as previously described [20]. $\boldsymbol{Rosa26\text{-}^{Cre\text{-}ERT2}}$:$\boldsymbol{Gcgr^{flox/flox}}$ mice received intraperitoneal (ip) injections of tamoxifen (TMX, Sigma; T5648) to disrupt *Gcgr* expression (TMX-treated mice, defined onwards as "conditional KO, cKO"). TMX was initially dissolved in 100% ethanol, diluted in olive oil and injected at 50 mg/kg for 5 consecutive days [20,22]. Control mice received oil injections (oil-treated mice).

2.2. Glucose tolerance test

6-h fasted mice received ip glucose (2 g/kg, 20% wt/vol D-glucose [Sigma] in phosphate buffered saline (pbs)). Blood samples were collected immediately before and 15, 30, 45, 60, 90, and 120 min after injection. Blood glucose was determined with a handheld glucometer.

2.3. Peptides

The *Gcgr* agonist IUB288, the insulin receptor antagonist S961 and the GLP1R antagonist Jant4 were chemically synthesized as previously described [23,24], diluted in pbs and injected as indicated.

2.4. STZ treatment

Mice were fasted overnight and received an injection of STZ (150 mg/kg, ip, Sigma–Aldrich, MO) freshly dissolved in sodium citrate buffer (pH = 4.5), within the first two h following the onset of the light phase. After the injection, food was returned to the mice, and they had access to a **10%** sucrose solution for 72 h. When indicated, mice received a second injection of STZ seven d later as previously described [5] at a dose of 100 mg/kg, ip.

2.5. Rapamycin treatment

Rapamycin (LC Labs, Cat #R-5000) was initially dissolved in DMSO, further diluted in pbs containing 1% Tween 80 and injected at 10 mg/kg, ip every other day.

2.6. Gene expression analysis

RNA was extracted from frozen liver samples using RNAqueous-mini kit (Qiagen). cDNA was synthetized with the SuperScript® III First-Strand Synthesis kit (Invitrogen, Life Technologies) after DNAse I treatment (Invitrogen, Life Technologies), and qPCR was performed using commercially available gene-specific Taqman® FAM-labeled probes following the manufacturer's instructions (Invitrogen, Life Technologies). Actb was used as housekeeping gene and the relative quantification was performed using the delta–delta Ct method.

2.7. Inmunoblot

Frozen liver tissue was homogenized in lysis buffer containing Halt protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL USA), 0.5 mM PMSF and 0.1 mM benzamidine (Sigma–Aldrich, St. Louis, MO, USA) using a Tissuelyser (Quiagen). The samples were rocked at 4 °C for 30 min, passed through a 28-g syringe needle and centrifuged for 15 min at 4 °C and 23,000 × g, and the supernatants were collected in a fresh tube. Protein concentrations were measured using a Pierce BCA method protein assay kit (Thermo Fisher Scientific Inc). 50 μg were separated by electrophoresis in denaturing conditions using 4–15% polyacrylamide gels (Biorad), and the proteins were transferred overnight to polyvinylidene difluoride (PVDF) membranes previously incubated in methanol for 3 min. The membranes were cut, blocked in 5% nonfat dried milk (20 mm Tris, pH 7.6; 0.9% NaCl; 0.1% Tween 20) and independently incubated overnight at 4 °C with rabbit antibodies against ser473-phospho-AKT (1:1000, Cell Signaling, # 9271) or beta actin (1:2000, Cell Signaling, #4967). Membranes were then washed and incubated with secondary antibodies (antirabbit-horseradish peroxidase coupled, 1:10,000; Cell Signaling), washed and developed by enhanced chemiluminescence (ECL2 Plus, Thermo Fisher Scientific Inc) and x-ray films (Denville Scientific). The membrane containing pAKT was then washed three times, blocked and *incubated* overnight at 4 °C with an antibody against total AKT AKT (1:2000, #9272 Cell Signaling Technology, MA) and revealed as described above. Films were scanned and densitometry was assessed using ImageJ 1.48v (http://imagej.nih.gov/ij).

2.8. Inmunoassays

C-peptide (Crystal Chem, #90050) and FGF21 (EZRMFGF21–26 K; Millipore, Billerica, MA) were analyzed individually by ELISA assay and total GLP-1 was determined using the Meso Scale Assay System (Cat# K150JVC, Mesoscale Discovery, Rockville, MD) in EDTA-collected plasma.

2.9. Statistical analysis

All data are presented as mean ± SEM. Differences between two groups were analyzed using unpaired two-tailed t-tests. Differences due to genotype x treatment were analyzed using a 2-way ANOVA followed by the Sidak test for post hoc comparisons. Analyses of genotype or treatment x time used 2-way repeated measurements (RM) ANOVA followed by the Sidak post hoc test when all subjects had completed the experiment. Otherwise, differences were analyzed using multiple t-test comparisons with the Holm-Sidak correction (alpha 0.05). The comparison of survival curves was performed using the Mantel–Cox test. The statistical analyses were performed using

GraphPad Prism, version 7 (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered significant.

3. RESULTS

3.1. Impact of congenital or temporally controlled Gcgr deletion on glucose tolerance and the response to a glucagon receptor agonist

We first tested the suitability of our *Gcgr*$^{f/f}$ mouse model to recapitulate the glycemic phenotype reported using other congenital "KO" strains. When tested at 10–14 wk of age, mice with a congenital loss of GCGR signaling exhibited lower baseline blood glucose levels and significantly improved glucose tolerance (Figure 1A) compared to wildtype littermates and lacked a hyperglycemic response following a challenge with the *Gcgr* agonist agonist IUB288 (Figure 1B). These findings conform to previous *Gcgr* KO lines and demonstrate a functional absence of glucagon signaling, mostly attributable to loss of hepatocyte GCGR. Temporally induced, conditional *Gcgr* knockout, using tamoxifen in *Rosa26-*$^{Cre-ERT2}$*:Gcgr*$^{flox/flox}$ mice (cKO) demonstrated similar results to the congenital *Gcgr* knockouts. (Figure 1C,D). These results indicate that 7–10 days after an induced deletion of Gcgr there is a near complete absence of glucagon action. Thus, the reduced basal glucose and improved glucose tolerance exhibited by congenital *Gcgr* KO mice can be readily recapitulated in adult cKO mice within at least seven d of TMX-dependent *Gcgr* gene disruption.

