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GABA and insulin but not nicotinamide augment α- to β-cell transdifferentiation in insulin-deficient diabetic mice

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ABSTRACT

Aim: Poorly controlled diabetes is characterised by a partial or complete loss of pancreatic islet β-cells, which deprives the remaining islet cells of important β-cell-derived soluble signals, such as insulin or GABA. We aimed to dissect the role of the two signals in the development of islet α-cells, focusing specifically on α-/β-cell transdifferentiation and using the stem cell differentiation factor nicotinamide as a comparator.

Methods: Streptozotocin (STZ)-treated diabetic mice expressing a fluorescent reporter in pancreatic islet α-cells were injected with GABA (10 mg/kg once daily), nicotinamide (150 mg/kg once daily) or insulin (1U/kg three times daily) for 10 days. The impact of the treatment on metabolic status of the animals as well as the morphology, proliferative potential and transdifferentiation of pancreatic islet cells was assessed using biochemical methods and immunofluorescence.

Results: Metabolic status of STZ-diabetic mice was not dramatically altered by the treatment interventions, although GABA therapy did reduce circulating glucagon and augment pancreatic insulin stores. The effects of the exogenous agents on islet β-cells ranged from the attenuation of apoptosis (insulin, nicotinamide) to enhancement of proliferation (GABA). Furthermore, insulin and GABA but not nicotinamide enhanced the differentiation of α-cells into β-cells and increased relative number of 'bihormonal' cells, expressing both insulin and glucagon.

Conclusions: Our data suggest a role for endogenous insulin and GABA signalling in α-cell plasticity, which is likely to bypass the common nicotinamide-sensitive stem cell differentiation pathway.

1. Introduction

Severe diabetes is associated with dysfunction of pancreatic islets of Langerhans, which impairs the hormonal regulation of blood glucose levels resulting in prolonged hyperglycaemia. In healthy organisms, the two key pancreatic hormones, insulin and glucagon are secreted by the islet β-cells and α-cells, to ensure the removal or recruitment of glucose from/into the systemic circulation, respectively. Despite their opposing physiological roles, α- and β-cells have many common features related to glucose sensing machinery, which are likely to be inherited from a common progenitor [1,2]. Extreme β-cell-stress has been reported to exploit this link, by inducing a switch in the fate of islet α- [3,4], δ- [5] and non-islet pancreatic [6] cells towards the differentiation into functional β-cells. Inherent islet cell plasticity may therefore represent a mechanism for potential regeneration of the β-cell mass upon diabetes-induced loss [2].

The conventional view of the second largest islet cell population, α-cells, as the most appropriate cellular source for the β-cell regeneration [3,7,8] has been strengthened by recent studies ranging from therapeutically induced gain of β-cell properties by α-cells [9–12] to complete α- to β-cell transdifferentiation [4,12–15]. The yet unclear mechanism underlying α- to β-cell lineage transition [8,16] is likely to involve the altered paracrine regulatory islet environment of the remaining α-cells, such as loss of β-cell-derived local signals like insulin [17], GABA [18], IAPP [19], glutamate [20] etc. Controversially, the latter has been suggested to induce both an autocrine proliferative stimulus for β-cells [21] and a signal for α- to β-cell transdifferentiation [14]. As such, GABA signalling was reported to encourage the expression of insulin in adult pancreatic α- and duct-cells by downregulating Arx [14]. This was however questioned by a later study utilising similar lineage tracing

Abbreviations: T1D, type 1 diabetes; GABA, gamma aminobutyric acid, GLP-1, glucagon-like peptide- 1; YFP, yellow fluorescent protein; STZ, streptozotocin; GIP, glucose-dependent insulinotropic peptide; K_{ATP} channel, ATP-sensitive K^+ channel; DAPI, 4′,6-diamidino-2-phenylindole; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling; BSA, bovine serum albumin.

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methodology [13] and an analogous study in the rhesus monkey [22]. Likewise, pharmacological agonism of the GABA receptor by anti-malaria drugs of the artemisinin family induced α-cell plasticity in a cell line [15] but not in the primary islet model [23].

Earlier studies [7,24] suggest insulin signalling represents a safe-guard mechanism against α-cell transdifferentiation [4,25], which was, however, not attained by exogenous insulin that failed to block the α-cell transdifferentiation, in mouse models of severe diabetes [3,7,26]. Moreover, the suggested dependence of α-cell plasticity effects on insulin and GABA signalling may stem from a multitude of unaccounted factors, such as residual β-cell function or glycaemic status. Therefore, in the present study, we aimed to dissect the impact of insulin and GABA on islet cell plasticity in a mouse model of relatively severe β-cell stress, whilst controlling for the diabetic phenotype. β-Cell stress was established in transgenic mice expressing an inducible fluorescent α-cell label ($Glu^{CreERT2}$; ROSA26e-YFP), using multiple low-dose administration of streptozotocin (STZ). We compared the effects of GABA and insulin to that of a reputable stem cell differentiation agent nicotinamide, on metabolic control, pancreatic islet morphology, proliferative potential and islet cell transdifferentiation in these mice. Notably, nicotinamide has been confirmed to induce the differentiation of mesenchymal [27], embryonic [28,29] and bone marrow [30] stem cells towards the β-cell lineage.

2. Materials and methods

2.1. Animals

All experiments were carried out 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 maintained in an environmentally controlled laboratory 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.

2.1.1. $Glu^{CreERT2}$;ROSA26-eYFP mice

Ten-week old male $Glu^{CreERT2}$;ROSA26-eYFP transgenic mice were used to perform all studies. Originally developed on the C57BL/6 background at the University of Cambridge [31], the colony was subsequently transferred to the animal facility at Ulster and genotyped to confirm Cre-ERT2 and ROSA26eYFP gene expression (Table 1). Three days prior to STZ dosing, mice were injected with tamoxifen (Sigma-Aldrich, Dorset, UK; 7 mg/mouse; i.p.) to activate the tissue-specific expression of yellow fluorescent protein (YFP) in pancreatic islet α-cells (Fig. 1A).

