

## ORIGINAL ARTICLE

# Sitagliptin and Roux-en-Y gastric bypass modulate insulin secretion via regulation of intra-islet PYY

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**Aims:** The gut hormone peptide tyrosine tyrosine (PYY) is critical for maintaining islet integrity and restoring islet function following Roux-en-Y gastric bypass (RYGB). The expression of PYY and its receptors (NPYRs) in islets has been documented but not fully characterized. Modulation of islet PYY by the proteolytic enzyme dipeptidyl peptidase IV (DPP-IV) has not been investigated and the impact of DPP-IV inhibition on islet PYY function remains unexplored. Here we have addressed these gaps and their effects on glucose-stimulated insulin secretion (GSIS). We have also investigated changes in pancreatic PYY in diabetes and following RYGB.

**Methods:** Immunohistochemistry and gene expression analysis were used to assess PYY, NPYRs and DPP-IV expression in rodent and human islets. DPP-IV activity inhibition was achieved by sitagliptin. Secretion studies were used to test PYY and the effects of sitagliptin on insulin release, and the involvement of GLP-1. Radioimmunoassays were used to measure hormone content in islets.

**Results:** PYY and DPP-IV localized in different cell types in islets while NPYR expression was confined to the beta-cells. Chronic PYY application enhanced GSIS in rodent and diabetic human islets. DPP-IV inhibition by sitagliptin potentiated GSIS; this was mediated by locally-produced PYY, and not GLP-1. Pancreatic PYY was markedly reduced in diabetes. RYGB strongly increased islet PYY content, but did not lead to full restoration of pancreatic GLP-1 levels.

**Conclusion:** Local regulation of pancreatic PYY, rather than GLP-1, by DPP-IV inhibition or RYGB can directly modulate the insulin secretory response to glucose, indicating a novel role of pancreatic PYY in diabetes and weight-loss surgery.

## KEYWORDS

dipeptidyl peptidase IV, glucagon-like peptide-1, insulin, islets, peptide tyrosine tyrosine, Roux-En-Y gastric bypass

## 1 | INTRODUCTION

Peptide tyrosine tyrosine (PYY) is an appetite-regulating hormone that has been shown to play a critical role in the restoration of

impaired islet secretory function in diabetic rats and following Roux-En-Y gastric bypass (RYGB).<sup>1</sup> The physiological relevance of PYY in pancreatic islets has been demonstrated by dramatic disruption of islet structure and beta-cell function in mice following selective ablation of PYY-expressing islet cells and by restoration of beta-cell function by exogenous application of PYY in streptozotocin-treated

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mice.<sup>2,3</sup> Similarly, overexpression of PYY in mouse pancreatic beta-cells results in improved glucose tolerance.<sup>4</sup> Marked elevations in plasma PYY levels have been reported following RYGB in both humans and rats.<sup>1</sup> Moreover, PYY pre-treatment of islets isolated from severely diabetic GK rats has been shown to restore impaired insulin and glucagon responses to glucose.<sup>1</sup> Collectively, these findings demonstrate that PYY may play an important function in islet physiology, extending beyond its classical role as an appetite regulator.<sup>5</sup> PYY expression has been documented previously in islets from rodents and other mammals,<sup>3,6-8</sup> but its expression in human islets has not been explored. Recent reports suggest that acute administration of PYY reduces insulin secretion in isolated mouse islets and human/rodent beta cell lines.<sup>3</sup> On the other hand, insulinotropic effects of PYY have been reported in 2 transgenic mouse models as well as in mouse and human islets.<sup>1,2,4</sup> PYY signals through a cluster of receptors belonging to the neuropeptide Y (NPY) family, of which there are 4 subtypes; NPY1R, NPY2R, NPY4R and NPY5R. However, PYY receptors in islets have been studied only at the transcript level,<sup>3,9</sup> and whether or not they are actually present remains unknown. The proteolytic enzyme di-peptidyl peptidase IV (DPP-IV) is an abundantly occurring serine protease. The efficacy of DPP-IV inhibition therapy in diabetes is thought to result from the prolongation of active glucagon-like peptide-1 (GLP-1).<sup>10,11</sup> However, DPP-IV also degrades a number of endogenous peptides including PYY.<sup>12,13</sup> As DPP-IV is expressed both in human and mouse islets,<sup>14-16</sup> DPP-IV inhibition therapy could impact on islet PYY function. We have compared gene and protein expression of PYY and its receptors and DPP-IV in mouse and human islets and investigated potential roles of DPP-IV inhibition in pancreatic islet function. To further explore the role of PYY in islet physiology, we have also investigated changes in islet PYY content in diabetes and upon RYGB.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

Female NMRI mice (Charles River Laboratories, Harlow, UK), aged 12 to 16 weeks, were used for functional and histological islet studies. Adult male 16 to 20 week-old Wistar rats were used as normoglycaemic controls and age- and sex-matched diabetic GK rats (Taconic, Laven, Denmark) were used as a model of type 2 diabetes. All animal experiments were conducted in accordance with the UK Animals Scientific Procedures Act (1986).

### 2.2 | Human tissues

Human adipose tissue RNA for positive controls for gene expression was available through existing collaborations within the Oxford University MoIPAGE consortia. Adult human pancreas samples for histology and islet samples for secretion studies were obtained with local and regional ethical approval and clinical consent. Donor details are provided online in Table S1, Supporting Information.

### 2.3 | Islet isolation

Mouse and Wistar rat islets were isolated by collagenase type V (Sigma-Aldrich Ltd, Gillingham, UK) digestion as described previously.<sup>17</sup> For optimal yield, GK rat islets were isolated by liberase as before.<sup>1</sup> A typical islet yield per mouse, Wistar rat and GK rat was 250, 400 and 200, respectively. Islets from GLP-1 receptor knockout mice and wildtype controls were obtained from the University of Lausanne (Switzerland). Human islets were isolated in the Diabetes Research and Wellness Foundation Human Islet Isolation Facility by collagenase digestion (Serva, Heidelberg, Germany), using modified versions of published protocols.<sup>18</sup>

### 2.4 | Secretion studies

Islets from the dorsal part of the pancreas were hand-picked and size-matched on the basis of conformation.<sup>19</sup> Irregular-shaped and very large islets from the ventral area of the pancreas were excluded. Experiments were performed with 10 to 13 islets per tube, in triplicate. Islets were pre-incubated in Krebs-Ringer buffer (KRB) containing 2 mg/mL BSA and 1 mmol/L glucose for 1 hour at 37°C, followed by 1-hour test incubation in KRB supplemented with glucose as indicated; 20 mM glucose was used for glucose-stimulated insulin secretion (GSIS) unless otherwise described. Insulin content of the supernatant was determined by radioimmunoassay (Millipore UK Ltd, Livingstone, UK) as described previously.<sup>20</sup> To allow comparison of experiments, some secretion data are presented as mean % basal, where basal is secretion at 1 mM glucose.

