Pancreatic PYY Is Critical in the Control of Insulin Secretion and Glucose Homeostasis in Female Mice

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Insulin secretion is tightly controlled through coordinated actions of a number of systemic and local factors. Peptide YY (PYY) is expressed in α-cells of the islet, but its role in control of islet function such as insulin release is not clear. In this study, we generated a transgenic mouse model (*Pyy*$^{tg/+}$*/Rip-Cre*) overexpressing the *Pyy* gene under the control of the rat insulin 2 gene promoter and assessed the impact of islet-released PYY on β-cell function, insulin release, and glucose homeostasis in mice. Our results show that up-regulation of PYY in islet β-cells leads to an increase in serum insulin levels as well as improved glucose tolerance. Interestingly, PYY-overproducing mice show increased lean mass and reduced fat mass with no significant changes in food intake or body weight. Energy expenditure is also increased accompanied by increased respiratory exchange ratio. Mechanistically, the enhanced insulin levels and improved glucose tolerance are primarily due to increased β-cell mass and secretion. This is associated with alterations in the expression of genes important for β-cell proliferation and function as well as the maintenance of the β-cell phenotype. Taken together, these data demonstrate that pancreatic islet-derived PYY plays an important role in controlling glucose homeostasis through the modulation of β-cell mass and function. **(*Endocrinology* 156: 3122–3136, 2015)**

Type 2 diabetes has become one of most detrimental health problems worldwide. It is characterized by peripheral insulin resistance and hyperglycemia (1, 2). It is well acknowledged that significant loss of β-cell mass and function that no longer compensates for metabolic load contributes to the etiology of diabetes (3). There are 4 principal types of cells within pancreatic islets, α-, β-, δ-, and pancreatic polypeptide cells. β-cells are the sole source of insulin production, whereas α-cells produce glucagon in response to reduced circulating glucose level to counteract insulin's action. Insulin release in healthy individuals is tightly controlled by circulating nutrients particularly glucose, and concerted action of hormones within islets is critical in maintaining normoglycemia and glucose homeostasis.

In addition to glucagon, pancreatic α-cells are also known to express peptide YY (PYY), a peripherally released member of the neuropeptide Y family that is also expressed in the gut (4, 5). The role of gut-released PYY in the regulation of glucose and energy homeostasis has been well established. Indeed, administration of gut-derived PYY3–36, the major circulating form of PYY, reduces food intake in rodents and humans (6–8). Importantly, obese individuals have reduced levels of circulating PYY3–36 that could contribute to the hyperinsulinemia and the obesity observed in these individuals (9). Furthermore, PYY knockout (*Pyy*$^{-/-}$) mice exhibited greater adiposity associated with a significant increase in both nonfasted and glucose-induced insulin levels compared with

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Abbreviations: Arc, arcuate nucleus; CMV, cytomegalovirus; ER, endoplasmic reticulum; GSIS, glucose-stimulated insulin secretion; GTT, glucose tolerance test; *Ins2*, insulin 2; ITT, insulin tolerance test; K_{ATP}, ATP-sensitive K^+; KRB, Krebs-Ringer buffer; Pparγ, proliferator-activated receptor-γ; PVN, paraventricular nucleus; PYY, peptide YY; RER, respiratory exchange ratio; *Sur1*, sulfonylurea receptor 1; Vdcc, voltage-dependent Ca^{2+} channel; VO_2, oxygen consumption rate; WAT, white adipose tissue; WT, wild type.

normal controls (9), clearly suggesting a role for reduced PYY signaling in the development of obesity and hyperinsulinemia. In contrast to the greater efforts in investigating the effects of gut-released PYY on satiety, body weight regulation, and glucose metabolism, the role of islet-derived PYY in the regulation of glucose homeostasis and islet function remains largely unexplored. Interestingly, although PYY is mainly expressed in glucagon-producing α-cells within the islets, it is indeed coexpressed in all islet cell types, including β-cells at early developmental stages, indicative of an important role for local PYY in islet development (10–12). In agreement with this notion, Bewick and colleagues (13) have recently revealed that loss of endogenous PYY function in adult cells using diphtheria toxin administration leads to severe hyperglycemia, which is accompanied by disrupted islet morphology as well as significant reduction in insulin level. The results from this study strongly suggest a potential role of islet PYY in the regulation of β-cell function and survival.

PYY signals through a set of different Y receptors to exert its actions. In general, Y receptors preferentially associate with the Gi/o G proteins to act in an inhibitory fashion, leading to reduction in cAMP levels in target cells (14). Although PYY binds to all known Y receptors, only Y1 receptors have been detected in the islet β-cells (5, 14). Thus, the presence of Y1 receptors in the islets further indicates the potential for a direct effect induced by locally produced PYY. In fact, a possible direct action of pancreatic PYY in insulin secretion is indicated by a study showing that PYY administration inhibits insulin secretion from isolated islets of wild-type (WT) mice in response to glucose (10), and the action is mediated via the Y1 receptor (13). By contrast, islets isolated from $Pyy^{-/-}$ mice produce significantly more insulin than WT (9). Taken together, these data demonstrate a clear link between PYY and the control of β-cell function and insulin secretion. However, how the islet-derived PYY influences these processes and subsequent whole-body glucose metabolism is not clear.