2.2. Diabetes model and antidiabetic medications

As we aimed to dissect the direct effects of insulin, GABA and nicotinamide on islet morphology and cell transdifferentiation, on background of sustained hyperglycaemia, we designed the experiments to exclude the effects mediated by changes of insulin sensitivity or blood glucose, which may affect islet composition and function [32,33]. Thus, three days after the tamoxifen injection, insulin-deficient diabetes [34] was induced by a 5-day injection course of low-dose STZ [35] (Sigma-Aldrich, Dorset, UK; 50 mg/kg body weight daily, i.p.) (Fig. 1A), dissolved in 0.1 M sodium citrate buffer (pH 4.5). The control group of animals was injected with the STZ-free buffer. The animals that underwent STZ injections and developed hyperglycaemia (non-fasting blood glucose > 10 mM [36]) were then divided into 4 groups (n = 6) and administered, intraperitoneally, saline vehicle (once a day 'STZ' group), insulin (1 U/kg, 3 times per day; Sigma-Aldrich, Poole, UK, 'insulin' group), GABA (10 mg/kg, once a day; TCI, Oxford, UK, 'GABA' group) or nicotinamide (150 mg/kg, once a day; Sigma-Aldrich, Poole, UK, 'nicotinamide' group) for 10 successive days (Fig. 1A). The doses above were selected on the basis of non-ameliorating the diabetic phenotype induced by multiple low-dose STZ (e.g. blood glucose levels), in order to elucidate direct effects on islet cell plasticity. This manoeuvre however cannot rule out the secondary effects mediated by stem cells, duct cells, immune cells, nervous system, insulin sensitive cells that might be affected by the STZ treatment. Food and fluid intake, blood glucose and body weight were assessed at regular intervals. Non-fasting plasma insulin and glucagon were determined at the termination of the study.

Table 1
Primers used to confirm $Glu^{CreERT2}$;ROSA26-eYFP mouse genotype.

Primers	Sequence
β-catenin (Housekeeping control, 220BP)	Forward: AAGGTAGAGTGATGAAAGTTGTT Reverse: CACCATGTCCTCTGTCTATTC
iCre002/003 fragment (Cre lines, 537BP)	Forward: GACAGGCAGGCCTTCTCTGAA Reverse: CTTCTCCACACCAGCTGTGGA
GLUCre-ERT2 (759BP)	Forward: CCACCTTCTAGAATGTGCCTG Reverse: CATCTGCATGCAAAGCAATATAGC
EYFP (442BP)	Forward: GACGTAAACGGCCACAAGTT Reverse: GGATCTTGAAGTTCGCCTTG

2.3. Blood glucose and hormone measurements

Blood samples were collected from the tail vein of the mice into ice-chilled heparin-coated microcentrifuge tubes. Blood glucose was measured using a portable Ascencia meter (Bayer Healthcare, Newbury, Berkshire, UK). For plasma insulin and glucagon measurements, blood was collected in fluoride/heparin-coated tubes (Sarstedt, Numbrecht, Germany), centrifuged for 10 min at 2,200*g* after which the plasma was removed and stored at −20 °C. For hormone quantification in tissues, samples underwent acid–ethanol extraction (HCl: 1.5% v/v, ethanol: 75% v/v, H_2O: 23.5% v/v). Insulin concentrations were assessed by an in-house radioimmunoassay [37]. Plasma glucagon and pancreatic glucagon content were measured using glucagon ELISA (EZGLU-30K, Merck Millipore), or RIA kit (250-tubes GL-32K, Millipore, USA), respectively.

2.4. Immunohistochemistry and imaging

Pancreatic tissue samples were cut longitudinally, fixed with 4% PFA for 48 hr at 4 °C, embedded and processed for antibody staining as described [34]. Tissue sections (7 μm) were blocked with 2% BSA, incubated with respective primary antibodies overnight at 4 °C, and, subsequently, with appropriate secondary antibodies (Table 2). For nuclear staining, a final incubation was carried out with 300 nM DAPI (Sigma-Aldrich, D9542), at 37 °C. To assess cell proliferation and/or apoptosis, co-staining of mouse anti-insulin or guinea pig anti-glucagon with rabbit anti-Ki-67 or TUNEL reaction mixture was used (Table 2). YFP, a marker of the α-cell lineage, was detected with a rabbit anti-GFP antibody (1:1000; Abcam, ab6556) (Table 2), which is reactive against all variants of *Aequorea Victoria* GFP, including YFP. Slides were imaged on an Olympus BX51 microscope, equipped with a 40x/1.3 objective. We included all the islets visible on the slide in the morphometry analysis (typically 70–100 per treatment group, as indicated in the figure legends), with at least 50 cells analysed within each islet cross-section in the per-cell studies (Fig. 2B, Fig. 3, Fig. 4). The multi-channel fluorescence was recorded using DAPI (excitation 350 nm/emission 440 nm), FITC (488/515) and TRITC (594/610) filters and a DP70 camera controlled by $Cell^F$ software (Olympus, UK). Images were analysed using ImageJ software. All counts were determined in a blinded manner.