In a separate experiment, we characterized the impact of acute GCGR deletion on baseline insulin signaling, hepatic gene expression and on circulating levels of FGF21 and GLP-1. TMX-treated cKO mice exhibited a significant reduction in baseline glucose as early as Day 5 (Figure 2A). This reduction persisted until Day 14, when the mice were euthanized for plasma and liver tissue collection. The lower glucose in the TMX-treated cKO mice was associated with a significant reduction in plasma c-peptide and with significantly lower hepatic phosphorylated (serine 473) AKT, compared to oil-treated controls (Figure 2B, Suppl. Fig 1). These data suggest that lower blood glucose after acute disruption of *Gcgr* was not caused by increased circulating insulin or increased insulin receptor signaling in the liver. We measured the hepatic expression of *Gcgr* and genes involved in the control of amino acid metabolism (Oat, Mnmt, Got1 and Cth), insulin action (Irs1, Irs2, Igfbp1), glucose metabolism (Pck1, g6pc, Slc2a2, and Gck), lipid metabolism (Srebf1, Pkl, Fasn) and FGF21 expression (Ppara, Ppgc1a and Fgf21) using standard qPCR. *Gcgr* expression was significantly disrupted in TMX-treated cKO mice (Figure 2C). Consistent with

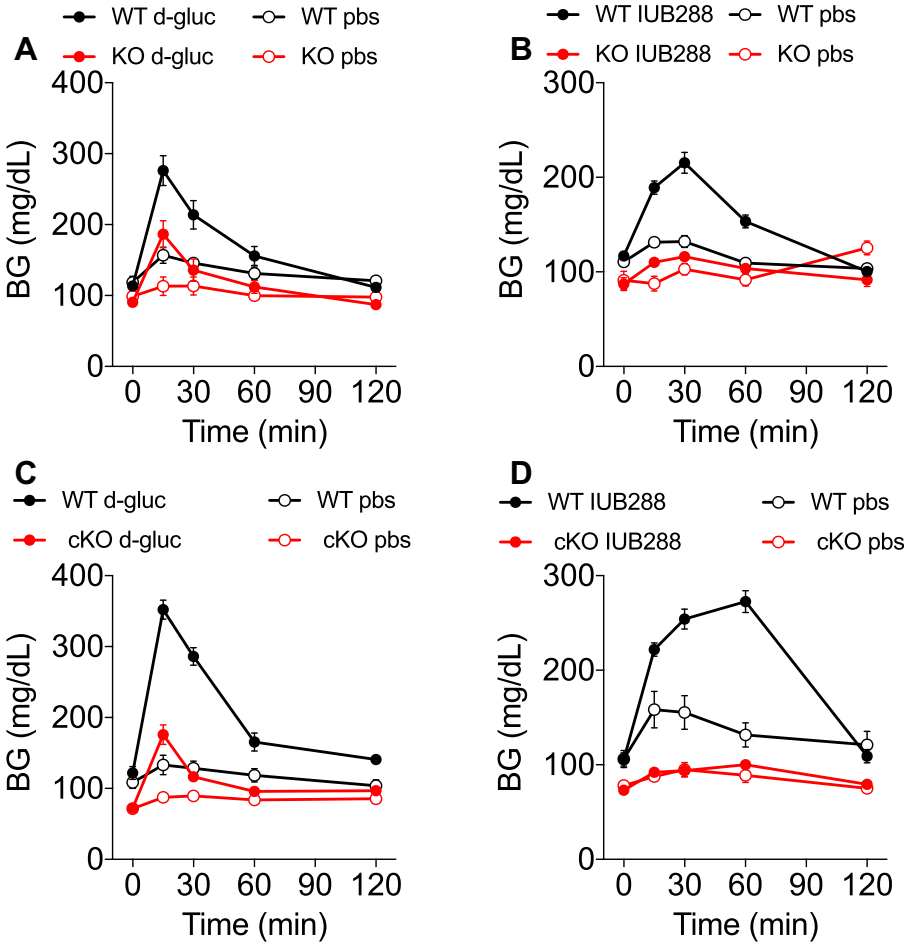

Figure 1: Glucose control in male mice with congenital or tamoxifen-induced disruption of Gcgr expression. (A,C) Glucose tolerance (D-glucose, 2 g/kg, ip.; solid symbols) and **(B,D)** challenge with the *Gcgr* agonist IUB288 (30 nmol/kg,ip.; solid symbols) in either 10–14 wk-old congenital *Gcgr*$^{-/-}$ (KO, red symbols) or *wildtype* (WT) littermate male mice (black symbols) (A,B); or in 10–14 wk-old *Rosa26-*$^{Cre-ERT2}$*:Gcgr*$^{flox/flox}$ mice treated with 5 consecutive daily ip injections of tamoxifen to induce GCGR deletion (cKO, red symbols) or oil as vehicle (WT, black symbols), 7 (C) or 10 d (D) earlier. Open symbols depict blood glucose levels of simultaneously tested groups receiving phosphate buffered saline (pbs). (A) $P < 0.05$ (genotype) *WT d-gluc* vs. *KO d-gluc*; (B) $P < 0.05$ (treatment) *WT IUB288* vs. *WT pbs*; (C) $P < 0.05$ (genotype) *WT d-gluc* vs. *cKO d-gluc*; (D) $P < 0.05$ (treatment) *WT IUB288* vs. *WT pbs*. Data are presented as mean ± SEM. (A,B) n = 9–6; (C–D) n = 6. 2-way RM ANOVA followed by Sidak test.