For the acute experiments, islets were treated with sitagliptin (Stratech Scientific Ltd, Newmarket, UK), or with PYY (1-36) or PYY (3-36) (Bachem, Bubendorf, Switzerland) for 1 hour during glucose stimulation as described above. For the chronic experiments, human or mouse islets were treated for 72 hours with sitagliptin (100 nM; Stratech Scientific Ltd) in RPMI culture media (10 mM glucose) in the presence or absence of exendin (9-39) (1 µM) or anti-PYY antibody (1/500) (ab22663, Abcam, Cambridge, UK) or PYY (100pM or 100 nM; Bachem) or the NPY1R blocker BIBP-3226 (1 µM) (Tocris, Bristol, UK). Secretion studies were conducted subsequently as described above.

### 2.5 | RNA extraction and gene expression

Total RNA was extracted from rat islets using the phenol-chloroform-guanidinium-thiocyanate method.<sup>21</sup> Briefly, 100 islets were homogenized in Tri Reagent (Life Technologies, Paisley, UK) before RNA isolation using chloroform and isopropanol precipitation. RNA quantity was determined spectrophotometrically using Nanodrop technology. Turbo DNase (Life Technologies) was used to remove any residual genomic contamination before 1 µg of RNA was reverse transcribed in a random primed single-strand synthesis reaction.

Gene expression analysis was performed using Taqman technology. cDNA was diluted 1:10 using 0.01 M Tris-HCl before amplification with inventoried gene specific assays (Table S2, Supporting Information) or assays against the housekeeping genes *Hprt1*, *B2m* or *Ppia* for human and rat studies, and *Gapdh*, *B2m*, *B-Actin* and *Gusb* for mouse studies. All samples were amplified in triplicate using gene

expression mastermix (Life Technologies) and were run in parallel with a standard curve to permit determination of assay efficiency. Amplification was performed using an ABI7900HT thermalcycler at 95°C – 10 minutes, 50 cycles (95°C – 15 seconds, 60°C – 1 minute). Gene expression levels were calculated using the  $2^{-\Delta\Delta CT}$  method modified by Pfaffl<sup>22</sup> and are presented in arbitrary units (au).

## 2.6 | Histological analysis

Pancreases or isolated islets from Wistar rats, GK rats and human subjects were fixed in 4% formalin, paraffin embedded and cut into 5- $\mu$ m sections. For immunofluorescence, sections were dewaxed and rehydrated as described above. All samples were subjected to antigen retrieval by heating in 0.01 M sodium citrate solution before immunolabelling. Sections were incubated overnight using antibodies targeting PYY (ab22663), insulin (in house), glucagon (G-2654, Sigma, Gillingham, UK), somatostatin (sc-25262, Santa Cruz, Insight Biotechnologies, Wembley, UK), PP (ab77192), NPY1R (ab91262, Abcam, Cambridge, UK), NPY2R (ab31894, Abcam), NPY4R (HPA027863, Sigma), NPY5R (ab32886, Abcam) or DPP-IV (ab28340, Abcam). The tyramide amplification system was used as a secondary antibody system to improve visualization of PYY in human samples, all NPYR proteins and DPP-IV (ThermoFisher, Loughborough, UK). All other proteins were visualized using fluorescently- and HRP-labelled secondary antibodies for immunolocalization as follows; anti-rabbit 488 (A11034, Life Technologies, Loughborough, UK), anti-mouse TRITC (F5262, Sigma), anti-guinea pig 648 (ab150187, Abcam), anti-rabbit HRP (P0448, Dako, Cheadle, UK). Images were visualized using a BioRad (Radiance 2100) confocal microscope.

## 2.7 | Electron microscopy

Samples for immuno electron microscopy were fixed in 2.5% glutaraldehyde, post-fixed in 2% uranyl acetate and embedded in London Resin Gold (Agar Scientific, Stansted, UK). Ultrathin sections were cut onto nickel grids for immunogold labelling. Anti PYY (Abcam) binding was visualized with Protein A Gold (10 nm) (British Biocell International, Cardiff, UK). Sections were contrasted with 2% uranyl acetate and lead citrate and viewed in a Joel 1010 electron microscope (Jeol, Tokyo, Japan) equipped with a digital camera (Gatan, Pleasanton, California).

## 2.8 | DPP-IV activity in islets

Islets were isolated from NMRI mice and disrupted by sonication in 10 mM Tris-HCl buffer. The islet homogenate was centrifuged at 204 000G for 75 minutes at 4°C. The pellet was solubilized in 10 mM Tris-HCl buffer containing 0.01% Triton X-100 and 1  $\mu$ g/mL aprotinin. DPP-IV activity was determined using the H-Gly-Pro-AMC (Bachem, I-1225) in the presence and absence of 5 nM sitagliptin. The islet sample was incubated with 10  $\mu$ M H-Gly-Pro-AMC for 120 minutes at 37°C. The amount of AMC liberated was compared with a 1  $\mu$ M AMC standard.

## 2.9 | Roux-en-Y gastric bypass model

Sham or RYGB surgery was performed on anesthetized rats as previously described.<sup>1</sup> Briefly, the intestine was transected 10 cm distal to the ligament of Treitz, creating a distal and a proximal end. The proximal end was anastomosed to the intestine and a gastric pouch was created (2%-3% of total stomach). The distal end of the intestine was anastomosed to the gastric pouch in an end-to-side fashion. For sham operation the animals were opened through a midline incision and the viscera were gently manipulated. Experiments were approved by the Norwegian national animal research authority (FDU).

## 2.10 | Measurements of PYY and GLP-1 in islets and in medium

Islets were lysed in acid ethanol and stored at -80°C until determination of hormones. Total PYY was measured in islets and in the medium used for culture by radioimmunoassay (Phoenix Pharmaceuticals, Burlingame, California) and total GLP-1 was assayed using the highly sensitive Total GLP-1 (v2) kit (K150JVC-1, Mesoscale Discovery, Rockville, Maryland), as described previously.<sup>1</sup> The secretion protocol did not include an inhibitor to prevent the degradation of GLP-1<sup>23</sup> and, therefore, samples were not optimized for measurement of intact GLP-1.

