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The glucagon receptor antagonist desHis^{1}Pro^{4}Glu^{9}-glucagon(Lys^{12}PAL) alters alpha-cell turnover and lineage in mice, but does not cause alpha-cell hyperplasia

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ABSTRACT

Objective: Glucagon receptor (GCGR) antagonism elicits antihyperglycemic effects in rodents and humans. The present study investigates whether the well characterised peptide-based GCGR antagonist, desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL), alters alpha-cell turnover or identity in mice.

Methods: Multiple low-dose streptozotocin (STZ) treated (50 mg/kg bw, 5 days) transgenic *Glu^{CreERT2};ROSA26-eYFP* mice were employed. STZ mice received twice daily administration of saline vehicle or desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL), at low- or high-dose (25 and 100 nmol/kg, respectively) for 11 days.

Results: No GCGR antagonist induced changes in food or fluid intake, body weight or glucose homeostasis were observed. As expected, STZ dramatically reduced ($P < 0.001$) islet numbers and increased ($P < 0.01$) alpha-to beta-cell ratio, which was linked to elevated ($P < 0.05$) levels of beta-cell apoptosis. Whilst treatment with desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) decreased ($P < 0.05$-$P < 0.001$) alpha- and beta-cell areas, it also helped restore the classic rodent islet alpha-cell mantle in STZ mice. Interestingly, low-dose desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) increased ($P < 0.05$) alpha-cell apoptosis rates whilst high dose decreased ($p < 0.05$) this parameter. This difference reflects substantially increased ($P < 0.001$) alpha-to beta-cell transdifferentiation following high dose desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) treatment, which was not fully manifest with low-dose therapy.

Conclusions: Taken together, the present study indicates that peptidic GCGR antagonists can positively influence alpha-cell turnover and lineage in identity in multiple low-dose STZ mice, but that such effects are dose-related.

1. Introduction

It is recognised that elevated glucagon levels in the diabetic state leads to an increase in hepatic glucose production and glycogen breakdown that further provoke hyperglycaemia (Unger, 1978). In keeping this with, glucagon receptor (GCGR) blockade is considered a possible therapeutic approach for diabetes. On the other hand, GCGR activation can reduce body weight by decreasing food intake and increasing metabolic rate. Moreover, when combined with glucagon-like peptide-1 receptor (GLP-1) activation, GCGR mediated increases in blood glucose appear to be largely offset (Lafferty et al., 2021a). However, unlike GCGR activation that necessitates modulation of other receptor pathways to yield benefits, blockade of GCGR can exert independent benefits in diabetes (Lafferty et al., 2021a). That said, safety concerns such as uncontrolled hypoglycaemia (Kelly et al., 2015), negative alterations of serum lipids and liver enzymes (Guan et al., 2015; Kazda et al., 2016), hyperaminoacidaemia (Galsgaard et al., 2018) as well as hyperplasia of pancreatic islet alpha-cells (Larger et al., 2016) have hindered clinical progression of this class of drugs. However, while previous studies have employed either small molecule (Guzman-Perez et al., 2013; Mu et al., 2012; Pettus et al., 2020), monoclonal antibodies (Kim et al., 2012) or antisense oligonucleotides (Morgan et al., 2019) to induce GCGR antagonism, specific and selective peptidergic GCGR antagonists may represent a more acceptable approach to annul GCGR signalling.

In that regard, our previous preclinical work highlights the potential of peptide-based agents as a safe and effective method of GCGR blockade in diabetic rodents (Irwin et al., 2013; O'Harte et al., 2013; Franklin et al., 2014; McShane et al., 2014; Lafferty et al., 2022), in agreement with others (Yang et al., 2021). For example, desHis^{1}Pro^{4}Glu^{9}-glucagon, which incorporates removal of the His^{1} residue as well as Gly^{4} to Pro^{4}

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and Asp[9] to Glu[9] amino acid substitutions of native glucagon, residues known to be essential for GCGR activation (Unson et al., 1989; Ahn et al., 2001; Hruby, 1982), annuls glucagon signalling *in vitro* and *in vivo* (O'Harte et al., 2013). We have previously demonstrated desHis[1]Pro[4]-Glu[9]-glucagon to be effective in managing diet-induced obesity in high-fat fed (HFF) mice, both alone and when combined with GLP-1, or especially glucose-dependent insulinotropic peptide (GIP), receptor agonists (McShane et al., 2016; Franklin et al., 2022). Further modification of desHis[1]Pro[4]Glu[9]-glucagon, through covalent attachment of palmitic acid to Lys[12] via a γ-glutamyl spacer molecule, to generate desHis[1]-Pro[4]Glu[9]-glucagon (Lys[12]PAL), does not impede bioactivity of the molecule and prolongs circulating half-life (O'Harte et al., 2013). Moreover, sustained administration of this acylated peptidic GCGR antagonist corrects various aspects of genetically- and dietary-induced obesity-related diabetes in obese-diabetic (*ob/ob*) and HFF mice, respectively (O'Harte et al., 2014). More recently, this acylated GCGR antagonist was established to improve glucose tolerance and pancreatic insulin stores in a single high-dose streptozotocin (STZ) induced diabetic mouse model (Franklin et al., 2022).