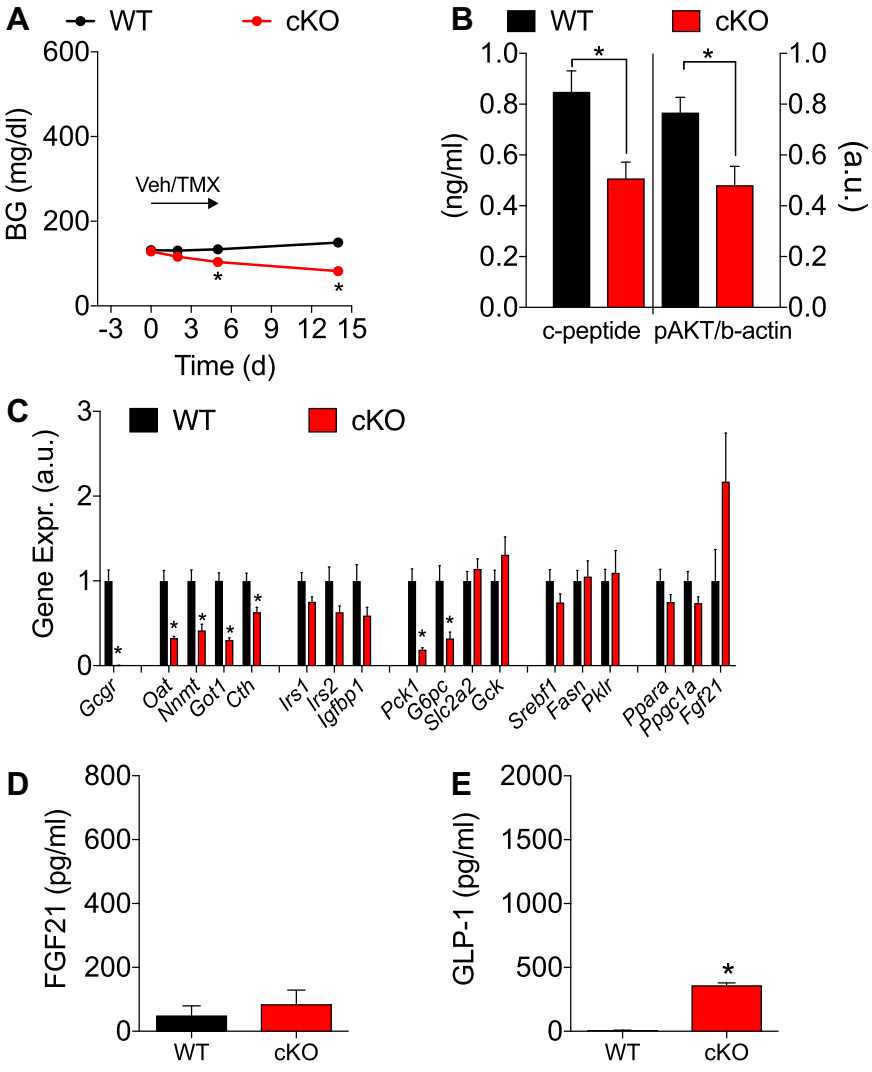

Figure 2: Effect of tamoxifen-induced disruption of Gcgr expression in adult male mice. Blood glucose (A), plasma c-peptide and hepatic pAKT (B), hepatic gene expression (C), plasma FGF21 (D) and plasma GLP-1 (E) of 10–14 wk old mice receiving tamoxifen to disrupt *Gcgr* expression (cKO) in normoglycemic male mice. Data are presented as mean ± SEM. n = 15 (A,D,E) or 6 (B,C). *P < 0.05 cKO vs corresponding oil-treated WT control. 2-way RM ANOVA followed by Sidak test (A) or T-test (B,C,E).

previous findings [18], loss of GCGR signaling led to significantly reduced Oat, Mnmt, Got1 and Cth expression. Pck1 and G6pc expression were also significantly reduced. Fourteen days after *Gcgr* deletion there was a trend towards up-regulation of Fgf21 expression that did not reach statistical significance. Consistent with this, plasma FGF21 levels were similar between groups (Figure 2D). In contrast, TMX-treated cKO mice had significantly increased plasma GLP-1 (Figure 2E), in keeping with findings previously reported on $Gcgr^{-/-}$ mice [2]. Altogether, these data demonstrate the development of compensatory changes within 2 weeks of loss of GCGR signaling.

3.2. Impact of temporally controlled Gcgr deletion on glycemia in STZ-treated mice

An interesting characteristic of congenital $Gcgr^{-/-}$ mice is the maintenance of normoglycemia following beta-cell destruction with STZ [5]. To confirm this finding in our line of congenital $Gcgr^{-/-}$ mice they were treated with standard diabetogenic doses of STZ. This treatment that caused frank diabetes in control mice, while the group with congenital *Gcgr* KO had minimal hyperglycemia or loss of body weight, and increased survival (Figure 3).

Since acute disruption of *Gcgr* expression recapitulated the glycemic phenotype of congenital loss of *Gcgr* in normal mice, we next tested whether inducible removal of GCGR would also provide protection from hyperglycemia and death in adult mice with STZ diabetes. To this end, we compared the glycemic effect of STZ in two groups of 10–14 wk-old adult male mice. The first group (Figure 4A–E) received STZ, and 7 days later TMX (or vehicle) to induce *Gcgr* deletion. The second group (Figure 4F–J) received TMX, or vehicle, and 6 wk later STZ treatment [5]. The group of mice with *Gcgr* deletion after induction of insulinopenic diabetes had lower blood glucose compared to oil controls, a difference that became statistically significant after Day 4 post-TMX (Figure 4A). Interestingly, STZ-TMX-treated cKO mice did not recover normoglycemia, and the average reduction in glucose levels was only of 81 ± 9 mg/dl compared to STZ-oil-treated controls. This contrasts with the maintenance of normoglycemia exhibited by congenital $Gcgr^{-/-}$ mice, which had 271 ± 5 mg/dl lower glucose compared to $Gcgr^{+/+}$ controls (Figure 3A). Interestingly, STZ-TMX-treated cKO mice exhibit lower food intake and decreasing body weight (Suppl. Fig 2a,b) that could have contributed to the modest decrease in blood glucose. Plasma c-peptide and hepatic pAKT levels in mice with *Gcgr* knockout