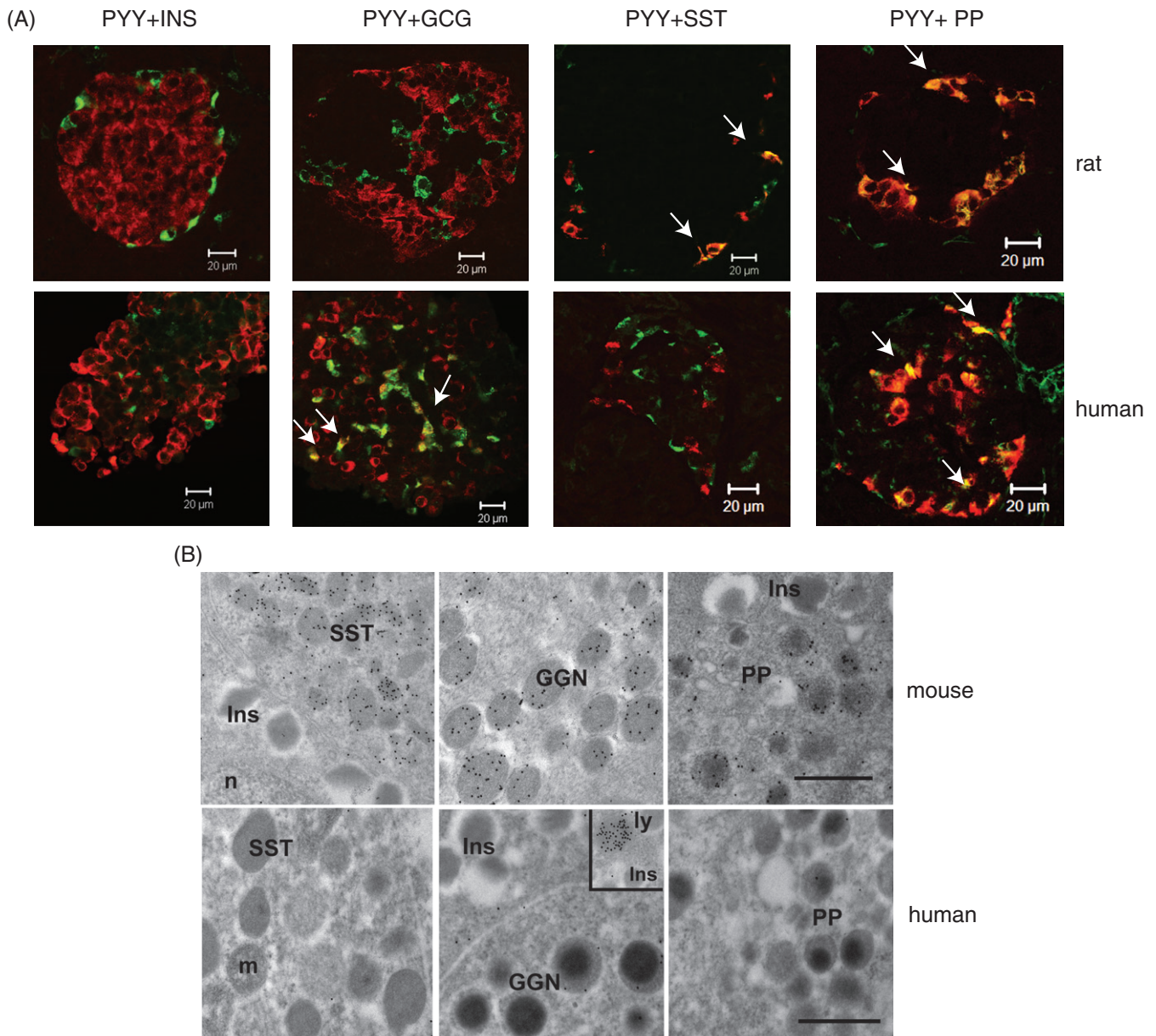
## 2.11 | Statistical analysis

Unpaired Student's *t* test, two-tailed and assuming unequal variance, was used to determine significance between diabetic and control animals, and between islets incubated with different treatments. For all statistical analyses, *P* < .05 was regarded as significant.

## 3 | RESULTS

### 3.1 | PYY is expressed in rodent and human islets

Using tissue from rats and healthy human subjects, we confirmed that PYY is detectable at both the mRNA (Figure S1A, Supporting Information) and protein level in islets (Figure 1A). To localize PYY to islet cells, sections of rat and human pancreas were labelled with antibodies against insulin, glucagon, somatostatin and PYY. Antibody efficiency was confirmed by detection of PYY-positive cells within rat ileum and human duodenum (Figure S1B, Supporting Information). In rat islets, no co-localization of PYY with either insulin or glucagon was observed, but co-localization with somatostatin and PP was seen in most specimens, suggesting that in rats PYY is located predominantly within delta and gamma cells (Figure 1A, upper panel). In contrast, a different pattern of expression was observed in human islets (Figure 1A, lower panel). No co-localization was observed with insulin or somatostatin. However, PYY immunoreactivity was co-localised with glucagon and PP. The specificity of the antibody was confirmed by blocking antibody binding by pre-incubation with exogenous PYY; no labelling was observed (Figure S1C, Supporting Information). No cross-reactivity was seen between the antibodies to PP and PYY; however, in rat samples most PP cells were immuno-positive for PYY. Additional analysis of PYY localization by electron microscopy (Figure 1B) showed that, in mouse islets, PYY (upper panel) is expressed in somatostatin and PP-positive



**FIGURE 1** PYY protein localization in rat, mouse and human islets. A, Representative confocal microscopy images from rat (upper panel) and human sections (lower panel) stained with PYY (green) and insulin, glucagon, somatostatin or PP. B, Immunogold labelling for PYY on mouse islets (upper panels) and human islets (lower panels). The density of gold labelling was much higher in mouse islets compared to human islets. In the mouse, labelling was present over glucagon granules (GGN, electron dense with no halo and an additional very dense core), somatostatin granules (SST, electron pale and slightly angular) and pancreatic polypeptide granules (PP, similar to glucagon but smaller and more variable in size) but not on insulin granules (Ins, dense core and clear halo). In insulin-containing  $\beta$ -cells primary and secondary lysosomes (Ly, inset in middle panel) were densely labelled with immunogold for PYY. In human islets no convincing labelling of any granule type was found. Lysosomes were identified by their pale electron density and different sizes, usually bounded by a clear membrane. M, mitochondrion; n, nucleus. Scale bars, 0.5  $\mu$ m

cells and a subpopulation of glucagon-positive cells. A lower amount of PYY was detected in human islets (lower panel), confirming the presence of PYY in alpha cells and a small percentage of PP cells. However, dense labelling for PYY was found in lysosome in human beta-cells.

### 3.2 | PYY receptors are expressed in rodent and human islets

*NPY1R* and *NPY4R* mRNA expression was detected in rat islets (Figure S2A, Supporting Information), which is consistent with

previous reports.<sup>5</sup> The presence of *NPY1R* immunoreactivity was identified in rat islets (Figure 2) and this receptor was localized exclusively to beta-cells. However, immunolabelling was not positive for *NPY4R* in islets, although the antibody labelled specific tracts of rat jejunum and subcutaneous adipose tissue (Figure S4A and B, Supporting Information). Neither *NPY2R* nor *NPY5R* mRNA was expressed in rat islets. These staining data show that *NPY1R* localizes to the beta-cells in rat islets.

In human islet samples we detected mRNA expression of *NPY1R*, *NPY4R* and *NPY5R* but not *NPY2R* (Figure S3, Supporting

Information), in agreement with previous reports.<sup>5</sup> Antibodies to both NPY1R and NPY4R labelled insulin-positive beta-cells in the human pancreas (Figure 3A and B). No co-localization of either NPY1R or NPY4R with glucagon or somatostatin was observed. NPY5R was not detected in human islets (data not shown), despite its presence at the mRNA level. Using human subcutaneous adipose tissue, we were able to confirm that antibodies to NPY1R, NPY4R and NPY5R showed positive immunolabelling in human tissues (Figure S4C, Supporting Information).

### 3.3 | Time and dose-dependent effects of PYY on islet secretory function

The time-dependent effects of PYY application on GSIS were examined in mouse islets. Incubation of islets for 1 hour or up to 24 hours with PYY (100 nM) did not affect GSIS. However, 72-hour pre-treatment of islets with PYY (100 nM) resulted in a significant elevation in GSIS (Figure 4A), without significant change in the basal secretion compared to islets without PYY incubation. PYY pre-treatment also resulted in a marked increase in total insulin content in rodent and human islets (Figure S6, Supporting Information).