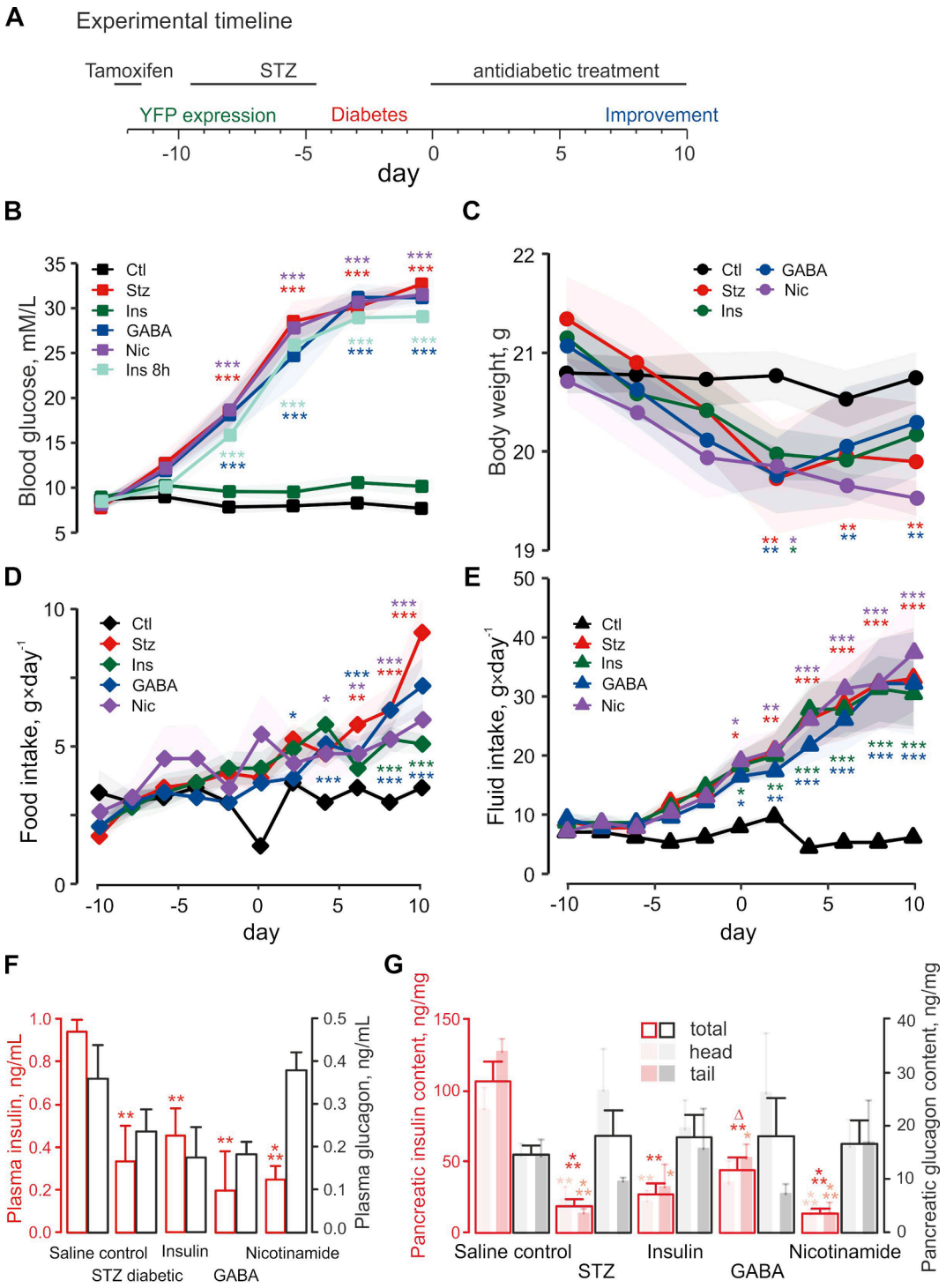

Fig. 1. Insulin, GABA and nicotinamide partially rescue the diabetic phenotype of the STZ-treated mice. *A:* Experimental timeline. Antidiabetic treatment starts on day 0. Tamoxifen was employed to induce the tissue-specific expression of YFP in α-cells. Low multiple-dose STZ (50 mg/kg body weight daily, i.p.) is administered for five days to induce insulin-deficient diabetes. The ability of 10-days treatment with GABA (10 mg/kg once daily), nicotinamide (150 mg/kg once daily) or insulin (1U/kg three times daily) to improve the diabetic phenotype is then determined. *B, C, D, E:* Fasting blood glucose (*B*), body weight (*C*), food (*D*) and fluid (*E*) intake of $Glu^{CreERT2}$;ROSA26-eYFP STZ-diabetic transgenic mice (n = 6), following administration of antidiabetic drugs, as indicated. 'STZ', streptozotocin; 'Ins', insulin; 'GABA', γ-amino-butyric acid; 'Nic', nicotinamide; 'Ctl', saline control; 'Ins 8 h', insulin for 8 h. *F:* plasma insulin (red) and glucagon (black) (n = 6). *G:* pancreatic insulin (*red*) and glucagon (*black*) content (n = 6). *F, G* measurements were taken on day 10, in different groups of mice, as indicated. *p < 0.05, **p < 0.01 and ***p < 0.001 compared to saline control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Primary and secondary antibodies used for immunohistochemistry.

Primary antibody	Dilution	Source
Mouse anti-insulin	1:1000	Abcam, Cambridge, UK: ab6995;
Guinea pig anti-glucagon	1:200	Raised in-house: PCA2/4
Rabbit anti-GFP	1:1000	Abcam, ab6556
Rabbit anti-Ki-67	1:200	Abcam, ab15580
TUNEL enzyme	1:10	Roche Diagnostics Ltd, UK
Secondary antibody	Dilution	Source
Goat anti-mouse	1:400	Alexa Fluor 488, Invitrogen, UK
Goat anti-mouse	1:400	Alexa Fluor 594, Invitrogen, UK
Goat anti-guinea pig	1:400	Alexa Fluor 488, Invitrogen, UK
Goat anti-guinea pig	1:400	Alexa Fluor 594, Invitrogen, UK
Goat anti-rabbit	1:400	Alexa Fluor 488, Invitrogen, UK
Donkey anti-rabbit	1:500	Alexa Fluor 594, Invitrogen, UK

2.5. Data analysis and statistics

Statistical analysis was performed using PRISM 5.0 (GraphPad, USA) or R [38]. Values are expressed as mean ± SEM, the sample size for each of the experiments is specified in the respective figure legends. Comparative analysis between experimental groups were carried out using independent-samples Student's *t*-test or (for > 2 samples) a one-way ANOVA with Bonferroni's post-hoc. The difference between groups was considered significant for $p < 0.05$. For correlative and cluster analysis, the data representing each individual treatment was expressed as a fold of respective STZ group, to account for the variability of the diabetic phenotype.