Given it is now accepted that fully mature pancreatic islet cells display genetic plasticity and can undergo complete lineage alteration both in response to hyperglycaemia (Migliorini et al., 2014) and directly following treatment with antidiabetic agents (Tanday et al., 2020a), it is important to investigate this phenomenon in relation to peptide-based GCGR blockade. This is especially insightful taking into consideration the potential impact of GCGR antagonism on alpha-cell hyperplasia (Galsgaard et al., 2018). As such, we employed the well-characterised transgenic *Glu*^{CreERT2};*ROSA26-eYFP* islet alpha-cell lineage tracing mouse model, that permits observation of alpha-to beta-cell trans-differentiation events through selective fluorescent tagging of pancreatic alpha-cells (Campbell et al., 2020a). To examine effects of GCGR blockade on islet cell plasticity, the present study investigated the dose-dependent (25 and 100 nmol/kg bw) effects of twice daily injection of the GCGR antagonist, desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) in multiple low-dose STZ treated *Glu*^{CreERT2};*ROSA26-eYFP* transgenic mice. Notably, this is the first investigation to assess the impact of peptide-based GCGR blockade on alpha-cell identity and islet cell transdifferentiation.

2. Materials and methods

2.1. Peptide synthesis

desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) was purchased from GL Biochem Ltd. (Shanghai, China) at greater than 95% purity. To characterise the peptide in-house, purity was confirmed using high performance liquid chromatography (HPLC) analysis and molecular weight by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, as described previously (Lafferty et al., 2020).

2.2. Animals

Full details of the generation and characterisation of transgenic *Glu*^{CreERT2};*Rosa26-eYFP* mice is provided by Campbell et al. (2020). All mice were bred in-house with PCR genotyping for each colony employed as previously described (Campbell et al., 2020a). All experiments were conducted under the UK Animals (Scientific Procedures) Act 1986 & EU Directive 2010/63EU and approved by the University of Ulster Animal Welfare and Ethical Review Body (AWERB). Animals were used at 12 weeks of age and were maintained in an environmentally controlled unit at 22 ± 2 °C with a 12 h dark and light cycle and given *ad libitum* access to standard rodent diet (10% fat, 30% protein and 60% carbohydrate; Trouw Nutrition, Northwich, UK) and drinking water.

2.3. Experimental protocols

Glu^{CreERT2};*Rosa26-eYFP* female mice (n = 6) received twice-daily (09:00 and 17:00 h) treatment with either saline vehicle (0.9% (w/v) NaCl) or desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) at low and high doses (25 nmol/kg bw and 100 nmol/kg bw, respectively) 1 day prior to commencement of multiple low-dose (50 mg/kg bw, i.p. for 5 consecutive days) streptozotocin (STZ) injection, and for a subsequent 10 days. Twice-daily 25 nmol/kg treatment was selected based on previous studies showing positive metabolic effects of desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) at this dose in normal and diabetic rodents (Franklin et al., 2014; Lafferty et al., 2022; McShane et al., 2016; O'Harte et al., 2014). The twice-daily 100 nmol/kg dose was then employed as a high dose regimen, with the current study representing the first observations with desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) at this dosage. A multiple low-dose STZ model was preferred since previous investigations confirm detrimental effects of this approach on islet cell lineage in *Glu*^{CreERT2};*Rosa26-eYFP* mice associated with increased alpha-cell population and enhanced alpha-to-beta cell ratio (Mohan et al., 2021; Lafferty et al., 2021b; Sarnobat et al., 2020, 2022). An additional separate group of saline treated *Glu*^{CreERT2};*Rosa26-eYFP* female mice (n = 6), that did not receive STZ or desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) injections, were employed for comparative purposes. All mice were monitored for 3 days prior to commencement of peptide therapy interventions. In addition, all *Glu*^{CreERT2};*Rosa26-eYFP* animals were administered tamoxifen (7 mg/mouse bw, i.p.) 2 days prior to the first STZ injection, to induce expression of the alpha-cell fluorescent lineage marker protein, as previously described (Tanday et al., 2020a, 2020b). Throughout the study, body weight, circulating blood glucose, cumulative food and fluid intake were assessed at regular intervals. At termination, pancreatic tissues were excised and immediately fixed in 4% paraformaldehyde for 48 h at 4 °C for histological analysis (Mohan et al., 2021).

2.4. Immunohistochemistry

Fixed tissues were processed and embedded in paraffin wax blocks using an automated tissue processor (Leica TP1020, Leica Microsystems), and 5 μm sections cut on a microtome (Shandon Finesse 325, Thermo Scientific). Slides were dewaxed by immersion in xylene and rehydrated through a series of ethanol solutions of reducing concentration (100–50%). Heat-mediated antigen retrieval was then conducted in citrate buffer (pH 6.0 at 90 °C for 20 min and allowed to cool for a further 20 min). Sections were blocked in 4% bovine serum albumin solution before overnight incubation (4 °C) with appropriate primary antibodies including insulin (1:400; Abcam, ab6995), glucagon (1:400; raised in-house, PCA2/4), GFP (1:1000; Abcam, ab5450), Ki67 (1:500; Abcam, ab15580) or TUNEL (100 μl reaction mix per slide; Roche, *In situ* cell-death fluorescein Kit, cat#: 11684795910). To note, YFP, the specific marker of alpha-cell lineage in *Glu*^{CreERT2};*Rosa26-eYFP* transgenic mice, was detected by the anti-GFP antibody (Abcam, ab5450) which is reactive against all variants of *Aequorea Victoria* GFP, including YFP. Slides were then rinsed in phosphate buffered saline and incubated for 45 min at 37 °C with appropriate Alexa Fluor secondary antibodies (1:400; Invitrogen, Alexa Fluor 498 or 594, Invitrogen). All slides were finally incubated with DAPI for 15 min at 37 °C, and then mounted for imaging using a fluorescent microscope (Olympus model BX51) fitted with DAPI (350 nm) FITC (488 nm) and TRITC (594 nm) filters and a DP70 camera adapter system (Lafferty et al., 2020).