Dose-dependent effects of PYY peptides on insulin secretion were also examined in mouse islets. A low, but physiologically-relevant, concentration of PYY (100 pM) enhanced GSIS and there was

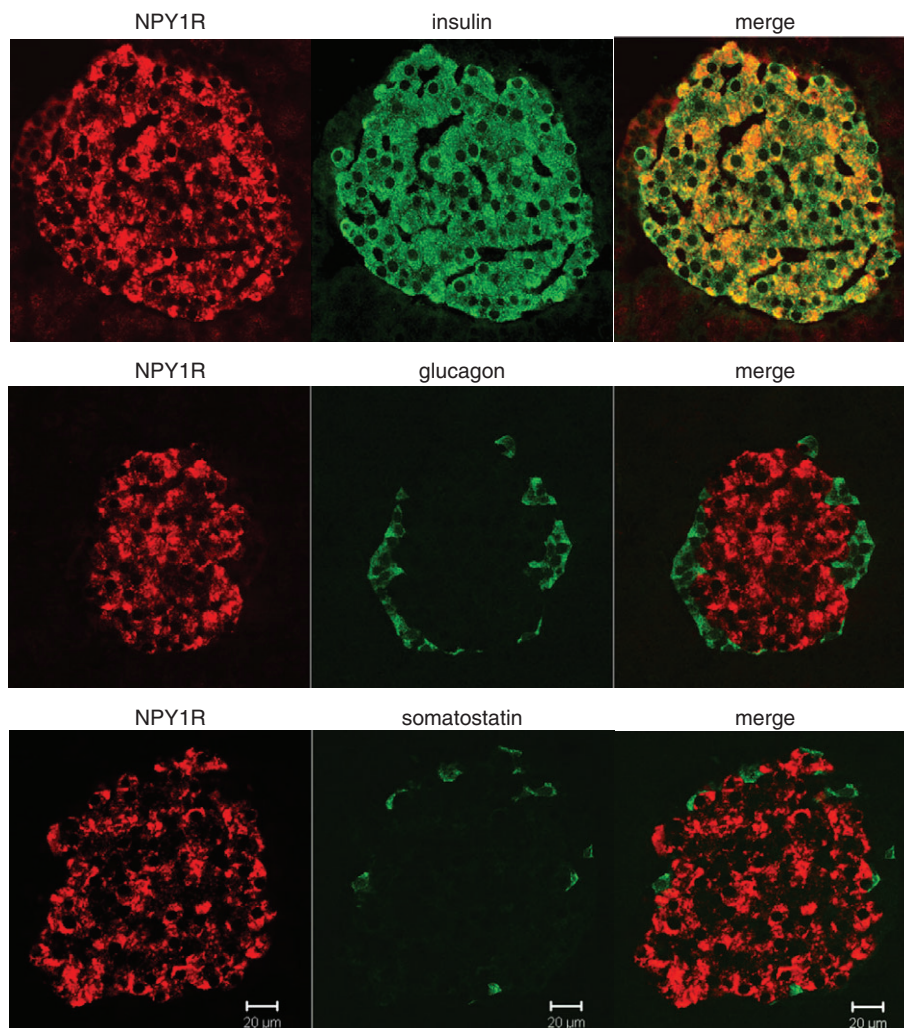
no further enhancement at a higher concentration (100 nM) (Figure 4B).

Similarly, in human islets from healthy donors, acute (1 hour) application of PYY, as either the full length or the truncated peptide, had no significant effect on GSIS (Figure 4C).

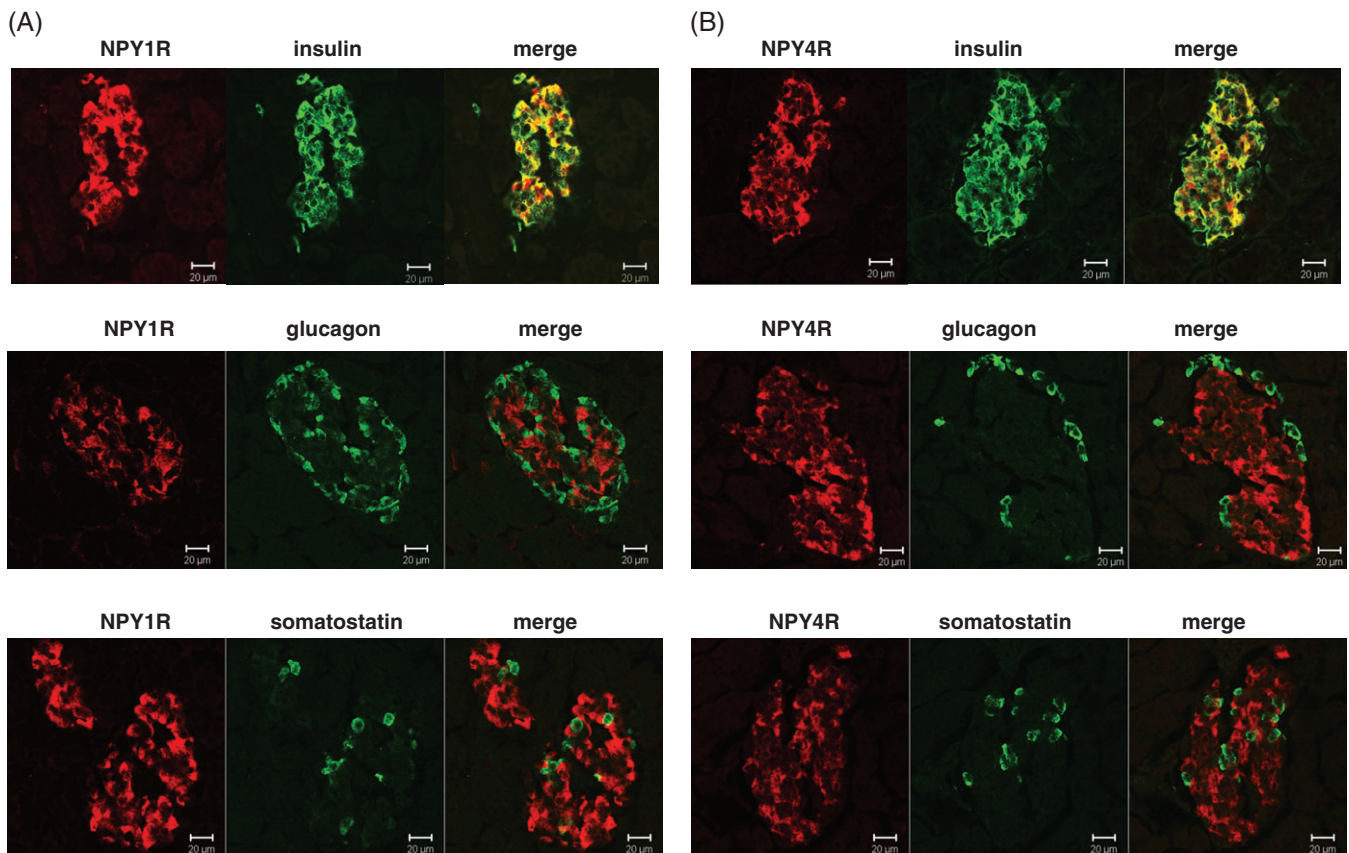
We have reported previously that chronic PYY pre-treatment can enhance GSIS in non-diabetic human islets.<sup>1</sup> However, the chronic effect of PYY on diabetic human islets has not been investigated. Chronic PYY treatment (72 hours) of human islets from 2 donors with type 2 diabetes resulted in potentiation of GSIS (Figure 4D).