In this study, we generated a novel islet-specific PYY overexpression mouse model to delineate the functional role of islet-derived PYY in the regulation of glucose/energy homeostasis and β-cell function. The precise underlying mechanisms were also explored. Findings from this study provide novel insights into the understanding of the action of islet PYY in the control of local β-cell function as well as whole-body glucose and energy homeostasis.

Materials and Methods

Animals

All research and animal work was conducted under the regulation of Garvan Institute/St Vincent's Hospital Animal Ethics Committee and were in agreement with the Australian Code of Practice for the Care and Use of Animals for Scientific purpose. Mice were housed under conditions of controlled temperature (22°C) and illumination (12-h light, 12-h dark cycle, lights on at 7 AM). All mice were fed on a chow diet ad libitum (8% calories from fat, 21% calories from protein, 71% calorie from carbohydrate and 10.87 kJ/g; Gordon's Specialty Stock Feeds). Details of generation of the PYY transgenic mice ($Pyy^{tg/+}$) were published previously (15). Briefly, in the $Pyy^{tg/+}$ line, the PYY gene is separated from the cytomegalovirus (CMV) promoter by the insertion of a lacZ-Neo cassette and a transcription termination signal that is flanked by loxP sites. Deletion of the loxP-flanked cassette by Cre then allows the removal of the terminal signal and allows for the expression of the *Pyy* gene in a tissue-specific fashion.

Pyy transgene construct and generation of pancreas-specific PYY overexpression model

In order to gain better insights into the specific function of pancreatic islet PYY, a transgenic mouse model expressing the *Pyy* gene under the control of the rat insulin 2 (*Ins2*) gene promoter ($Pyy^{tg/+}$/*Rip-Cre*) has been generated. This was achieved by crossing our PYY transgenic mice ($Pyy^{tg/+}$) with transgenic mice expressing the Cre-recombinase (*Cre*) gene under the control of the rat Ins2 promoter (B6.Cg-Tg(INS2-cre)25Mgn/Jausb strain, referred to as *Rip-Cre*) (16). *Cre*-mediated recombination resulted in pancreatic islet-specific overexpression of the *Pyy* gene. WT, $Pyy^{tg/+}$, and *Rip-Cre* littermates were used as controls to accommodate any possible influences that this alteration may already have on PYY function (17, 18).

Determination of food intake, body weight, energy expenditure, and body composition

Female mice (8–12 per group) were housed individually throughout the whole experiment, and body weight was measured once a week. At 10–11 weeks of age, spontaneous food intake and fasting-induced food intake was measured, respectively. Oxygen consumption rate (VO_2), carbon dioxide output, and physical activity were measured at 12 weeks of age using an open circuit eight-chamber indirect calorimeter (Oxymax series; Columbus Instruments) with airflow of 0.6 L/min as previously described (19). Energy expenditure and respiratory exchange ratio (RER) was calculated. Whole-body lean mass was measured at 14 weeks of age using dual-energy x-ray absorptiometry (Lunar PIXImus2 mouse densitometer; GE Medical Systems).

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

At 12 weeks of age, mice were fasted for 6 hours before ip injection of a 10% D-glucose solution (1.0 g/kg body weight) (19, 20). Blood samples were obtained from the tail tip at the indicated times, and glucose levels were measured using a glucometer (AccuCheck II; Roche). ITT was carried out to determine insulin-induced hypoglycemia in mice at 13 weeks of age. Briefly, the mice were injected with insulin (1 IU/kg body weight) ip to induce hypoglycemia after being fasted for 6 hours, then blood samples were collected from the tail tip at the indicated times, glucose levels were measured using a glucometer as shown in the results.

Tissue collection and analysis

At the completion of the study, mice of 15 weeks of age were killed by cervical dislocation between 12 and 3 PM, trunk blood was collected, allowed to clot at room temperature, centrifuged, and the resultant serum were stored at −20°C for subsequent analysis. Pancreas and white adipose tissue (WAT) depots (right inguinal, right retroperitoneal, right epididymal, and mesenteric) obtained from *Pyy*$^{tg/+}$*/Rip-Cre* and control *Rip-Cre* mice were collected, weighed, and fixed in 4% paraformaldehyde in PBS for further analysis. Pancreatic islets from *Pyy*$^{tg/+}$*/Rip-Cre* and *Rip-Cre* control mice were isolated for ex vivo glucose-stimulated insulin secretion (GSIS) assay and gene expression analysis as described below.