2.5. Image analysis

Islet parameters, including islet, beta and alpha cell areas were analysed using the Cell^{F} imaging software and the closed loop polygon tool (Olympus Soft Imaging Solutions). For transdifferentiation cells co-expressing both insulin and GFP (insulin^{+ve}, GFP^{+ve} cells), cells expressing GFP without insulin (insulin^{-ve}, GFP^{+ve} cells), cells

expressing glucagon without GFP (glucagon^{+ve}, GFP^{-ve} cells) along with cells co expressing GFP and glucagon (glucagon^{+ve}, GFP^{+ve} cells) were analysed, as appropriate. In addition, islet cell apoptosis was determined using co-expression of TUNEL with either insulin or glucagon. Similarly, islet cell proliferation was also assessed using Ki67 staining and co-expression with either insulin or glucagon. All cell counts were determined in a blinded manner with >50 islets analysed per treatment group.

2.6. Biochemical analyses

Blood samples were collected from the cut tail vein of animals. Blood glucose was measured using a portable Ascencia Contour blood glucose meter (Bayer Healthcare, Newbury, Berkshire, UK).

2.7. Statistics

Data were analysed using GraphPad PRISM 5.0, with data presented as mean ± SEM. Comparative analyses between groups of mice were conducted using a one-way ANOVA with a Bonferroni *post hoc* test or a two-way repeated measures ANOVA with a Bonferroni *post hoc* test, as appropriate. Results were deemed significant if $P < 0.05$.

3. Results

3.1. Effects on body weight, food and fluid intake

Twice daily administration of desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) at a dose of either 25 or 100 nmol/kg for 11 days had no significant effect on body weight, cumulative food or fluid intake in multiple low-dose STZ mice (Fig. 1A–D). However, STZ mice did present with expected reductions ($P < 0.05$) of percentage body weight change and reduced fluid intake change in *Glu^{CreERT2};Rosa26-eYFP* mice (Fig. 1B,D). Notably, parameters in all STZ mice treated with desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) were not significantly different from lean controls (Fig. 1A–D).

3.2. Effects on circulating glucose and glucose tolerance

Twice daily administration of 25 or 100 nmol/kg desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) had no effect on circulating blood glucose in multiple low-dose STZ mice (Fig. 2A and B). Glucose levels were elevated in all STZ mice when compared to normal controls on study termination (8.3 ± 1.2 *vs.* 15.1 ± 2.4 mmol/l; respectively), but the increase just failed to reach significance with a p value of 0.06 (Fig. 2B). Similarly, multiple low-dose STZ significantly ($P < 0.01$) increased blood glucose concentrations during a 120 min glucose tolerance test, when compared to normal mice (Fig. 2C and D). desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) treatment, at either dose, had no impact on glucose tolerance in STZ *Glu^{CreERT2};Rosa26-eYFP* mice (Fig. 2C and D).

3.3. Effects on pancreatic islet morphology

Representative images of pancreatic islets co-stained for insulin and glucagon highlight STZ-induced islet distortion in *Glu^{CreERT2};Rosa26-eYFP* mice, as well as the subsequent impact of twice daily desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) treatment for 11 days at either 25 or 100 nmol/kg (Fig. 3A–D). As expected, STZ dramatically ($P < 0.01$-$P < 0.001$) reduced islet number in *Glu^{CreERT2};Rosa26-eYFP* mice, that was unaffected by concurrent low or high dose desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) intervention (Fig. 3E). Islet area was decreased ($P < 0.05$-$P < 0.01$) by low- and high-dose desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) treatment when compared to normal control mice (Fig. 3F), and by 25 nmol/kg desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) when compared to saline treated multiple low-dose STZ control mice (Fig. 3F). Effects on beta-cell area essentially mirrored observations on overall islet area, as desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) STZ mice presented with reduced ($P < 0.001$) beta-cell area when compared to normal control mice (Fig. 3G). STZ treatment increased ($P < 0.001$) alpha-cell area, an effect that was fully reversed by both desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) interventions (Fig. 3H). However, alpha-to beta-cell area ratio was elevated ($P < 0.001$) in all STZ mice (Fig. 3I). Furthermore, multiple low dose STZ increased ($P < 0.01$) the number of glucagon positive alpha-