### 3.4 | DPP-IV is present in islets and is inhibited by sitagliptin

DPP-IV was identified in mouse and human islets by immunoreactivity on pancreatic sections. In mouse islets, DPP-IV was found mostly in islet beta-cells (Figure S5A, Supporting Information). However, in human islets from healthy subjects, immunoreactivity for DPP-IV was found co-localized with glucagon, but not with insulin (Figure S5B, Supporting Information). To demonstrate DPP-IV activity in islets, an assay was developed to measure enzyme activity. DPP-IV activity was present in mouse islets and was significantly inhibited by 5 nM sitagliptin (Figure S5C, Supporting Information).



**FIGURE 2** NPY receptor expression in rodent islets. Representative confocal microscopy images of NPY1R staining in rat islets co-stained with insulin, glucagon or somatostatin



**FIGURE 3** NPY receptor expression in human islets. Representative confocal microscopy images of A, NPY1R and B, NPY4R staining in human islets co-stained with insulin, glucagon or somatostatin

### 3.5 | DPP-IV inhibition improves GSIS via PYY (1-36) action

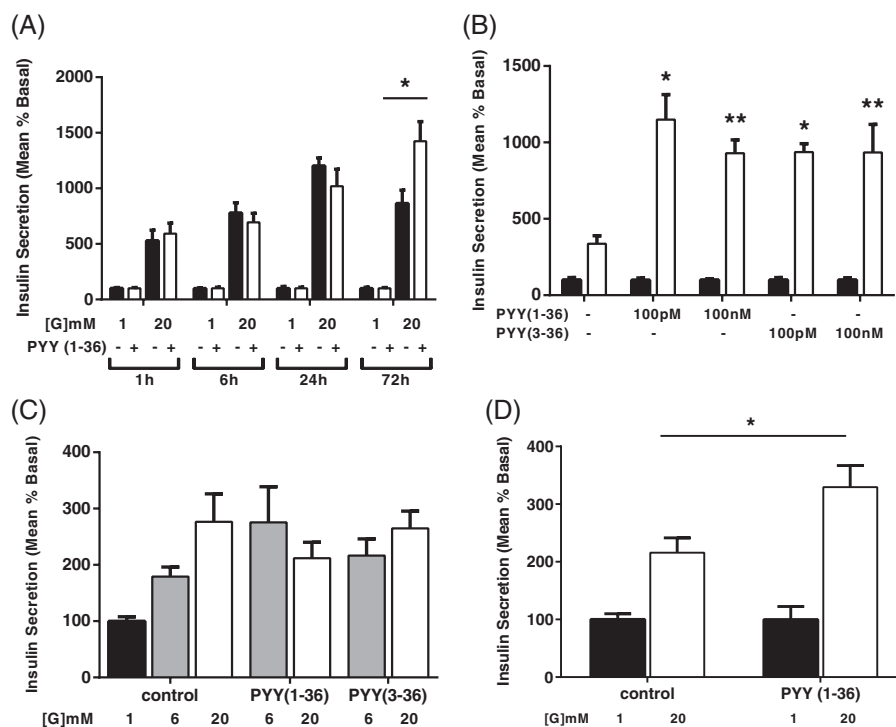
Mouse and human islets treated acutely (1 hour) with sitagliptin, at concentrations ranging from nM to  $\mu$ M, showed no improvement in GSIS (Figure 5A and B). However, when mouse and human islets were pre-cultured (72 hours) with 100 nM sitagliptin, determined to be the optimal concentration in these islets, there was a significant improvement in secretion at 20 mM glucose without a significant change in basal secretion compared to control islets (Figure 5C and D). These data suggest that islet function is enhanced by chronic exposure to a local factor which is normally degraded by DPP-IV.

To determine whether the beneficial effects of DPP-IV inhibition on GSIS were due to locally-produced GLP-1 or PYY, experiments were performed on rodent islets with sitagliptin in the presence of either the GLP-1R antagonist, exendin (9-39; 1  $\mu$ M), or an antibody specific to PYY. In the presence of exendin (9-39), the potentiating effect of sitagliptin pre-treatment (72 hours) on GSIS was not significantly affected. When islets were cultured concomitantly with sitagliptin plus the anti-PYY antibody, insulin secretion at 20 mM glucose was significantly reduced compared to islets treated with sitagliptin alone (Figure 5E). Addition of exendin (9-39) or the anti-PYY antibody on its own had no effect on GSIS (data not shown). Total PYY and GLP-1 were both secreted into the medium by islets during culture conditions. However, sitagliptin application increased total PYY release only, while no changes in GLP-1 were detected in the same samples (Figure S7, Supporting

Information). On the other hand, co-application of sitagliptin and exendin (9-39) did not increase total PYY release, potentially explaining why the potentiating effect of sitagliptin on GSIS was not fully retained in this condition. To further test that PYY, as compared to GLP-1, is targeted by local DPP-IV action, the effects of sitagliptin on islets from GLP-1 receptor knockout (GLP-1R KO) mice were investigated. These mice do not respond to GLP-1 with potentiated GSIS.<sup>24</sup> However, chronic treatment with sitagliptin resulted in enhanced GSIS and the response was similar to that observed in islets from wildtype mice (Figure 5F). In addition, immunoneutralization of PYY resulted in a complete reversal of sitagliptin-mediated GSIS in islets from both the wildtype and GLP-1R KO mice, demonstrating an important role of islet-derived PYY in the enhancement of insulin secretion in response to DPP-IV inhibition (Figure S8, Supporting Information).

To determine the receptor bound by PYY, the effects of the NPY1R blocker BIBP3226 were tested in the presence of sitagliptin in mouse islets. Application of the antagonist completely reversed the potentiating effects of sitagliptin (Figure 5G). Taken together, these data suggest that the PYY may be an important islet-derived factor which is responsible for enhanced GSIS upon sitagliptin treatment.

Full-length PYY (1-36) is cleaved by DPP-IV to form the degradation product, PYY (3-36), which is unlikely to be affected by sitagliptin. However, both recombinant versions of PYY potentiate GSIS to a similar extent. To determine whether, in islets, both endogenous PYY forms modulate insulin secretion equally, mouse islets were treated chronically with PYY (1-36) or PYY (3-36), with or without sitagliptin.