3. Results

3.1. STZ-induced diabetic phenotype was partially rescued by insulin but not GABA or nicotinamide therapy

Multiple low dose STZ administration in $Glu^{CreERT2}$; ROSA26-eYFP mice resulted in a significant increase in food (9.1 ± 1.2 g × day^{-1}) and fluid (33.1 ± 8.6 g × day^{-1}) intake when compared to control mice (3.5 ± 0.1 g × day^{-1} and 5.1 ± 0.4 g × day^{-1} respectively, $p < 0.05$) (Fig. 1D,E), an effect that was unaltered by either insulin, GABA or nicotinamide treatment. As expected, STZ administration also reduced body weight (19.8 ± 0.3 vs 20.7 ± 0.6 g in the control group, $p < 0.05$), which was not rescued by any of the experimental treatment regimens over the 10-day observation period (Fig. 1C). Severe STZ-induced hyperglycaemia ($p < 0.001$) was unaltered by GABA or nicotinamide (Fig. 1B), but insulin normalised blood glucose levels (Fig. 1B). However, it should be noted that this parameter was routinely monitored 1 h post injection; when determined 8 h post-injection ('Ins 8 h' data series in Fig. 1B) any beneficial glucose-lowering actions were absent. Circulating (Fig. 1F) and pancreatic (Fig. 1G) insulin concentrations were significantly ($p < 0.01$ to $p < 0.001$) reduced in all STZ-treated animals. The administration of GABA, however, resulted in a significant increase ($p < 0.05$) of insulin content on the STZ background (Fig. 1G). At the same time, neither plasma (Fig. 1F), nor pancreatic (Fig. 1G), glucagon

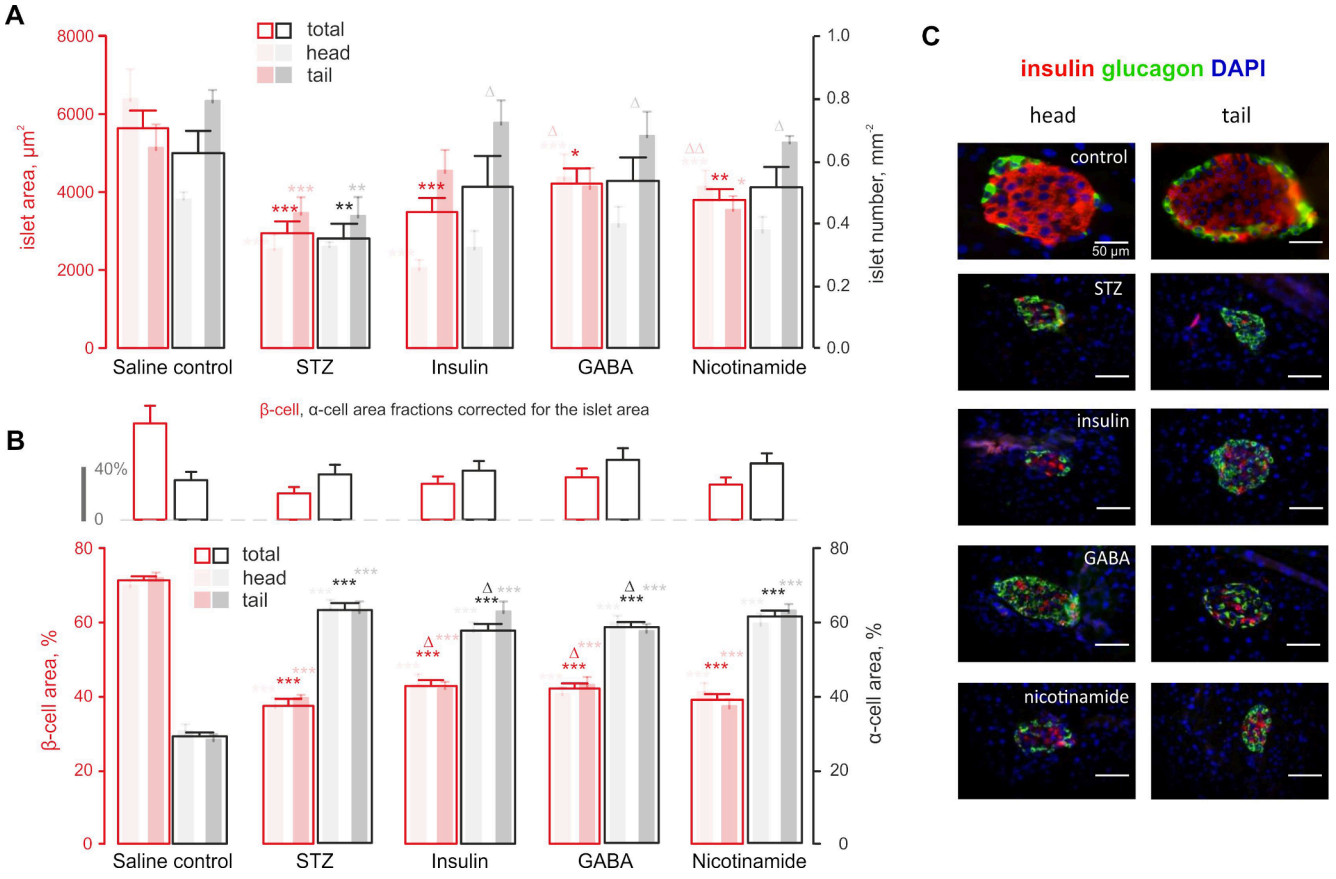

Fig. 2. STZ-induced diabetic phenotype is associated with changes in the islet size and composition. *A:* Islet area (shades of red, n = 150 islets from 6 mice) and number (shades of black, n = 150 islets from 6 mice) in response to the administration of STZ to $Glu^{CreERT2}$;ROSA26-eYFP transgenic mice and subsequent treatment with antidiabetic drugs, as indicated. *B:* (bottom): β- (red, n = 150 islets from 6 mice) and α-cell (black, n = 150 islets from 6 mice) percentage among the islet cells (*B*). Shaded bars in *A, B* represent the origin of the islets within the pancreatic tissue, head or tail, as indicated. (top): the area of β- and α-cells corrected for the change of the total islet area. *C:* Representative immunostaining of mouse pancreatic sections for DAPI (blue), glucagon (green) and insulin (red). **$p < 0.01$ and ***$p < 0.001$ compared to saline control group. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$ compared to streptozotocin treatment group. Scale bars: 50 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