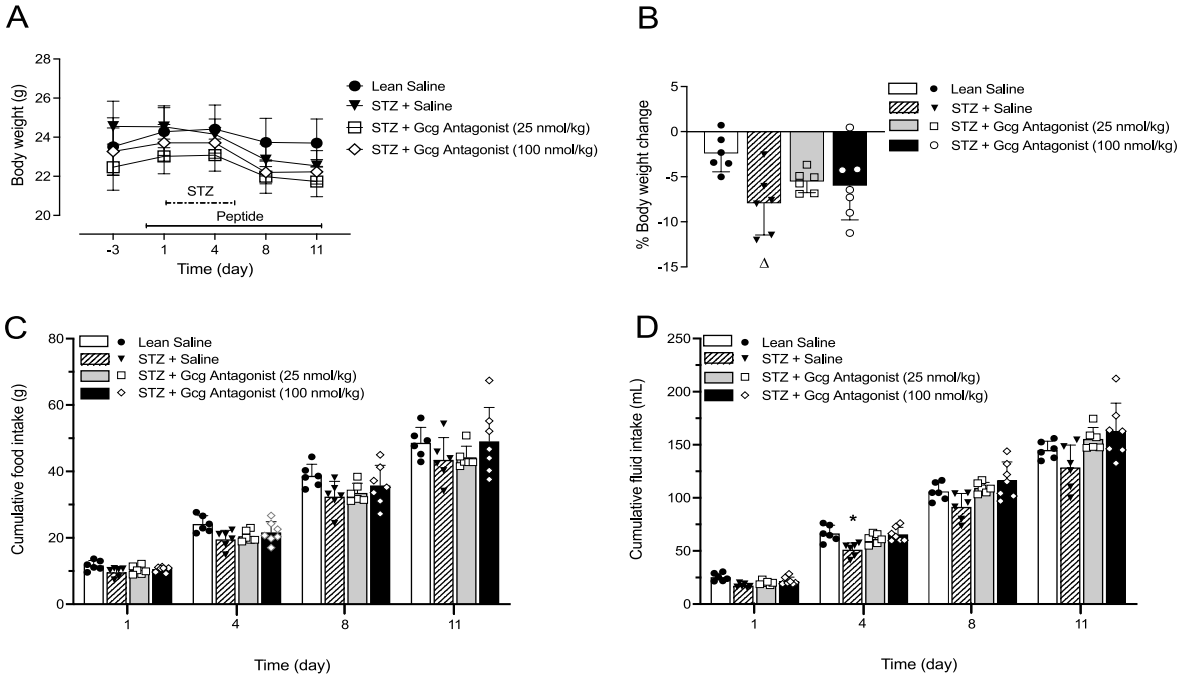

Fig. 1. Effects of twice-daily administration of the GCGR antagonist desHis^{1}Pro^{4}Glu^{9}-glucagon (Lys^{12}PAL) on body weight (A), % body weight change (B), cumulative food (C) and fluid (D) intake in multiple low-dose STZ *Glu^{CreERT2};Rosa26-eYFP* mice. Parameters were measured 3 days prior to, and 11 days during, twice daily treatment with saline vehicle or desHis^{1}Pro^{4}Glu^{9}-glucagon (25 or 100 nmol/kg bw). Values are mean ± SEM (n = 6). *$P < 0.05$ compared to normal control mice.

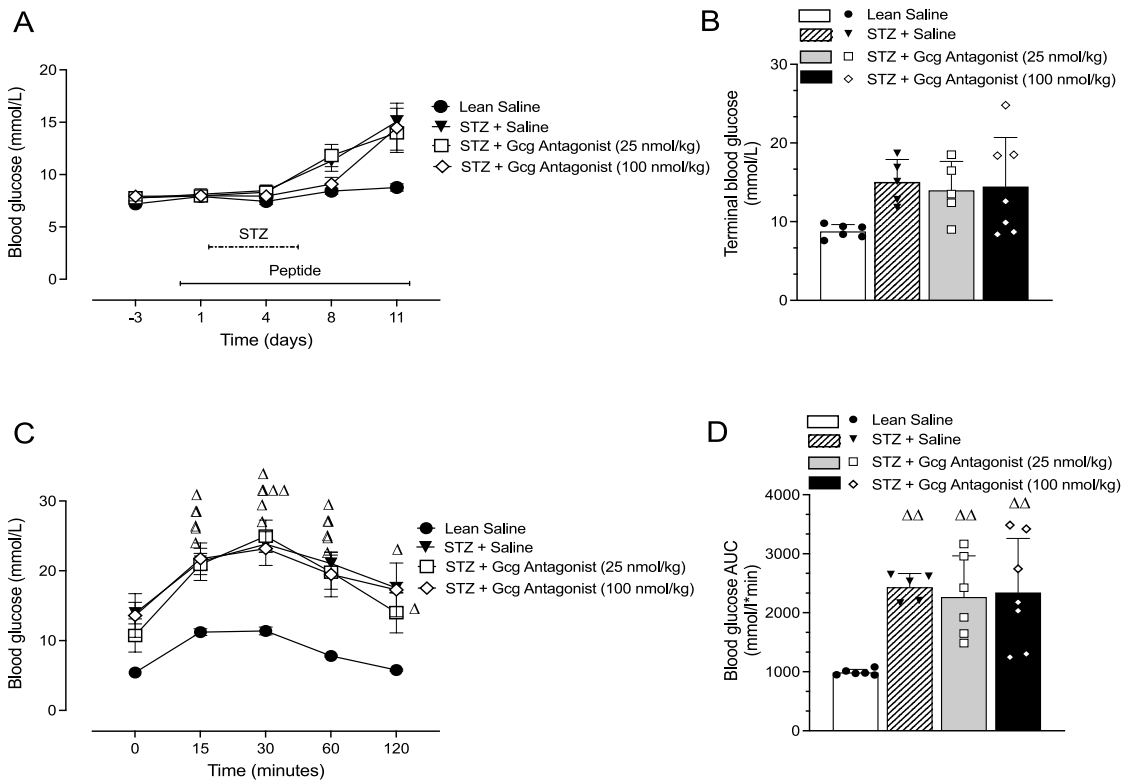

Fig. 2. Effects of 11 days twice-daily administration of the GCGR antagonist desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) on circulating blood glucose (A), terminal, non-fasting blood glucose (B) and glucose tolerance (C,D) in multiple low-dose STZ *Glu*^{CreERT2};*Rosa26-eYFP* mice. Glucose tolerance was investigated following glucose administration (18 mmol/kg bw; i.p.) in overnight fasted (16 h) mice (C), with respective 0–120 min area under the curve data also provided (D). Values are mean ± SEM (n = 6). $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ compared to normal control mice.

cells within the centre of islets, which was not observed in mice treated with 25 nmol/kg desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) (Fig. 3J).

3.4. Effects on islet cell turnover

Beta-cell proliferation rates were not significantly altered by multiple low-dose STZ injection in *Glu*^{CreERT2};*Rosa26-eYFP* mice (Fig. 4A). However, STZ mice presented with reduced ($P < 0.01$) alpha-cell proliferation, which was absent in all GCGR antagonist treated mice (Fig. 4B). STZ increased ($P < 0.05$) beta-cell apoptotic rates, with neither dose of the GCGR antagonist able to combat this (Fig. 4C). Interestingly, high-dose GCGR antagonist reduced ($P < 0.05$) alpha-cell apoptosis when compared to saline treated STZ mice, whereas low-dose desHis[1]-Pro[4]Glu[9]-glucagon (Lys[12]PAL) increased ($P < 0.05$) the rate of alpha-cell apoptosis when compared to normal mice (Fig. 4D).