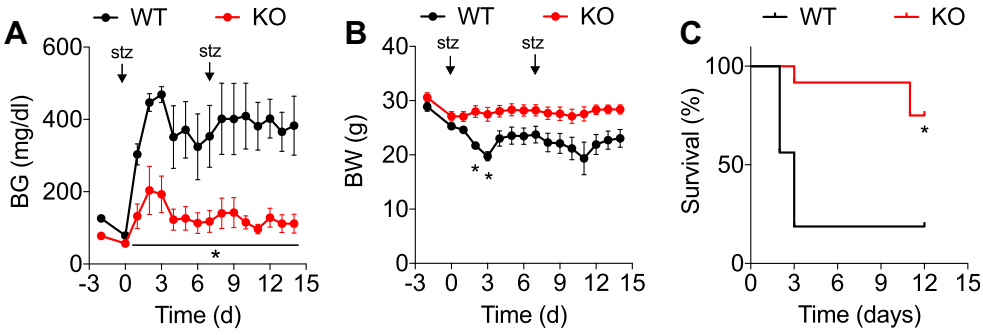

Figure 3: Effect of STZ on congenital *Gcgr*$^{-/-}$ mice. Blood glucose (A), body weight (B) and survival (C) of congenital *Gcgr*$^{-/-}$ (KO) male mice and their *wildtype* (WT) littermates following STZ administration (150 and 100 mg/kg, ip., at Days 0 and 7, respectively). Data are presented as mean ± SEM (A,B) or as percentage survival (F). n = 16 (*Gcgr*$^{+/+}$) −11(*Gcgr*$^{-/-}$). (A) P < 0.05 *KO* vs *WT* at all time points; (B) *P < 0.05 *KO* vs *WT*, multiple t-test comparison with Holms-Sidak correction. (C) *P < 0.05 *KO* vs *WT*; Log-rank Mantel—Cox test.

after diabetes (Figure 4B, Suppl. Fig 1) were low and did not differ from controls. Gene expression analysis confirmed the disruption of *Gcgr* expression in the tamoxifen-induced knockouts, and Oat, Mnmt, Got1, and Cth gene expression was significantly reduced in the absence of GCGR (Figure 4C). Expression of Pck1 and g6pc was comparable in diabetic *Gcgr* knockout mice and their controls (Figure 4C). However, loss of GCGR caused reduction of Irs1 and Ppara expression, and increased expression of the lipogenic genes Pkl and Fasn. The increase in expression of lipogenic genes was associated with a significant increase in plasma triglycerides and free fatty acids in the STZ-TMX-treated cKO mice compared to controls (Suppl. Fig 2c,d) and a trend towards elevated plasma ketone bodies (Suppl. Fig 2e).

In the group of mice that had inducible deletion of *Gcgr* 6 wk before the injection of STZ, the course of diabetes was much different. As expected, oil-treated control mice exhibited a rapid onset of hyperglycemia after the first dose of STZ that was modestly enhanced by a second injection 7 d later (Figure 4D). In contrast, glucose levels in TMX-STZ-treated cKO mice remained significantly lower after both STZ injections, with levels not differing very much from normoglycemia (Figure 4D). The average glucose during the last 7 d post-STZ was lower by 276 ± 7 mg/dl compared to levels in oil-STZ-treated controls, and within the range of that seen in congenital STZ-KO mice (Figure 3A). Despite having lower glucose, c-peptide and hepatic pAKT levels in TMX-STZ-treated cKO mice were similar to those of controls (Figure 4E, Suppl. Fig 1). In agreement with the findings in the previous two experiments, *Gcgr* expression remained undetectable, and the expression of the genes involved in amino acid metabolism (*Oat*, *Mnmt*, *Got1* and *Cth*) was significantly reduced in TMX-STZ-treated cKO mice (Figure 4F). Consistent with the loss of GCGR signaling [25,26], these mice exhibited significantly reduced *Pck1*, *G6pc*, *Ppgc1a*, and I*gfbp1* expression (Figure 4F). In contrast, they exhibited significantly elevated expression of *Gck* (>10-fold), the lipogenic genes *Srebf1*, *Pkl* and *Fasn*, as well as *Fgf21* (>50-fold), compared to WT, oil-STZ-treated, mice.

We measured plasma FGF21 and total GLP-1 at the end of both experiments to compare the impact of acute versus chronic GCGR deletion in STZ-treated mice. Unlike the marked (>10-fold) increase in hepatic Fgf21 gene expression (Figure 4C), acute GCGR disruption after STZ administration did not translate to greater circulating FGF21 levels (Figure 4G). On the other hand, those mice had substantially (>100-fold) elevated GLP-1 plasma levels compared to controls (Figure 4H). This raises the possibility that those mice are exposed also to high levels of active GLP-1, a potential contributor to the improved glucose homeostasis characteristic to congenital GCGR KO mice [6,27]. In contrast, plasma FGF21 levels were significantly increased in STZ-treated mice with previous chronic GCGR deletion (Figure 4G). Likewise, plasma total GLP-1 levels were massively elevated in those mice, with nearly 3-fold greater levels than the group with short-term *Gcgr* deletion (Figures 2E and 4H).

Collectively, these data suggest that in contrast to congenital loss of *Gcgr*, acute disruption of *Gcgr* in adult mice provides only a fraction of the protection against hyperglycemia in insulinopenic conditions. Full protection can be recapitulated with more prolonged exposure to *Gcgr* deletion (at last 6 wk), likely due to compensatory mechanisms including increased circulating FGF21 and GLP-1.

3.3. Contribution of mTOR signaling to the improved glycemic control of Gcgr-deficient mice

A feature of chronic loss of GCGR signaling is mTOR-dependent alpha-cell hyperplasia, driven by hyperaminoacidemia. Exposure to increased amino acid levels raises the possibility that increased signaling through mTOR in other tissues indirectly influences whole body glucose control in *Gcgr* deficient mice. Furthermore, glucagon signaling in the liver opposes the stimulatory effect of insulin on mTORC1 [28,29], suggesting that chronic loss GCGR signaling my result in sustained increased mTOR signaling even during conditions of low insulin action. This effect would be consistent with recent data indicating that interruption of GCGR signaling abolished the hyperglycemia induced by insulin receptor blockade [15]. To determine whether this response is mediated by increased mTOR signaling after chronic loss *Gcgr* (Figure 4F), we administered rapamycin (rapa, 10 mg/kg, ip., alt.d.) to adult *Rosa26*^{ERTcre}:*Gcgr*$^{f/f}$ mice, beginning 3 d after the first TMX injection. Similar dosing has proven efficacious at reducing mTOR signaling and preventing alpha-cell hyperplasia [17,18,30]. Neither loss of *Gcgr* following TMX nor chronic rapa treatment affected food intake (Figure 5A). Rapa also did not affect body weight in oil-treated mice, but it led to some weight loss in TMX-treated cKO mice that became statistically significant after the 4th wk of treatment when compared to TMX-treated cKO mice receiving vehicle (Figure 5B). At Wk 5, baseline glucose was significantly lower in TMX-treated cKO mice compared to oil-treated controls, but it was not significantly affected by rapa (Figure 5C). We then acutely blocked insulin action by administering three consecutive hourly injections of the insulin receptor antagonist S961 (300 nmol/kg, ip each). S961 injections led to significant hyperglycemia in mice with intact GCGR, and this effect was significantly amplified in those that also received rapamycin