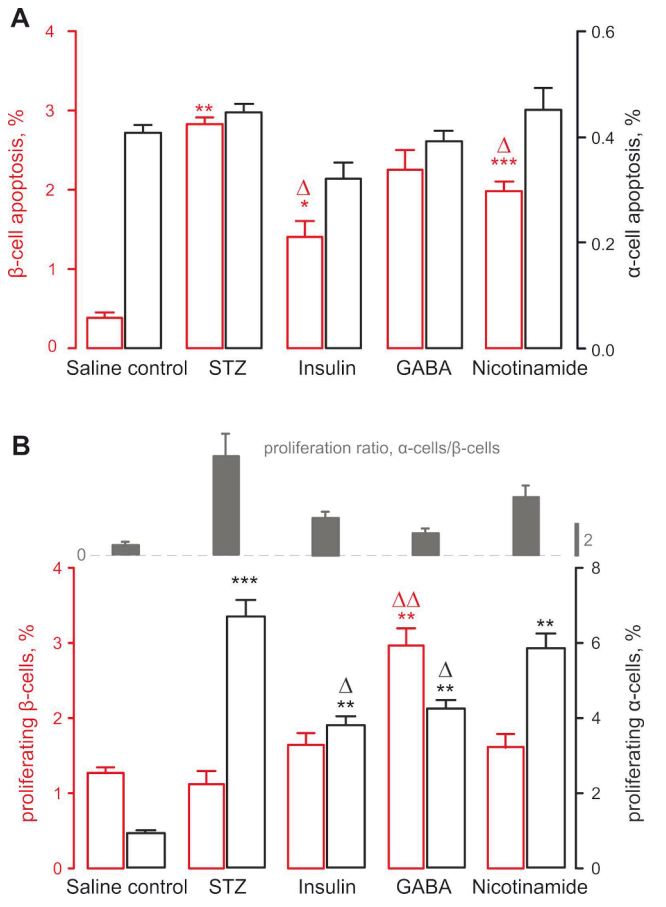

Fig. 3. Antidiabetic drugs decrease β-cell apoptosis and decrease proliferation of α-cells. Percentage of β-cells (red, n = 60 islets from 6 mice) and α-cells (black, n = 60 islets from 6 mice) undergoing apoptosis (*A*), as determined by TUNEL staining, or proliferation (*B*), Ki-67 staining, in response to the administration of STZ to $Glu^{CreERT2}$;ROSA26-eYFP mice and subsequent treatment with antidiabetic drugs, as indicated. Grey bars in *B* represent the ratio of the fractions of proliferating α- and β-cells. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to saline control group. $^{\Delta}p < 0.05$ and $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared to STZ-treatment group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

were significantly affected by either STZ or any of the treatments.

3.2. The drugs had a mild effect on the islet composition and morphology

Altered STZ-induced pancreatic hormone content was associated with a significant ($p < 0.01$ to $p < 0.001$) decrease in islet area (Fig. 2A) and average islet number (Fig. 2A), both in the head and the tail of the pancreas ($p < 0.05$). Islet area was increased by all treatment interventions (Fig. 2A), but this parameter was still significantly ($p < 0.05$ to $p < 0.001$) reduced when compared to the control group of mice. GABA, insulin and nicotinamide did restore islet numbers towards lean control levels, with this effect particularly evident in tail of the pancreas (Fig. 2A). In keeping with reduced overall islet area, STZ treatment also induced a significant ($p < 0.001$) decrease in relative β-cell area (Fig. 2B, C), with a respective increase in relative α-cell area (Fig. 2B,C). The latter, however, did not reflect any significant α-cell expansion and was fully due to the depletion of β-cells (Fig. 2B, inset). Although none of experimental treatments were able to fully counter the negative impact of STZ on α- and β-cell areas, insulin and GABA did partially ($p < 0.05$) reverse this effect (Fig. 2B,C).

3.3. Insulin and nicotinamide attenuate β-cell apoptosis while GABA enhances the proliferation of β-cells

We observed a 7-fold (2.9 ± 0.1 vs 0.4 ± 0.1% in control mice, $p < 0.05$) increase in the percentage of apoptotic β-cells in STZ-treated mice (Fig. 3A). Insulin and GABA, but not nicotinamide, induced a significant ($p < 0.05$) partial rescue of β-cell apoptosis (Fig. 3A). α-Cell apoptotic rate was unaffected by STZ or any of the treatment regimens (Fig. 3A). STZ treatment also induced a 7-fold (6.7 ± 0.5 vs 0.9 ± 0.1% in control mice, $p < 0.05$) increase in the proliferation of α-, but not, β-cells (Fig. 3B). Interestingly, GABA doubled ($p < 0.01$) the percentage of proliferating β-cells when compared to STZ-diabetic mice (Fig. 3B). In addition, treatment with insulin or GABA, but not nicotinamide, significantly ($p < 0.05$) decreased α-cell proliferation (Fig. 3B).

3.4. Insulin and GABA enhance α-/β-cell transdifferentiation

The expression of YFP in $Glu^{CreERT2}$; ROSA26-eYFP transgenic mice, induced tamoxifen, was limited to α-cells. We were unable to detect any significant change in the number of YFP+ cells in any of the experimental groups. A small percentage of YFP+ cells that did not express glucagon (0.3 ± 0.1%) was apparent in the control group through co-detection with anti-glucagon and YFP-antibodies, which was increased 4-fold by the STZ treatment (1.3 ± 0.4%) (Fig. 4A). In the head of the pancreas, this transition was further potentiated ($p < 0.05$) by GABA, but not insulin or nicotinamide (Fig. 4A). However, expression of YFP+/glucagon- cells was not altered by GABA when considering the pancreas as a whole (Fig. 4A). Interestingly, the percentage of the YFP + insulin + cells was tripled in STZ-diabetic mice (0.8 ± 0.1% vs. 2.3 ± 0.2%) when compared to control mice (Fig. 4B), which was further increased by the treatment with insulin (3.9 ± 0.2%; $p < 0.05$) or GABA (5.1 ± 0.1%; $p < 0.01$) (Fig. 4B). Surprisingly, nicotinamide had no statistically significant effect on the commitment of the α-cells towards a β-cell lineage (Fig. 4B). The islet cell transdifferentiation process was reflected by an increased ($p < 0.05$) percentage of insulin/glucagon positive bihormonal cells in insulin or GABA treated STZ mice (Fig. 4B). Of note this islet cell population was largely unaffected by either the STZ treatment alone or subsequent administration of nicotinamide (Fig. 4B).