3.5. Effects on alpha-cell lineage

Alpha-cell lineage was investigated by co-staining islets with glucagon or insulin alongside the lineage marker GFP. The number of insulin^{+ve},GFP^{+ve} islet cells was elevated ($P < 0.01$-$P < 0.001$) in STZ treated *Glu*^{CreERT2};*Rosa26-eYFP* mice administered the highest dose of GCGR antagonist when compared to all other groups (Fig. 5A). Populations of insulin^{-ve},GFP^{+ve} cells were unaltered in all mice (Fig. 5B). Whilst numbers of glucagon^{+ve},GFP^{+ve} cells were not significantly affected in multiple low-dose STZ mice (Fig. 5C), twice-daily administration of desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) reduced ($P < 0.01$-$P < 0.001$) these cell numbers (Fig. 5C). In good agreement with this finding, glucagon^{+ve},GFP^{-ve} cell numbers were significantly elevated ($P < 0.01$) in mice receiving low-dose desHis[1]Pro[4]Glu[9]-glucagon treatment (Fig. 5D).

4. Discussion

Unlike sustained GCGR antagonism with low molecular weight compounds (Kelly et al., 2015; Kazda et al., 2016), similar application of peptidic GCGR antagonists does not appear to induce alpha-cell hyperplasia (Franklin et al., 2014). This was confirmed in the current study, that employed a rodent model of insulin deficiency through multiple low-dose STZ administration and subsequent twice daily injection of the characterised and long-acting GCGR antagonist, desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) (O'Harte et al., 2013), at either 25 or 100 nmol/kg. Accordingly, our observations correspond well with previous investigations of chronic peptide-based GCGR antagonism in high-fat-fed mice (McShane et al., 2014), obese diabetic (*ob/ob*) mice (O'Harte et al., 2014) and in mice given a single high dose of STZ (Lafferty et al., 2022). Furthermore, while previous studies documenting anti-hyperglycaemic efficacy of peptidic GCGR antagonists employed male mice (O'Harte et al., 2013; Lafferty et al., 2022; Franklin et al., 2022), the current work extends these findings to female mice.

It is established that female mice are less prone to STZ-induced hyperglycaemia and typically display improved glucose tolerance when compared to male counterparts (Leiter, 1982). To add to this, our previous investigations suggest that *Glu*^{CreERT2};*ROSA26-eYFP* mice are inherently more resistant to STZ-induced beta-cell toxicity than other strains of mice (Lafferty et al., 2021b). Thus, although multiple low-dose STZ increased alpha-cell area and related alpha-to beta-cell ratio, as well as substantially decreasing islet cell numbers, there was no significant modification to the area of surviving islets. However, these mice did present with phenotypical signs of STZ-induced metabolic dysregulation, such as declining body weight and impaired glucose tolerance (Furman, 2015). Treatment with desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) was unable to positively impact body weight, food or fluid intake, which largely agrees with other studies employing this GCGR antagonist