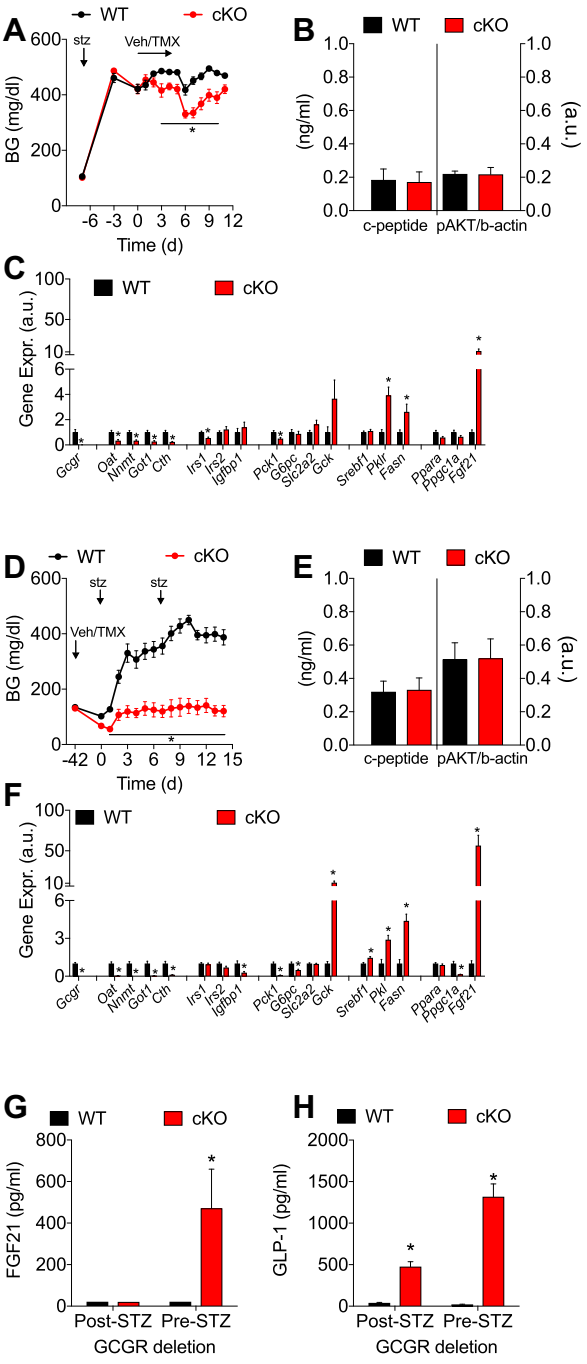

Figure 4: Effect of tamoxifen-induced disruption of Gcgr expression in adult STZ-treated male mice. Blood glucose (A,F), plasma c-peptide and hepatic pAKT (B,E) and hepatic gene expression (C,F) of 10–14 wk old *Rosa26-*$^{Cre\text{-}ERT2}$*:Gcgr*$^{flox/flox}$ male mice receiving tamoxifen to disrupt *Gcgr* expression (cKO) compared to littermates oil-treated WT controls. **(A–C)**: effect of acute disruption of *Gcgr* in adult mice previously treated with STZ (150 mg/kg, ip). **(D–F)**: effect of chronic tamoxifen induced disruption of *Gcgr* for 6 wk prior administration of STZ (150 and 100 mg/kg, ip., at Days 0 and 7, respectively). (G) Comparison of FGF21 and (H) total GLP-1 levels in plasma collected at the end of both experiments. Data are presented as mean ± SEM. n = 10–11 (A,D,E), n = 14 (F,I,J) or n = 6 (B,C,G,H). *P < 0.05 TMX-treated cKO vs corresponding oil-treated WT control. Multiple t-test with Holms-Sidak correction (A); T-test (C,F,G,H); 2-way RM ANOVA followed by Sidak test (F).

(Figure 5C), suggesting that mTOR signaling compensates for acute loss of insulin action. Blocking insulin action with S961 also increased glucose in GCGR knockout mice, but the effect was greatly attenuated compared with controls; both the maximum increase over baseline (82 ± 5 vs 110 ± 12 mg/dl, P < 0.05) and the area under the curve were significantly lower in the TMX-treated groups oil-treated mice given rapamycin or vehicle (Figure 5D). These findings suggest that GCGR deletion does not depend on mTOR signaling to mitigate hyperglycemia due to reduced insulin action.

3.4. Contribution of GLP-1 signaling to the reduced baseline glucose levels following Gcgr deletion in STZ-treated mice

We next investigated the factors contributing to the modest protection from hyperglycemia when *Gcgr* deletion is induced after STZ-induced diabetes (Figure 4A). We observed that plasma GLP-1 levels, but not c-peptide or FGF21, were significantly increased in the STZ-TMX-treated cKO mice. To determine the contribution of increased GLP-1 to the protection from hyperglycemia, we repeated the experiment including an additional group of STZ-TMX-treated cKO mice receiving daily injections (500 nmol/kg ip) of the GLP1R antagonist Jant4 [24]. Consistent with the previous experiment (Figure 4A), TMX-treated cKO mice exhibited a modest but significant reduction of hyperglycemia after the completion of TMX treatment compared to oil-treated controls (Figure 6A), although those levels remained far higher (332 ± 6 mg/dl) than normoglycemia. Interestingly, glucose levels in STZ-TMX-treated cKO mice receiving Jant4 did not differ from those of STZ-oil-treated mice, at least during the first 9 d after TMX injection (Figure 6A). However, over time, glucose gradually decreased to levels significantly different from controls (Figure 6A). Neither TMX nor Jant4 significantly impacted food intake (Figure 6B). However, and consistent with our previous experiment (Figure 3B), acute *Gcgr* deletion in STZ mice resulted in significant BW loss compared to STZ-oil-treated WT controls, a decrease that was prevented by the daily administration of Jant4 (Figure 6C). When considering our previous results (Figures 2E and 4E), these data suggest that the rapid increase in circulating GLP-1 plays a significant role in the protection from hyperglycemia and in the body weight loss elicited by acute deletion of *Gcgr* in STZ mice. In addition, these data also suggest a negligible direct contribution of GCGR signaling (or lack or thereof) to blood glucose levels even under insulinopenic conditions.