4. Discussion

We report the antiproliferative effect of the two key β-cell signals, insulin and GABA, on neighbouring α-cells in STZ-diabetic mice. In our hands, the exogenous addition of both factors on a background of severe β-cell stress, drove α-cell transdifferentiation towards the β-cell lineage to ultimately augment β-cell mass, an effect that could not be recapitulated by a dedicated factor involved in the differentiation of stem cells into β-cells, namely nicotinamide [28,29].

4.1. Diabetic phenotype of the mice and islet morphology

As expected, multiple low-dose STZ injection induced a severe diabetic phenotype including increased fluid intake, overt hyperglycaemia alongside decreased body weight and increased energy intake [39,40]. The diabetic phenotype was consistent and progressive throughout, with the three treatment agents, insulin, GABA and nicotinamide employed at relatively low doses, failing to substantially alter this. As expected, the predicted antihyperglycaemic effect of insulin [41], was observed shortly after injection but was not sustained, as can be verified by the lack of overall effect on body weight and intake of fluid and food, as well as plasma insulin assayed at the end of the experimental course. Notably, neither circulating nor pancreatic glucagon were affected by STZ-induced expansion of the α-cell population, and this likely partly reflects the large size of the 'resting' α-cell pool [42,43]. STZ treatment decreased islet size, number and β-cell/α-cell ratio [34], although decreased number of islets could be attributed to the limits of

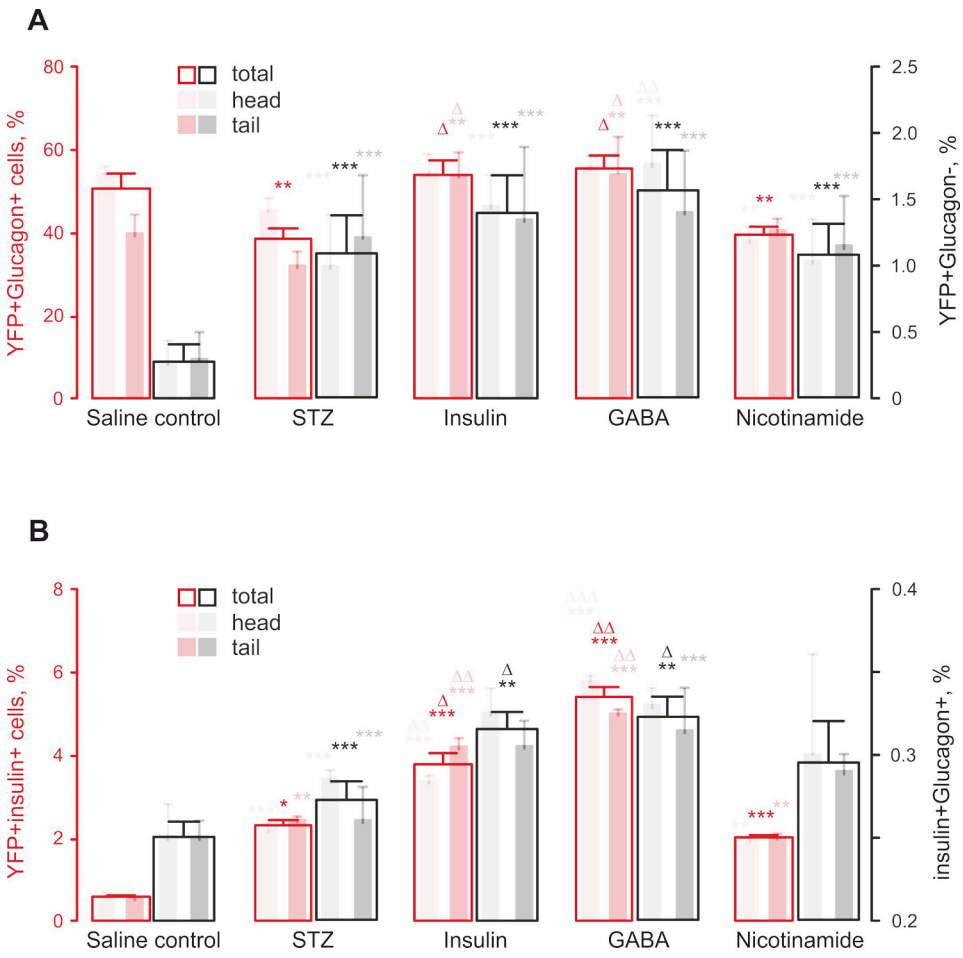

Fig. 4. GABA and insulin, but not nicotinamide, promote α-cell transdifferentiation into β-cells. Percentage of the YFP-expressing cells detected within α- (*A*, red, n = 60 islets from 60 mice) and non-α-cells (*A*, black, n = 60 islets from 60 mice) as well as insulin positive β-cells (*B*, red, n = 60 islets from 60 mice) after administration of STZ and subsequent treatment with antidiabetic drugs, as indicated, visualised at the termination of the study (Day 10). Percentage of the double-positive (insulin + glucagon+) cells detected at the same time-point (*B*, black, n = 60 islets from 60 mice). *p < 0.05, **p < 0.01 and ***p < 0.001 compared to saline control group. $^{\Delta}$p < 0.05 and $^{\Delta\Delta}$p < 0.01 compared to streptozotocin treated group. Scale bars: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

microscopic detection of these organoids. Whilst attenuation of STZ mediated reductions of islet β-cell fraction was predictable for each treatment intervention [4,14,15,25], the ability of nicotinamide to counteract STZ-induced diminution of islet size was mediated by increases in both α- and β-cells, or may also have stemmed from a non-islet cell source, such as duct cells [44,45], which was not investigated in the current study.