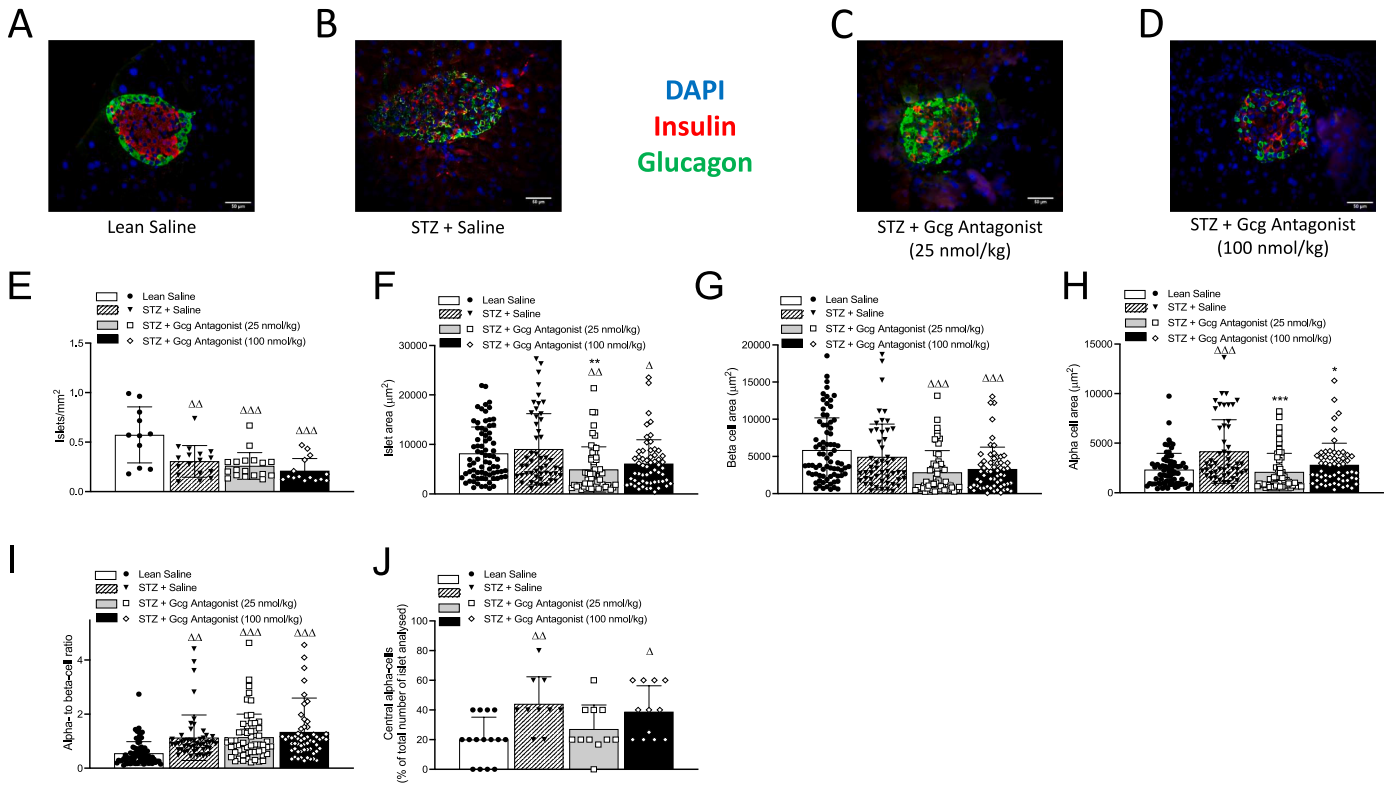

Fig. 3. Effects of 11 days twice-daily administration of the GCGR antagonist desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) on pancreatic morphology in multiple low-dose STZ *Glu*^{CreERT2};*Rosa26-eYFP* mice. Representative images (40X) of stained islets are provided in (A–D). Number of islets/mm^2 (E), islet- (F), beta- (G) and alpha-cell (H) areas as well as alpha/beta-cell ratio (I) and the number of centrally stained alpha-cells (J) were assessed using Cell^F imaging software and the closed loop polygon tool. Values are mean ± SEM for n = 6 mice, with a minimum of 50 islets per group analysed. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to multiple low dose STZ group. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ compared to normal control mice.

(Franklin et al., 2022). We also observed no obvious influence of the treatment interventions on circulating glucose or glucose tolerance in multiple low-dose STZ mice, even at an elevated dose of 100 nmol/kg bw, that slightly contrasts with previous findings in high fat fed mice (Franklin et al., 2022). However, metabolic benefits of GCGR blockade are understood to be dependent on residual beta-cell function (Damond et al., 2016), with the multiple low-dose STZ model employed here being a possible significant factor in this regard (Saini et al., 1996). Thus, peptide-based GCGR blockade has been demonstrated to improve glycaemic status in models of diabetes where beta-cell function is still partially intact (McShane et al., 2014; Lafferty et al., 2022; O'Harte et al., 2014). In harmony with this, beta-cell apoptosis was elevated in this STZ-induced mouse model and desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) treatment was unable to counter that effect. In addition, unlike our previous studies (Franklin et al., 2022), treatment intervention with the GCGR antagonist was commenced prior to diabetes induction.

In complete contrast to the notion that GCGR blockade leads to pancreatic alpha-cell hyperplasia (Larger et al., 2016; Okamoto et al., 2015), desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL), at either dose, fully reversed the detrimental effect of STZ to increase alpha-cell area. Moreover, previous studies employing twice daily injection of desHis[1]-Pro[4]Glu[9]-glucagon (Lys[12]PAL) in normal mice also confirm no changes in islet morphology or pancreatic insulin and glucagon content (Franklin et al., 2014). In good support, GCGR blockade did not influence alpha-cell proliferation in the current study. Overall, desHis[1]Pro[4]-Glu[9]-glucagon (Lys[12]PAL) intervention decreased both alpha- and beta-cell areas, but more importantly appeared to re-establish the alpha-cell mantle characteristic of healthy, functional rodent islets, which was abolished by STZ injection (Furman, 2015). The related glycaemia-independent mechanism for this restoration of normal rodent pancreatic morphology is unknown, but it is believed that the alpha-cell mantle is essential for appropriate paracrine interactions between islet cells and overall functionality of the rodent islet (Arrojo E Drigo et al., 2019). Interestingly, low-dose desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) increased alpha-cell apoptosis, with an opposing effect observed at the higher dose. This slight disparity can be explained by cell lineage changes to regulate alpha-cell numbers in STZ *Glu*^{CreERT2};*Rosa26-eYFP* mice treated with high dose desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL). Indeed, a GCGR antagonist monoclonal antibody was recently shown to provoke alpha-to beta-cell transdifferentiation in *db/db* and high-fat fed mice (Cui et al., 2022). Our data fundamentally support these recent observations of increased alpha-to beta-cell lineage change with peptide-based GCGR blockade, to help preserve functional pancreatic islets under STZ insult. The ability of desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) to induce transdifferentaition of glucagon-positive islet cells towards a beta-cell phenotype was more obvious with the highest dose employed, suggesting possible dose-dependent effects of the GCGR antagonist on islet cell lineage.

This dose-dependency in relation to desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) effects on alpha-cell apoptotic rate and lineage alteration is intriguing. As such, elevated doses decrease apoptotic rate but promote lineage transition of alpha-cells, whereas low dose appears to exert comparatively opposing effects. Whether this is directly or indirectly related to increased protection of alpha-cells from metabolic stress (Marroqui et al., 2015), disturbed islet paracrine interactions (English and Irwin, 2019) or the differential processing of proglucagon gene, yielding products such as GLP-1 under diabetic milieu (Campbell et al., 2020b), remains to be established. That said, despite increased trans-differentiation of alpha-to beta-cells with high dose desHis[1]Pro[4]Glu[9]--glucagon (Lys[12]PAL) in the STZ *Glu*^{CreERT2};*Rosa26-eYFP* mice, overall beta-cell area was decreased, possibly due to the net effect of the small