4. DISCUSSION

The engagement of multiple compensatory mechanisms limits assessment of the direct contribution of GCGR signaling to glycemic control in models of chronic loss of GCGR signaling. By temporally controlling the disruption of *Gcgr*, we found that acute loss of *Gcgr* in a model of insulinopenic diabetes provides only modest protection from hyperglycemia and that a large component of this is mediated by the GLP-1R. These data suggest that loss of GCGR signaling and the effects typically attributed to acute glucagon action, glycogenolysis and gluconeogenesis, have only a mild impact on hyperglycemia associated with insulinopenia. Instead, it appears that the engagement of compensatory mechanisms over time, including increased GLP-1R signaling, plays the critical role in protection against diabetes that accompanies GCGR knockout.

Glucagon is a cornerstone of the physiological control of blood glucose and is generally believed to be the principle mediator of counter-regulation, opposing the effect of insulin and preventing hypoglycemia. Thus, defective stimulation of glucagon secretion is acknowl-

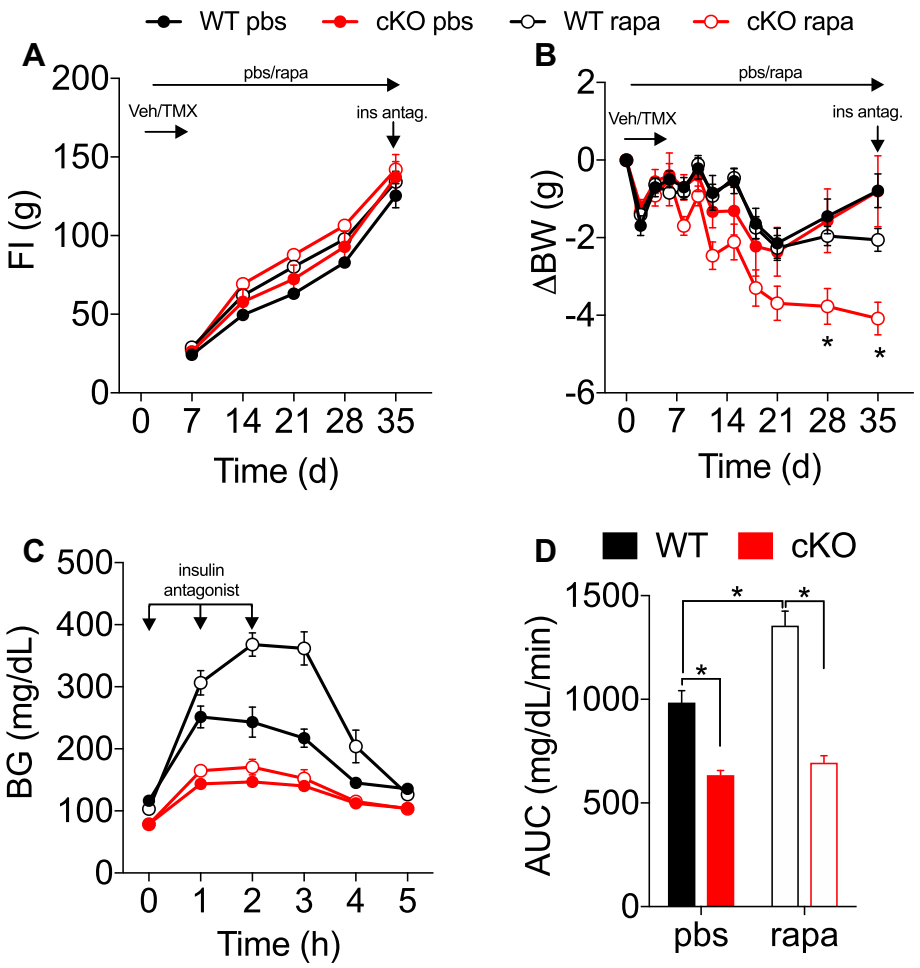

Figure 5: Effect of chronic rapamycin treatment in mice with tamoxifen-induced disruption of Gcgr expression. Cumulative food intake (A) and body weight change (B) in 10–14 wk-old *Rosa26-*$^{Cre\text{-}ERT2}$*:Gcgr*$^{flox/flox}$ male mice receiving either oil as vehicle (WT, black symbols) or tamoxifen to disrupt *Gcgr* expression (cKO, red symbols). Mice from each group received either rapamycin (10 mg/kg, ip., alt.d., open symbols) or pbs (solid symbols). Blood glucose (C) and calculated area under the curve (D) following three consecutive injections of the insulin antagonist S961 (200 nmol/kg, ip., each) 5 wk after the first TMX injection. Data are presented as mean ± SEM. (B) 2-way RM ANOVA, Sidak, post hoc test *P < 0.05 cKO-rapa-treated vs WT-rapa-treated control. (C) P < 0.05 WT-rapa vs WT-pbs controls; P < 0.05 cKO-pbs vs. WT-pbs controls main group effect, 2-way RM ANOVA. (D) *P < 0.05, 2-way ANOVA, Sidak, post hoc test.