**FIGURE 4** Time and dose-dependent modulation of insulin secretion by PYY. A, Insulin secretion from mouse islets cultured without (black bars) or with (white bars) 100 nM PYY (1-36) for the indicated time points. Secretion was measured from islets stimulated with 1 mM or 20 mM glucose. Data are presented as percentage of basal secretion (mean  $\pm$  SEM as percentage of content; 1 hour, control:  $0.0196 \pm 0.0045$ , with PYY:  $0.0190 \pm 0.0018$ ; 6 hours, control:  $0.073 \pm 0.016$ , with PYY:  $0.121 \pm 0.02$ ; 24 hours, control:  $0.0189 \pm 0.0063$ , with PYY:  $0.0198 \pm 0.0028$ ; 72 hours, control:  $0.043 \pm 0.006$ , with PYY:  $0.033 \pm 0.0003$ ). B, Insulin secretion from mouse islets chronically cultured (72 hours) with PYY (1-36) or (3-36) at the indicated concentration. Secretion was measured from islets stimulated with 1 mM (black bars) or 20 mM glucose (white bars). Data are presented as percentage of basal secretion (mean  $\pm$  SEM as percentage of content; control:  $0.478 \pm 0.073$ , with PYY (1-36; 100pM):  $0.244 \pm 0.033$ , PYY (1-36; 100 nM):  $0.278 \pm 0.023$ , PYY (3-36; 100pM):  $0.471 \pm 0.072$ , PYY (3-36; 100 nM):  $0.249 \pm 0.036$ ). C, Insulin secretion from human islets stimulated with 1 mM (black bars), 6 mM (grey bars) or 20 mM (white bars) glucose in absence or presence of 100 nM PYY (1-36). D, Insulin secretion from human islets isolated from 2 diabetic donors cultured for 72 hours with or without 100 nM PYY (1-36). Insulin release was measured from islets stimulated with 1 mM (black bars) or 20 mM glucose (white bars); for each diabetic donor, experiments were done in groups of 3 to 4, totalling 7 to 8 replicates and statistical analysis was performed on these. \* $P < .05$ , \*\* $P < .01$  for indicated comparisons

As observed previously, sitagliptin application on its own markedly enhanced GSIS by  $\sim 3$ -fold (Figure 5C). This stimulation index was comparable to responses obtained by PYY (1-36) or PYY (3-36) alone (Figure 4B). However, co-application of sitagliptin with PYY (1-36) significantly elevated GSIS by  $\sim 50\%$ . Addition of sitagliptin to PYY (3-36) had no additive effects on GSIS compared to PYY (3-36) alone (Figure 5H), thus indicating that the locally produced uncleaved form of PYY is the main modulator of islet function.

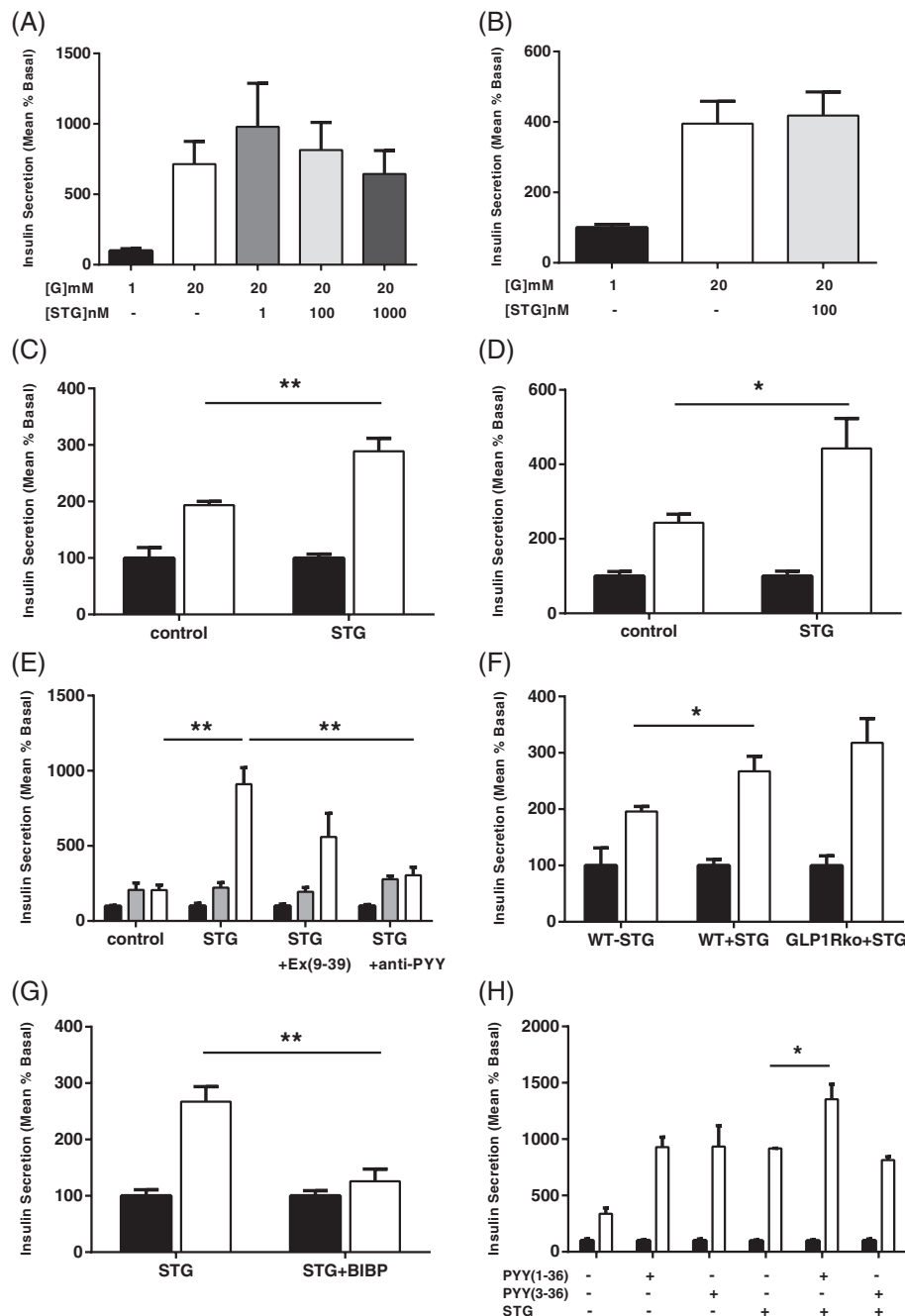
### 3.6 | Increased levels of PYY in islets contribute to diabetes remission after RYGB

Circulating PYY levels are known to be lower in type 2 diabetes in humans compared to non-diabetic individuals.<sup>25,26</sup> Consistently, total PYY concentrations in plasma were lower in diabetic GK rats compared to healthy Wistar rats (Figure 6A). We have reported previously that circulating PYY levels are markedly higher in GK rats following RYGB and are associated with restoration of islet function following the surgery.<sup>1</sup> However, it remains unknown whether RYGB can also affect PYY content levels in the pancreatic islets. PYY content was measured in islets obtained from healthy Wistar controls

and diabetic sham- and RYGB-operated GK rats. Islet PYY content was significantly lower in the diabetic GK rats, compared to the healthy Wistar control rats. In islets from the RYGB GK rats, total islet PYY content was increased to higher levels than in the Wistar rat islets (Figure 6B). On the other hand, a much smaller increase in GLP-1 content was observed in the same islets from RYGB GK rats. However, this increase in GLP-1 failed to reach the levels in the Wistar control islets (Figure S9, Supporting Information).