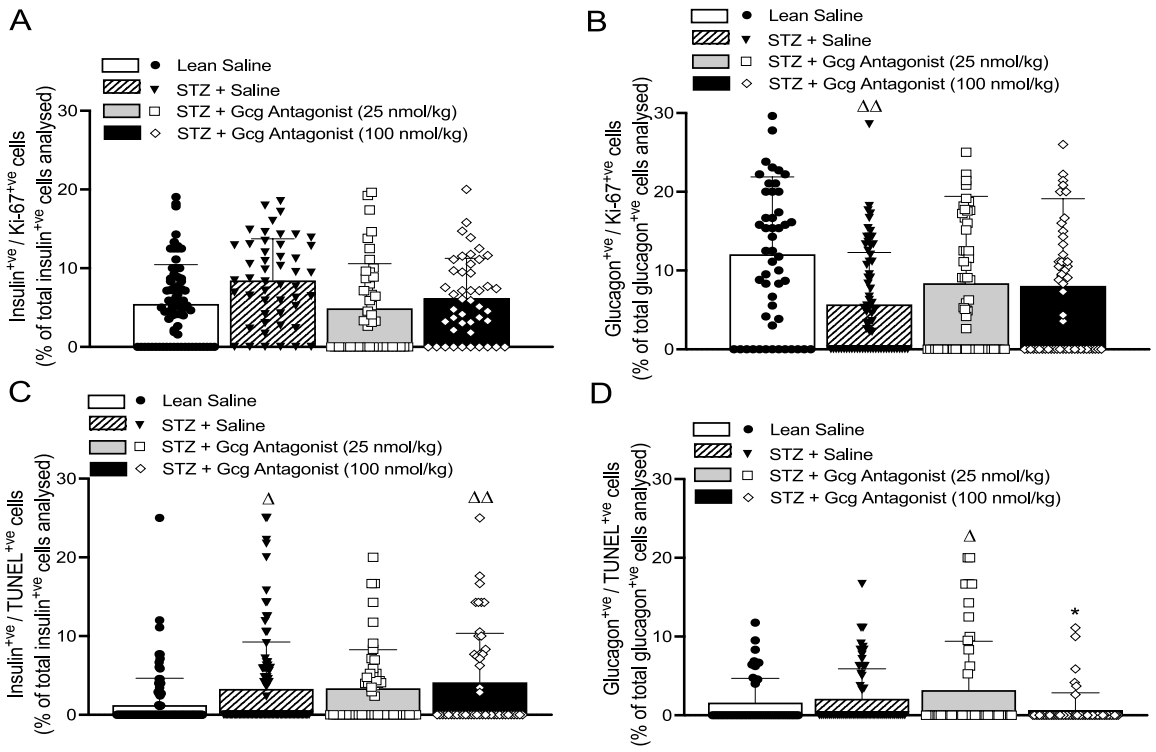

Fig. 4. Effects of 11 days twice-daily administration of the GCGR antagonist desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) on beta- and alpha-cell proliferation (A,B) and apoptosis (C,D) in multiple low-dose STZ *Glu*^{CreERT2};*Rosa26-eYFP* mice. Beta- and alpha-cell proliferation or apoptosis were assessed by co-staining of insulin or glucagon with Ki67 (A,B) or TUNEL (C,D), respectively. Values are mean ± SEM for n = 6 mice, with a minimum of 50 islets per group analysed. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ compared to normal control mice. $^{*}P <$ compared to mutiple low dose STZ group.