4.2. Effects of insulin, GABA and nicotinamide on apoptosis and proliferation of islet cells

Unsurprisingly, STZ dramatically enhanced β-cell apoptosis, but was without any substantial effect on the proliferation of these cells [34], in contrast to the earlier reports on increased β-cell proliferation in response to high doses of STZ [46]. In our hands, the relative increase in β-cell mass mediated by insulin treatment relied on the reduction of β-cell apoptosis, a finding which is both supported [47] and contested [48] by previous reports. However, recurrent short-lived post injection reductions of hyperglycaemia may also play some role in the β-cell antiapoptotic effects of insulin. Furthermore, GABA increased the proliferation of β-cells, in line with earlier reports [21,49,50]. The anti-apoptotic effect of nicotinamide [51] is mediated by inhibition of poly (ADP-ribose) polymerases (PARP) [52] or activation of a glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [53]; given the low dose of the drug used (150 mg/kg once daily), the former mechanism, sensitive to the concentrations of nicotinamide above 10 mM, is less likely to be involved. As the attenuation of apoptosis has been reported to correlate with the promotion of pancreatic differentiation by nicotinamide [54] it can arguably be used as a functional marker of intact downstream β-cell signalling in this mouse model. Of note, nicotinamide has been historically viewed as a pharmacological tool to counteract [55] or alleviate [56] the global type 1 diabetic phenotype in rodent but not in human [57] models.

4.3. Expression of insulin by YFP^{+} α-cells

STZ induced the expression of insulin (from 0.8 ± 0.1% to 2.3 ± 0.2%) and a loss of glucagon (from 0.3 ± 0.1% to 1.3 ± 0.4%) in cells bearing the YFP label, unequivocally highlighting a lineage shift of the original α-cell population in Glu^{CreERT2}; ROSA26e-YFP mice. STZ has been reported to alter the β-cell genotype [58] and hence can theoretically divert the Cre-based YFP expression pattern towards β-cells, however we believe the chance for that is negligible since tamoxifen induction of the YFP reporter occurred three days prior to STZ administration.

4.3.1. Expansion of the Insulin^{+}YFP^{+} fraction

Insulin and GABA, but not nicotinamide, further expanded the insulin + YFP+ cell fraction [3,14], although this effect could be considered somewhat counterintuitive. The exogeneic restoration of insulin and GABA from the state of their STZ-induced deprivation exacerbated the tendency for α-cell transdifferentiation towards mature insulin-expressing β-cells. At the same time, both signals could be viewed as perfect candidates for β-cell-derived safeguarding against this process. This finding leaves the driver of α-cell expansion upon the STZ treatment (Fig. 2B) unresolved, but regardless the presence of bi-hormonal islet cells and associated transdifferentiation events, it confirms that α-cells act as a pool for β-cell expansion, alongside other potential sources, such

as duct cells [59]. The lack of the effect of nicotinamide on α/β-cell lineage transition is likely to argue against the involvement of early common progenitors in this process, prompting for a further investigation of the role of this pool.

4.3.2. *Potential role of plasma membrane excitability*

Although the mechanisms discussed [52,53] suggest NAD as an intermediate for the nicotinamide effect, this variant of vitamin B_3, may directly affect K^+ conductance in islet cells. Whereas the molecular determinants [60] of the interaction between nicotinamide and K_{ATP} channel are unclear, the former has upregulated the basal activity of the latter in different tissues [61,62]. Opening of this ligand-gated ion channel [63] would inhibit β-cell activity [64], whereas, in α-cells, the modality of the effect would depend on the strength of the K^+ conductance agonism [65].

Insulin [66] and GABA [67,68] receptors have been reported on α-and β-cells. In line with its reputation of inhibitory neurotransmitter, GABA acutely attenuates the electrical activity of plasma membrane in α-cells [69], whereas in β-cells, an opposite effect was reported [70]. The difference, stemming, presumably, from anomalously high cytosolic levels of Cl^- in β-cells [71], may hint for a differential chronic effect on the two islet cells subpopulations, but not for a mechanism linking the membrane hyperpolarisation to transdifferentiation. Thus, speculatively, the opposite effect of long-term administration of nicotinamide and GABA on α-/β-cell transdifferentiation could reflect the opposite effect of the two compounds on the membrane excitability of the two islet cell types.

4.4. *Antidiabetic drugs targeting α-cell transdifferentiation: A meta-analysis*

Having previously reported on a set experimental results dissecting the effects of the existing and potential antidiabetic medications on the plasticity of pancreatic islet alpha-cells using $Glu^{CreERT2}$; ROSA26e-YFP