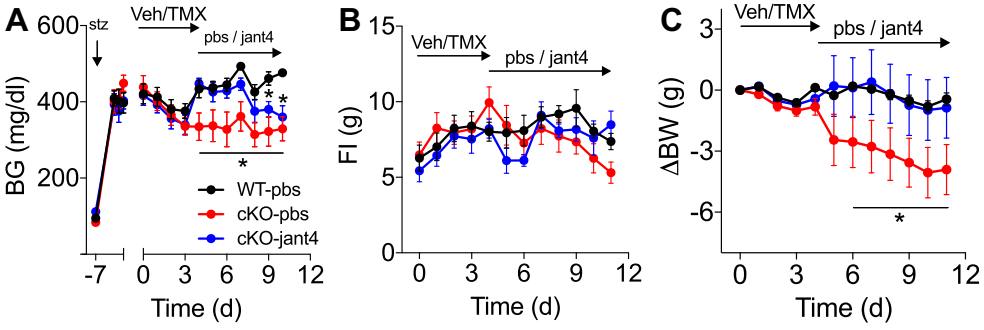

Figure 6: Effect of GLP-1R antagonism following acute disruption of Gcgr expression in STZ-treated mice. Blood glucose (A), daily food intake (B) and body weight (C) following acute disruption of *Gcgr* with tamoxifen (cKO) in 10–14 wk-old adult mice previously treated with STZ (150 mg/kg, ip). After the fourth tamoxifen injection, mice received daily injections of the GLP1R antagonist Jant4 (500 nmol/kg ip). Data are presented as mean ± SEM. n = 8–9. (B) *P < 0.05 vs WT-veh control, 2-way RM ANOVA, Sidak post hoc test.

edged as a key determinant of the incidence and severity of hypoglycemic episodes in T1DM patients [31]. On the other hand, pathophysiological conditions characterized by increased circulating levels of glucagon, including patients with glucagonoma, may result in hyperglycemia [32]. These findings support a beneficial effect of reducing glucagon receptor signaling as a potential antidiabetic treatment. Consistent with this, disruption of GCGR signaling in rodent models, either by or pharmacological or genetic means, results in

significant improvements in glycemic control. Indeed, congenital mouse models of loss of *Gcgr* expression (*Gcgr*$^{-/-}$ mice) exhibit reduced baseline blood glucose, improved glucose tolerance, and even more strikingly, the maintenance of normoglycemia and increased survival following the destruction of beta-cells with STZ [3,5]. However, they also exhibit resistance to body weight gain when fed a HFD [3], a finding that is somewhat counterintuitive considering that conditions characterized by increased endogenous glucagon levels [33,34] or treatment with *Gcgr* agonists [23] result in reduced food intake and increased weight loss. The apparently conflicting results are likely the result of the multiple compensatory mechanisms exhibited by congenital *Gcgr*$^{-/-}$ mice. Some of those compensations include increased circulating levels of proglucagon-derived peptides including glucagon and GLP-1 [2] as well as increased FGF21 [6], all factors with a significant contribution to glycemic control.

Previous attempts to minimize the contribution of the compensatory changes due to chronic loss of *Gcgr*, have used temporally-controlled reduction of glucagon levels by alpha-cell ablation, glucagon immunoneutralization, and the use of small molecule or antibody glucagon receptor antagonists [10,35]. Similar to our results, most of these acute disruptions of glucagon signaling did not mitigate the hyperglycemia of STZ diabetes [35]. In contrast to these previously described methods, we used the "cre-loxP" approach to temporally control the disruption of *Gcgr* in adulthood upon treatment with tamoxifen. In contrast to what occurs with alpha-cell deletion and pharmacologic approaches, mice with TMX-induced *Gcgr* deletion completely lack a hyperglycemic response to a *Gcgr* agonist, ensuring near total time-controlled loss of GCGR function in the liver. The literature points to the liver as the organ mediating most of the contribution of GCGR signaling to whole-body glucose control in mice. For example, liver-specific CGGR KO mice faithfully recapitulate the glucose metabolism and other phenotypical characteristics, including alpha cell hyperplasia, typical of mice with congenital global GCGR deletion [12]. Furthermore, re-expression of GCGR in the liver of congenital, global GCGR KO mice prevents the protection from hyperglycemia following STZ injection [4]. Importantly, our model recapitulates hallmarks of congenital deletion of *Gcgr*, including reduced baseline blood glucose, improved glucose tolerance and loss of a hyperglycemic response following a challenge with a *Gcgr* agonist, as soon as one week after disruption of *Gcgr* expression. The comparison of short versus long-term *Gcgr* deletion, specifically in STZ-treated mice, reveals a clear influence of the duration of *Gcgr* disruption in the protection from hyperglycemia. Thus, while the deletion of the *Gcgr* for 6 weeks provides near complete protection from hyperglycemia after STZ, short term *Gcgr* deletion in adult mice previously treated with STZ provides only comparatively modest protection. While the compensations to loss of GCGR were qualitatively similar in the 2 and 6 week knockout models based on our survey of relevant gene expression, there were quantitative difference as exemplified by the circulating levels of GLP-1 and FGF21. Moreover, the relative correction of hyperglycemia with acute and chronic *Gcgr* deletion did correspond with the marked reductions in *Pck1* and *G6pc* and increase in *Gck* expression in the 6 week knockout animals. These results suggest a gradual change in physiology after loss of GCGR signaling with a critical accumulation necessary for the full protection from insulinopenic diabetes.

Previous studies suggest that some of the compensatory mechanisms in response to *Gcgr* deletion act by increasing insulin action. Hence, *Gcgr*$^{-/-}$ mice exhibit increased insulin sensitivity [36], and treatment with a monoclonal antibody against *Gcgr* reduces insulin requirements in T1DM humans [37]. Our data suggest that factors other than different levels of basal plasma c-peptide (insulin) or insulin signaling measured as pAKT levels must contribute to the increased insulin action due to loss of GCGR signaling. Since suppression of mTOR signaling is one of the mechanisms whereby glucagon opposes the action of insulin in the liver ([28], we postulated that chronic *Gcgr* loss of function could result in insulin-like activity by promoting increased mTOR signaling. Indeed, some of the changes in gene expression seen following chronic loss of *Gcgr* are consistent with increased mTORC activity, including the reduction in Igfbp1 and the increase in Gck [30], as well as the increase in lipogenic genes [38] and the increase in Fgf21 [39]. In addition, a potential contribution of extrahepatic mTOR signaling was supported by the increase in plasma amino acid levels and the suppression of the hepatic expression of transporters and enzymes involved in amino acid metabolism exhibited by mice lacking glucagon [40] or *Gcgr* expression [18]. These were confirmed by our gene expression analysis. Amino acids are one of the principal activators of the mTOR pathway, and recent evidence strongly suggests that hyperaminoacidemia contributes to the alpha-cell hyperplasia exhibited by rodent models of reduced glucagon levels [11,41] or *Gcgr* signaling [2,12], as well as those exhibited by human subjects with *Gcgr* mutations [16,17,42]. Thus, alpha cell proliferation can be greatly blunted by chronic administration of the mTOR inhibitor rapamycin [16–18]. We adopted the rapamycin administration protocol used by those groups to determine the impact of chronic reduction of mTORC signaling on the development of compensatory mechanisms leading to improved glucose control in mice with long-term *Gcgr* deletion in conditions of acute reduction of insulin action achieved by repeated injections with the insulin antagonist S961. In contrast to the expected hyperglycemia exhibited by the S961-treated control counterparts [43], chronic treatment with rapamycin did not change blood glucose very much in *Gcgr* deficient mice. In fact, mice lacking GCGR had a minimal glycemic response to S961 alone, suggesting the contribution of an insulin-independent mechanism to glycemic control in mice with long-term *Gcgr* deletion. These results also suggest that additional mechanisms contributing to the improved glucose control exhibited by long-term deletion of *Gcgr* develop and function under conditions of reduced mTORC signaling and independently of differences in circulating insulin. This notion is consistent with the maintenance of normoglycemia and survival under insulinopenic conditions to a degree that cannot be explained solely by the action the residual insulin [5,7,10]. On the other hand, the fact that loss of *Gcgr* does not prevent hyperglycemia and death under conditions of complete loss of insulin [8,9] demonstrates that such additional mechanisms are impotent preventing hyperglycemia in conditions of nearly absent insulin.