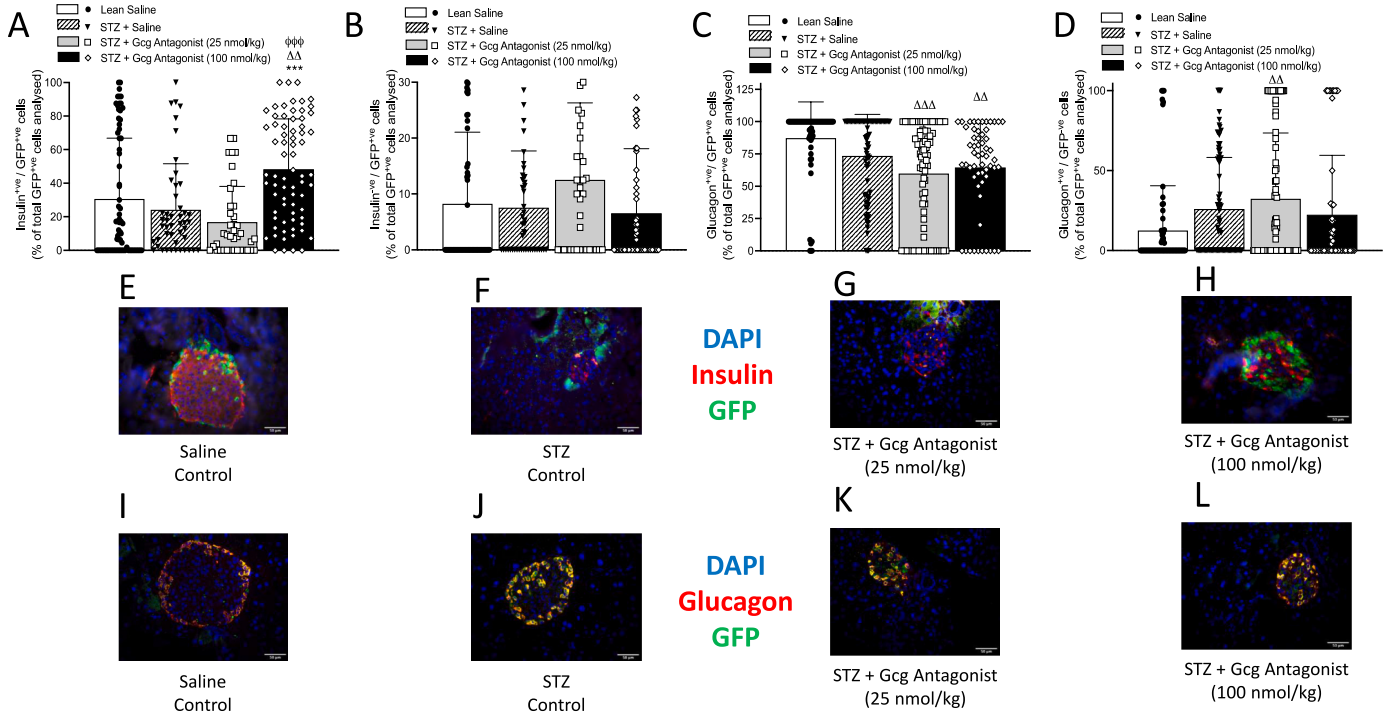

Fig. 5. Effects of 11 days twice-daily administration of the GCGR antagonist desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL) on alpha-cell lineage in multiple low-dose STZ, *Glu*^{CreERT2};*Rosa26-eYFP* mice. Specifically, insulin^{+ve},GFP^{+ve} (A), insulin^{-ve},GFP^{+ve} (B), glucagon^{+ve},GFP^{+ve} (C) and glucagon^{+ve},GFP^{-ve} (D) cell populations were assessed via immunohistochemistry. Representative images (40X) of islets showing (E–H) insulin (red) or (I–L) glucagon (red) as well as GFP (green) and DAPI (blue) immunoreactivity from each group of mice are provided. Values are means ± SEM (n = 5–6), with a minimum of 50 islets being analysed per group. $^{***}P < 0.001$ compared to normal control mice. $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ compared to multiple low dose STZ group. $^{\Phi\Phi\Phi}P < 0.001$ compared to low dose GCGR antagonist.

accompanying increase of apoptosis and decrease of proliferation. At the same time, regardless of the dose employed, it is clear that desHis[1]-Pro[4]Glu[9]-glucagon (Lys[12]PAL) exerts multiple direct effects on pancreatic islet cells to positively alter both turnover and lineage fate. It would also have been interesting to examine pancreatic islet hormone levels in the current setting, but unfortunately this was not possible as all pancreatic tissue samples were utilised for our in-depth investigations of islet morphology. In addition, earlier studies suggest that the metabolic benefits of GCGR blockade are associated with upregulated circulating GLP-1 levels (Lang et al., 2020), although we have previously observed no obvious added benefit of combined GLP-1 receptor activation alongside peptide-based GCGR antagonism (Franklin et al., 2022). Despite this, it might be interesting to consider circulating glucagon and GLP-1 levels in the current setting. In that regard, we have previously reported elevated pancreatic, but not circulating, glucagon concentrations following sustained peptidic GCGR antagonism in other rodent models of diabetes (Franklin et al., 2022; O'Harte et al., 2014).

In terms of translation of our findings to the human setting, it is worth noting that human islets contain a substantially higher proportion of alpha-cells than rodent islets (Henquin and Rahier, 2011), and that there are well-described differences in terms of alpha-cell location and secretory profile between species (Steiner et al., 2010), which may impact islet cell lineage events. That said, whilst most studies examining islet cell lineage transition have been conducted in rodent systems where cellular plasticity is believed to be more apparent (Tanday et al., 2020a), fully mature human islet cells also possess capability to alter their identity *in situ* (Moin and Butler, 2019). Certainly, improvement of metabolism following GCGR monoclonal antibody injection in a recently reported phase 2 clinical trial suggests optimism in terms of a clinically relevant therapeutic profile for GCGR antagonists (Pettus et al., 2022). It is also important to note that hypoglycaemic episodes were not evident in the current study, and mice displayed no signs of distress following twice-daily treatment with either 25 or 100 nmol/kg desHis[1]Pro[4]Glu[9]-glucagon (Lys[12]PAL). However, GCGR signalling is fundamentally involved in amino acid homeostasis (Wewer Albrechtsen et al., 2019; Elmelund et al., 2022) and the longer-term impact of peptidic GCGR blockade on circulating amino acid concentrations would also need to be considered in the clinical setting.

5. Conclusion

We reveal for the first time a potential beneficial a dose-dependent role for GCGR antagonism in driving alpha-to beta-cell transdifferentiation in mice with multiple low-dose STZ injection. Peptide-based GCGR therapies appear to be safe and do not induce alpha-cell hyperplasia that is witnessed with other GCGR antagonist modalities (Larger et al., 2016). Taken together, our observations support continued efforts to progress peptidic GCGR antagonists as a new class of drugs for the management of diabetes in humans.

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CRediT authorship contribution statement

Ryan Lafferty: Conceptualization, Methodology, Validation, Data curation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Neil Tanday:** Methodology, Validation, Data curation, Formal analysis, Investigation, Visualization. **Vaibhav Dubey:** Methodology, Validation, Data curation, Formal analysis, Investigation, Visualization. **Aimee Coulter-Parkhill:** Methodology, Validation, Data curation, Formal analysis, Investigation, Visualization. **Karthick Vishal:** Methodology, Validation, Data curation, Formal analysis, Investigation, Visualization. **R. Charlotte Moffett:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Finbarr O'Harte:** Conceptualization, Validation, Writing – original draft, Writing – review & editing, Funding acquisition. **Peter R. Flatt:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition. **Nigel Irwin:** Conceptualization, Methodology, Validation, Writing – original draft, Writing – review & editing, Visualization, Project administration, Funding acquisition.

Declaration of competing interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Data availability

Data will be made available on request.

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