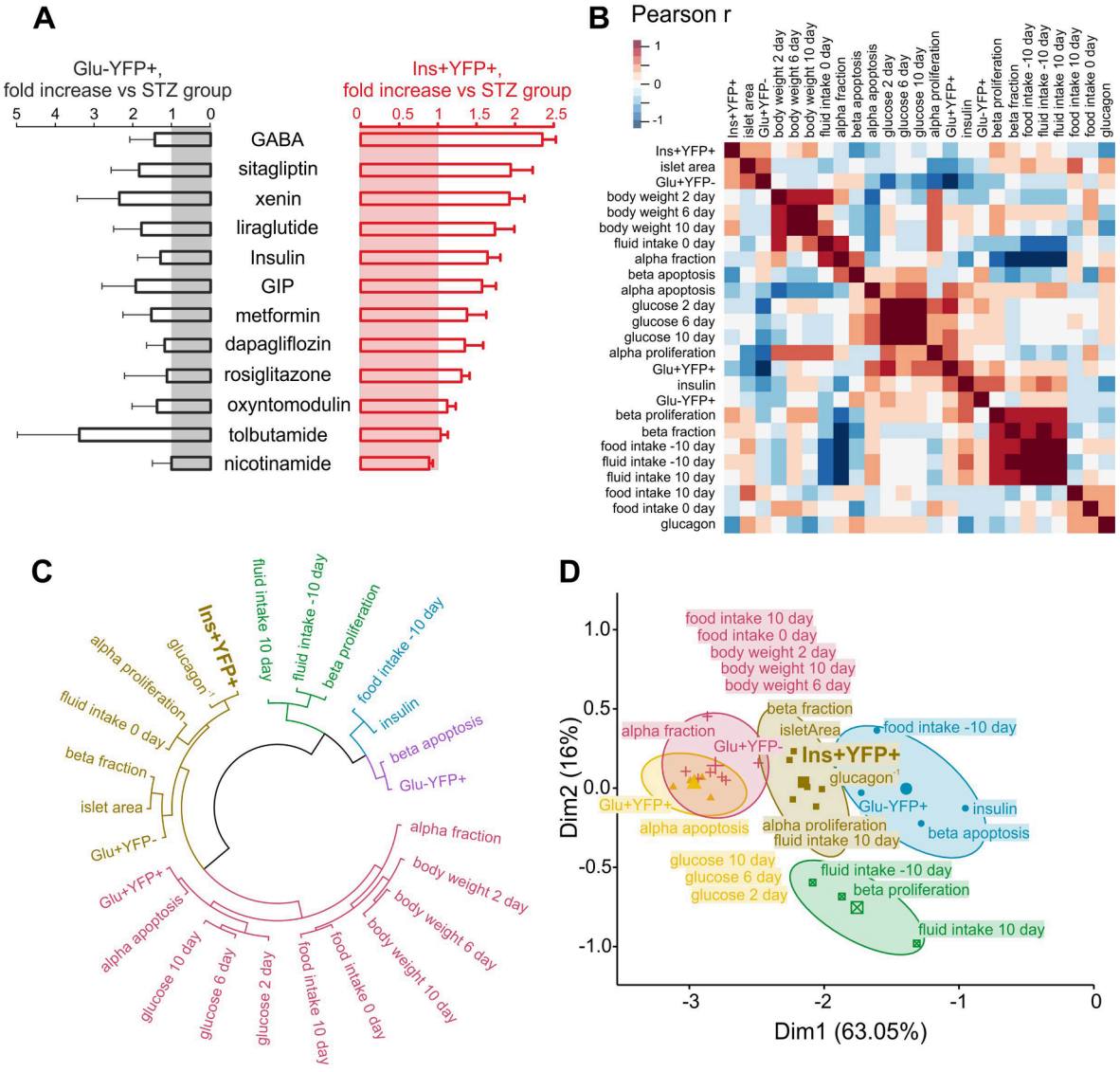

Fig. 5. Meta-analysis of the effects of insulin, nicotinamide, GABA (current study), liraglutide, sitagliptin, dapagliflozin [72], metformin, rosiglitazone, tolbutamide [40], GLP-1, GIP and xenin [39] on multiple characteristics in normal and STZ-diabetic $Glu^{CreERT2}$;ROSA26-eYFP transgenic mice. *A*: Fold increase in the percentage of YFP-expressing cells that do not express glucagon (black) or express insulin (red). *B*: Pearson correlations between various readouts reflecting mouse phenotype (body weight, blood glucose, fluid and food intake at three different time points during the experiment, plasma insulin and glucagon on the terminal day of the study) and islet cell development (percentages of YFP-expressing cells that express/not express insulin or glucagon as well as percentage of α-cells that do not express YFP) and biology (β- and α-cell fractions, islet size, proliferation and apoptosis of α- and β-cells). *C, D:* Hierarchical (*C*) and k-means (*D*) clustering of the animal and islet characteristics (as in *B*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mice [39,40,72], we performed a *meta*-analysis of the mean data from these works, supplemented with the respective outcomes from the current study (Fig. 5). Whilst data emanating from the control animals in each study associated closely, STZ treatment groups exhibited higher variability (data not shown), which suggested us to normalise the data from each of the treatments to the respective readout from the STZ group from the same study.

Every treatment we tested resulted in a statistically significant increase of the Ins + YFP+ pool, reflecting the transdifferentiation of α-cells into β-cells, on a background of the STZ treatment mice (Fig. 5A). Importantly, insulin and especially GABA signalling represented comparatively strong stimuli. Across the treatments, the size of the Ins + YFP+ fraction was statistically associated with several cellular or global characteristics, of which the strongest was the negative correlation with plasma glucagon levels ($r = -0.81$, $p < 0.01$, Fig. 5B). This observation was further confirmed by the hierarchical (Fig. 5C) and k-means clustering (Fig. 5D). Both characteristics associated closely with α-cell proliferation (Fig. 5C,D), whereas clustering with fluid intake on day 0 (the starting day for the antidiabetic treatment in mice with STZ-induced diabetes) may account for the variations in the diabetic phenotype. The two notable groups of statistically associative treatments were (i) the 'gut peptide' group including the GLP-1 agonist liraglutide, GIP, xenin, DPP-4 inhibitor sitagliptin and tolbutamide (the latter, due to the similarity of effects on β-cell apoptosis) as well as (ii) insulin and GABA from the present study. Notably, although the perceived beneficial effects of each agent on pancreatic islet architecture are likely mediated via contrasting mechanisms either direct or indirect, glycaemic status was generally not a confounding factor, which is important given that alterations in glucose levels can impact beta-cell identity [26].

4.5. Conclusions

Professional 'fast' signalling molecules from β-cells, insulin and GABA display variable effects on α-cells, depending on the route (endogenous from β-cells/exogenous) and the modality (acute/chronic) of their administration. In the current setting, exogenous GABA or insulin administration clearly augmented α-cell transdifferentiation towards a more β-cell phenotype in insulin-deficient STZ-diabetic mice, thereby advocating the use of the two compounds, as well as the insulinotropic agents, such as GLP-1 agonists, at earlier stages of diabetes. Whereas the specificity and the expression pattern of the respective receptors has been well characterised, the intracellular mechanisms linking these signalling pathways to islet cell transdifferentiation are yet to be established. Apart from potentially exploitable characteristics for diabetes therapy, we need to stress that the disruption of these signalling mechanisms (for instance, by STZ) may play a hitherto unrecognised role in the functional demise of islets in diabetes.

CRediT authorship contribution statement

Dipak Sarnobat: Investigation, Formal analysis, Data curation. **R. Charlotte Moffett:** Conceptualization, Methodology. **Peter R. Flatt:** Conceptualization, Supervision, Writing – original draft. **Nigel Irwin:** Supervision, Writing – original draft. **Andrei I. Tarasov:** Formal analysis, Data curation, Visualization, Writing – original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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