## 4 | DISCUSSION

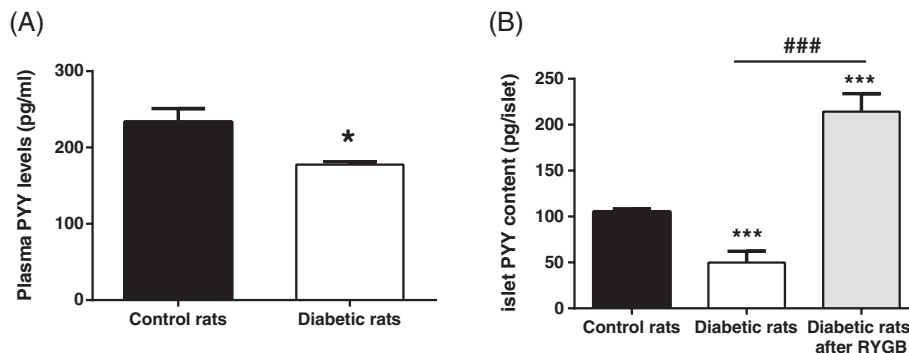
PYY is a classical appetite-regulating hormone secreted from the gut that exerts a major action in the brain. However, evidence from a number of recent studies demonstrates that PYY can also modulate insulin secretion,<sup>2,3</sup> as well as restore impaired islet function in diabetic rats following RYGB surgery.<sup>1</sup> The expression of PYY in islets, in addition to its main site of production in the gut and specific sites of action in the central nervous system, points to a potential autocrine role of the peptide in the pancreas. Moreover, PYY is a substrate for DPP-IV, which is highly expressed in islets, and its activity



**FIGURE 5** Effects of sitagliptin on insulin secretion are mediated by islet PYY. A and B, Insulin secretion from mouse (A) and human (B) islets stimulated acutely with 1 mM or 20 mM glucose, with varying doses of sitagliptin (STG). C and D, Insulin secretion from mouse (C) and human (D) islets chronically cultured with or without 100 nM sitagliptin. Secretion was measured from islets stimulated with 1 mM (black bars) or 20 mM glucose (white bars). Data are presented as percentage of basal secretion (mean  $\pm$  SEM as percentage of content; control:  $0.123 \pm 0.059$ , sitagliptin:  $0.112 \pm 0.031$  (for mouse islets), control:  $0.378 \pm 0.055$ , sitagliptin:  $0.558 \pm 0.123$  (for human islets). E, Insulin secretion from rodent islets chronically cultured in the presence of sitagliptin, sitagliptin + exendin (9-39) or sitagliptin + anti-PYY antibody. Secretion was measured in islets stimulated with 1 mM (black bars), 6 mM (grey bars) or 20 mM glucose (white bars). Data are presented as percentage of basal secretion (mean  $\pm$  SEM as percentage of content; control:  $0.105 \pm 0.004$ , sitagliptin:  $0.08 \pm 0.014$ , sitagliptin + exendin (9-39):  $0.09 \pm 0.012$ , sitagliptin + anti-PYY:  $0.09 \pm 0.008$ ). F, Insulin secretion at 1 mM (black bars) and 20 mM (white bars) glucose from islets isolated from wildtype (WT) and GLP-1 receptor knockout (GLP-1R KO) mice and cultured chronically with 100 nM sitagliptin. Data are presented as percentage of basal secretion (mean  $\pm$  SEM as percentage of content; WT with no sitagliptin:  $0.006 \pm 0.002$ , WT + sitagliptin:  $0.049 \pm 0.005$ , GLP-1R KO:  $0.046 \pm 0.008$ ). G, Insulin secretion from mouse islets chronically treated with sitagliptin in the absence or presence of 1  $\mu$ M BIBP. Data are presented as percentage of basal secretion (mean  $\pm$  SEM as percentage of content; sitagliptin:  $0.049 \pm 0.005$ , sitagliptin + BIBP:  $0.049 \pm 0.005$ ). H, Insulin secretion from mouse islets chronically treated with 100 nM PYY (1-36) or PYY (3-36) or 100 nM sitagliptin. Secretion was measured in islets stimulated with 1 mM (black bars) or 20 mM glucose (white bars). Data are presented as percentage of basal secretion (mean  $\pm$  SEM as percentage of content; control:  $0.478 \pm 0.073$ , PYY (1-36):  $0.278 \pm 0.023$ , PYY (3-36):  $0.249 \pm 0.036$ , sitagliptin:  $0.333 \pm 0.019$ , sitagliptin + PYY (1-36):  $0.344 \pm 0.026$ , sitagliptin + PYY (3-36):  $0.325 \pm 0.048$ ). \* $P < .05$ , \*\* $P < .01$  for indicated comparisons



**FIGURE 6** Islet PYY levels increase after RYGB. A, Plasma PYY levels in control rats ( $n = 4$ ) and diabetic GK rats ( $n = 7$ ). B, PYY content from islets isolated from control rats ( $n = 9$ ), diabetic sham-operated GK rats ( $n = 12$ ) and RYGB-operated GK rats ( $n = 11$ ). PYY content is presented in pg/islet. Data are presented as mean  $\pm$  SEM. \* $P < .05$  vs control rats, \*\*\* $P < .001$  vs control rat islets, ### $P < .001$  for indicated comparison



is subject to change under diabetic conditions.<sup>14</sup> This suggests that PYY may be prone to local regulation by islet DPP-IV, which can potentially impact on islet function under physiological and pathological conditions.

Several studies have confirmed pancreatic expression of PYY in rodents,<sup>3,6,8,27</sup> as well as cats, dogs, pigs and cows.<sup>7</sup> Whilst it is well-established that PYY does not co-localize with insulin-producing cells, controversial results have been reported concerning its expression in other islet cell types. Thus, PYY immunoreactivity in alpha-cells has been reported to be species-specific in the adult pancreas<sup>7</sup> and almost lost during development.<sup>8</sup> We show PYY gene expression in rat, mouse and human islets and, for the first time, demonstrate PYY co-localization with glucagon and PP in mature human islet cells, but with somatostatin and PP in rodent islets, probably indicating a species difference. These data are consistent with single-cell messenger RNAseq studies that demonstrate the expression of PYY in alpha-cells in human pancreatic islets.<sup>28–30</sup> Differences in cellular PYY localization between the rat and human samples were influenced by the differences in PYY content. Human islets contained much less PYY than rodent islets and this was evidenced by the observation that successful immunolabelling in human islets required signal amplification methods, and that the labelling of human EM sections (which is a less sensitive method than labelling for light microscopy) revealed little or no granule labelling. However, dense labelling for PYY in  $\beta$ -cells lysosomes indicated successful reactivity to the antibody and suggested that PYY had been internalized into  $\beta$ -cells during peptide signalling.

Similarly, we observed a different localization pattern between rodent and human islets for DPP-IV, in agreement with previous publications. The species specificity of PYY and DPP-IV expression, and its impact on islet physiology, remain unexplained and call for further investigation. Nevertheless, we found that application of PYY or DPP-IV inhibition by sitagliptin consistently modifies insulin response to glucose in rodent and human islets, thus suggesting a common mechanism that may be explained by a conserved receptor distribution.

NPY receptor mRNA expression in islets has been documented, but the actual presence of the receptors has not been shown.<sup>3,9</sup> Our staining data show that NPY1R localizes to beta-cells in rat islets, while NPY1R and NPY4R are expressed on human beta-cells. With our immunohistochemical detection system we were unable to detect NPY4R in the rat islets although the antibody positively identified this receptor in the neuronal tracts in the rat hypothalamus. These results

show that NPY1R receptor distribution is conserved in both rat and human islets. PYY-mediated effects on GSIS have been shown to occur via NPY1R in both species, suggestive of consistency in PYY signalling and action in rodent and human islets.<sup>1</sup> Both species display no detectable levels of NPY2R, which mediates the anorectic effect of PYY and is highly selective for PYY (3–36); this implies that the effects of PYY on islet secretory function are mediated through a pathway distinct from appetite regulation.