Congenital *Gcgr*$^{-/-}$ mice exhibit increased hepatic Fgf21 expression and circulating FGF21 levels. Previous studies by other investigators demonstrated that neutralization of circulating FGF21 with specific antibodies impairs glucose control in these mice [6]. In addition, the FGF21 analog LY2405319 lowers blood glucose in STZ mice [44], suggesting that high FGF21 levels may contribute to the protection from hyperglycemia exhibited by the STZ-treated *Gcgr*$^{-/-}$ mice. Tamoxifen-induced *Gcgr* deletion in STZ-treated mice also resulted in increased FGF21 levels, although only after long-term *Gcgr* deletion. Thus, FGF21 cannot account for the reduced baseline glycemia in normal and STZ mice with short-term *Gcgr* deletion.

Like FGF21, congenital *Gcgr*$^{-/-}$ mice also exhibit dramatically increased GLP-1 levels [2], and these can be recapitulated with treatment with antisense probes to reduce *Gcgr* expression [13]. In contrast to FGF21, we found significantly elevated plasma GLP-1 in mice with short-term *Gcgr* deletion, positioning it as a candidate to contribute to the improved glucose control exhibited by those mice. Thus, early evidence

suggests that increased plasma GLP-1 makes a meaningful contribution to the improved glycemia associated with reduced GCGR signaling. Hence, mice lacking glucagon [11] or *Gcgr* [2,45] exhibit a larger degree of improved glucose tolerance when compared to mice lacking proglucagon gene expression and consequently both glucagon and GLP-1 [40]. In addition, the double mutant $Gcgr^{-/-}$:$Glp1r^{-/-}$ mice lack the improved glucose tolerance exhibited by normoglycemic $Gcgr^{-/-}$mice [46]. Further, STZ-treated $Gcgr^{-/-}$:$Glp1r^{-/-}$ mice exhibit reduced degree of protection from baseline hyperglycemia compared to $Gcgr^{-/-}$ mice [27], and treatment with the GLP-1R antagonist exendin 9 during a glucose challenge significantly increases blood glucose in $Gcgr^{-/-}$ mice [6]. Consistent with these data, treatment with the GLP-1R antagonist Jant4 completely eliminated, albeit transiently, the modest protection from hyperglycemia due to acute *Gcgr* deletion in STZ-treated mice. The loss of efficacy of the antagonist preventing the blood glucose lowering is consistent with a steady increase in plasma GLP-1 levels that eventually overcome the fixed dose of antagonism. Alternatively, progressive development of GLP-1R-independent compensatory mechanisms may contribute to the gain of hyperglycemic protection. A genetic mouse model allowing simultaneous time-controlled deletion of both *Gcgr* and *Glp1r* should allow tracking the onset of those alternative mechanisms. Regardless, these findings position GLP-1R as one of the early compensatory mechanisms engaged following the loss of GCGR signaling that contribute to protection from hyperglycemia due to loss of *Gcgr*. The mechanisms whereby the increase in plasma GLP-1 levels may contribute to the blood glucose lowering are unclear but they may involve the temporary reduction in feeding observed in STZ-TMX-treated cKO mice. In addition, Jant4 prevented the body weight loss seen in STZ-mice with short-term *Gcgr* deletion. These results are consistent with a contribution of increased GLP-1R to the protection from obesity exhibited by $Gcgr^{-/-}$ mice [3]. It is well established that loss of *Gcgr* leads to increased circulating glucagon that exceed increases of GLP-1 [2]. Since glucagon exhibits weak agonism on the GLP-1R [47], it is possible that increased glucagon levels may indirectly regulate glycemia and body weight by acting as a weak GLP-1 mimetic. Although there is evidence of a beneficial role of GLP-1 pharmacology in improving glycemic control in T1DM subjects [48–50], the extent to which a contribution of GLP-1R to glycemic control in insulinopenic conditions may be clinically relevant remains to be convincingly demonstrated. Given the experience with GLP-1 mimetics to treat type-2 diabetes, the translation of these therapies to the pharmacological toolbox currently available to treat Type-1 Diabetes may provide meaningful improvements in glycemic control in those patients.

5. CONCLUSIONS

Temporally-controlled disruption of *Gcgr* reveals a lack meaningful contribution to the improvement of glycemic control in insulinopenic conditions attributable to the intrinsic loss of GCGR signaling. On the other hand, these data highlight the importance of compensatory systems, including GLP-1R signaling. Given the potential adverse effects exhibited by therapies aiming to block *Gcgr*, including adverse lipid profile and hepatic function [51–53], emphasis on identifying and developing the most effective of those compensatory mechanisms may provide safe and efficacious therapies for the treatment of T1DM.

DISCLOSURE STATEMENT

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CONFLICT OF INTEREST

None declared.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at https://doi.org/10.1016/j.molmet.2018.07.012.

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