We show that acute application of PYY to rodent or human isolated islets has no effect on basal or glucose-stimulated insulin secretion. We have reported previously that PYY pre-treatment enhances glucose-stimulated insulin secretion in rat and human islets.<sup>1</sup> In agreement with these findings, the potentiating effects of PYY peptides on GSIS in mouse islets were observed only following chronic application (>24 hours). Islets in culture over time (72 hours) undergo cellular stress which can affect insulin content. Thus, our results indicate that PYY pre-treatment preserves insulin content and secretory responses in cultured islets. Inhibitory effects of PYY ( $10^{-8}$  to  $10^{-6}$  M) on glucose-mediated insulin release have been reported recently under acute (1 hour) conditions in beta-cell lines and mouse islets.<sup>3</sup> Although the discrepancy in the effects of PYY on insulin release may be the result of differences in experimental conditions (eg, peptide source and concentrations), our data suggest that the glycaemic benefit of PYY results from its long-term, rather than acute, effects. The physiological relevance of PYY in the pancreatic islets and diabetes has been illustrated by several independent studies. Thus, drastic disruption of islet structure and beta-cell function has been demonstrated following selective ablation of PYY-expressing cells in islets and by restoration of beta-cell function by exogenously applied PYY in streptozotocin-treated mice<sup>2,3</sup> and immunoneutralization of PYY in rat islets treated with serum obtained from RYGB GK rats.<sup>1</sup> Importantly, we demonstrate that PYY can also enhance GSIS in human islets from diabetic donors, indicating that our observations in rodent islets can be extended to man. In support of this, chronic administration of PYY (3–36) in rats has been shown to induce a significant reduction in HbA1c level, as well as improvements in glycaemic parameters, whereas no effect on plasma glucose was observed following an acute (60 minutes) treatment with PYY.<sup>31</sup> However, a role for pancreatic polypeptide (PP) in this process cannot be excluded, and future studies should be undertaken to explore this hypothesis.

The beneficial effects of DPP-IV inhibitors as therapeutic agents for type 2 diabetes are thought to be primarily related to their ability to prolong the half-life of active GLP-1.<sup>10</sup> However, it is well-established

that GLP-1 is not the only substrate for DPP-IV action.<sup>32</sup> Thus, PYY in circulation is prone to rapid proteolysis by DPP-IV.<sup>33</sup> Consistently, Aaboe and colleagues have demonstrated that 12 weeks of sitagliptin therapy prevents PYY degradation, resulting in a significant increase in PYY (1-36) levels.<sup>34</sup> We confirm DPP-IV expression and activity in pancreatic islets in agreement with previous reports.<sup>14,16</sup> We also demonstrate that chronic treatment with the DPP-IV inhibitor sitagliptin leads to enhancement of GSIS in mouse as well as human islets. However, this effect is independent of endogenously-occurring pancreatic GLP-1, as it is preserved in the presence of the GLP-1 receptor antagonist and in islets from GLP-1R knockout mice. In contrast, inhibition of PYY action by immunoneutralization of the peptide or antagonism of NPY1R significantly blunts glucose-induced insulin stimulatory responses. These data demonstrate for the first time that DPP-IV has a role in intra-islet PYY degradation. Although these findings indicate that the modulating effect of the inhibition of DPP-IV activity in islets is the result of preservation of intact endogenous PYY, and not full length GLP-1, the role of other DPP-IV substrates such as GIP cannot be ruled out. Thus, the locally-produced, uncleaved form of PYY may be an important modulator of islet function. This is consistent with the expression pattern of NPY receptors in rodent and human islets, confirming a major role of NPY1R in maintaining glucose homeostasis.<sup>35</sup>

Overexpression of PYY in mouse islets has been associated with increased lean mass and improved energy metabolism and glucose tolerance,<sup>4</sup> suggesting that islet-derived PYY can also affect whole body physiology. Loss of PYY in the pancreas also results in impaired islet morphology and marked reductions in insulin secretion,<sup>2</sup> indicating that PYY plays a key role in maintaining islet integrity and beta-cell function. Interestingly, diabetic GK rats exhibit a characteristic disrupted islet morphology, which is significantly restored following RYGB, in association with improved insulin and glucagon secretion.<sup>1</sup> Circulating PYY levels are known to be lower in diabetic patients and strongly elevated following bariatric surgery. However, it is not known whether intra-islet PYY levels are also affected by diabetes and RYGB.

Consistent with the changes in plasma PYY, we show for the first time that islet PYY content is markedly lower in the diabetic GK rats, and is significantly increased following RYGB, in agreement with recent reports of improved islet secretory functions.<sup>1</sup> These results suggest that RYGB-induced restoration of impaired islet morphology and function may actually be associated with islet-derived PYY rather than PYY in circulation. Although it is not possible to differentiate the effects of circulating PYY from those of islet-derived PYY on islet function, the peptide is probably one of several factors that alter pancreatic islet function following RYGB. Importantly, gastric bypass does not restore GLP-1 in islets to levels that occur in healthy rats. The small increase in islet GLP-1 content may be the result of recovery in islet morphology post-surgery. This reiterates findings from several studies; that is, there is no elevation in fasting plasma GLP-1 levels within days or months after RYGB, corroborating a negligible effect of GLP-1 on islet secretion improvements.<sup>1,36-38</sup> Several mechanisms may account for the elevation of PYY in islets after bariatric surgery, including changes in bile acids and microbiota-derived metabolites. Quantitative and qualitative modification of these factors has been well documented post gastric bypass in rodents and

humans<sup>39-42</sup> and, in some cases, they have been shown to directly regulate PYY expression in gut cells.<sup>43,44</sup> Whether PYY content in islets is also affected by these factors remains an interesting avenue to explore. In addition, a reduction in plasma DPP-IV activity after gastric bypass has been reported in obese patients with type 2 diabetes,<sup>45</sup> supporting the hypothesis that a local decrease in pancreatic DPP-IV activity after surgery may enhance intra-islet PYY levels and subsequently affect glucose responses. Future studies are warranted to explore these possibilities and the relevance of pancreatic PYY in the surgical correction of diabetes.

Taken together our results indicate that local regulation of pancreatic PYY by DPP-IV inhibition or by gastric bypass can directly modulate islet function and insulin secretory responses to glucose. This may have a direct impact on the restoration of diabetes and may represent a novel therapeutic strategy for the treatment of the disease.

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## Conflict of interest

No potential conflicts of interest relevant to this article were reported.

## Author contributions

C. G. and L. M. designed and performed the experiments, analysed the data and wrote the manuscript. M. G., S. D. S., C. B. and J. D. performed some experiments and analysed data. D. B. provided GLP-1R KO islets and reviewed the manuscript. D.C. provided the RYGB rat model and reviewed the manuscript. A.C. performed some experiments, and reviewed and edited the manuscript. R. D. R. conceived of and performed the experiments, wrote and reviewed the manuscript.

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## SUPPORTING INFORMATION

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