Isolation of pancreatic islets and insulin secretion assays

Islets were isolated as previously described (21). Briefly, mice were sacrificed by cervical dislocation, and the common bile duct was cannulated and its duodenal end occluded by clamping. Liberase solution (3 mL at 0.25 mg/mL in Krebs-Ringer buffer [KRB]) was injected into the duct to distend the pancreas. The pancreas was excised, incubated at 37°C for 16 minutes, and mechanically disrupted in 15 mL of KRB. After pancreatic digestion, islets were purified using a Ficoll-paque (GE Healthcare) gradient. Islets were carefully handpicked under a microscope for RNA extraction and ex vivo GSIS assay. For insulin secretion assays, islets with similar size were cultured overnight in RPMI with 11 mmol/L D-glucose and 10% fetal calf serum (Invitrogen). Islets were preincubated for 1 hour in KRB containing 10 mmol/L HEPES, 0.1% BSA and 2 mM D-glucose. Batches of 5 islets, with duplicate per animal, were incubated at 37°C for 1 hour in 130-μL KRB containing 2 or 20 mM D-glucose as indicated in the text. Insulin release was determined by RIA (Linco/Millipore).

RNA extraction and quantitative real-time PCR

RNA was extracted from isolated islets using an RNAeasy kit (QIAGEN). RNA integrity was confirmed by pico chip (Agilent) and total RNA (0.5 μg) was reverse transcribed using a first-strand cDNA synthesis kit (QIAGEN). Real-time RT-PCR was performed as previously described (22). The value obtained for each gene product was normalized to the control gene *cyclophilin A* and expressed as a fold change of the value obtained for control islets. Primer sequences are listed in Supplemental Table 1.

Immunofluorescent staining for insulin, PYY, glucagon, and Ki67

Insulin, PYY, glucagon, and Ki67 immunofluorescence was performed on paraformaldehyde-fixed, paraffin-embedded pancreatic sections, as described previously (23). Briefly, whole pancreas were fixed in 4% PBS-buffered paraformaldehyde overnight at 4°C before being processed and embedded in paraffin. Sections were cut at 5 μm, deparaffinized, rehydrated, and incubated with a Target Retrieval Solution (DAKO Corp) in a pressure cooker. Slides were then washed in distilled water and incubated with 5% goat serum in PBS containing 1% BSA for 1 hour at room temperature. Subsequently sections were incubated overnight at 4°C in hydration chambers with the respective primary antibody. Slides were then washed in PBS and incubated with the respective secondary antibody for 1 hour at room temperature. The slides were washed in PBS and cover slipped with ProLong Gold Antifade mounting medium (Thermo Fisher Scientific, Inc). Sections were imaged via a Leica SP8 confocal microscope. Entire sections of the pancreas were scanned and imaged using the Leica LAS Power Mosaic (Leica Microsystems). β-cell mass, islet number and average islet size were measured using a digital image processing software (ImageJ) (23). Three sections separated by at least 150 μm were used for each mouse. β-cell mass was calculated from relative cross-sectional β-cell area and total pancreas weight (Table 1).

Immunohistochemical analysis of PYY expression in the brain

The mice were anesthetized with an overdose injection of sodium pentobarbitone (120 mg/kg, ip), and the brains were fixed by perfusion with 0.9% saline followed by ice cold 4% paraformaldehyde made in 0.1M PBS (pH 7.4). The brains were immediately removed and placed in 4% paraformaldehyde for 30 minutes and then in 30% sucrose solution in phosphate buffer overnight. Coronal slices of 30 μm thickness were mounted on glass slides (Menzel-Glaser) and washed in 1% H_2O_2 in 50% alcohol for 20 minutes to abolish endogenous peroxidase activity. Sections were incubated overnight at room temperature with our monoclonal PYY antibody generated in house (15).

After 3 washes in PBS-Triton X-100 for 10 minutes each, sections were incubated with the biotinylated secondary antibody (antimouse secondary antibody; Sigma-Aldrich) diluted 1:250 in PBS for 3 hours. Sections were then washed in PBS for 30 minutes and incubated with Avidin-Biotin-Peroxidase Vectastain (Vector Laboratories) for 30 minutes at room temperature. Sections were rinsed in PBS and treated with diaminobenzidine (DAKO) for 5 minutes. Slides were rinsed in water and dehydrated through to xylene. After cover slipping, twelve sections from each mouse were visualized for PYY immunoactivity within hypothalamic arcuate nucleus (Arc), paraventricular nuclei (PVNs), and the brainstem, which were defined according to the mouse brain atlas (24), using a Zeiss Axioplan light microscope equipped with the ProgRes digital camera (Carl Zeiss Imaging Solutions GmbH).

Statistical analysis

One- or two-way ANOVA, repeated measures ANOVA, or Student's *t* test was used to determine the significance of treatment effects and interactions (GraphPad Prism 5, version 5.0a; GraphPad Software, Inc). When there was a significant overall effect or interaction effect, Bonferroni post hoc tests were performed to identify differences among means. For all statistical analyses, $P < .05$ was regarded as significant.

Results

Generation of islet-specific *Pyy*-overproducing mice

The generation of classical transgenic mice overproducing PYY is hampered by the fact that increased levels of PYY during embryo development is lethal (25). Therefore an inducible approach had to be adapted to overcome

Table 1. Antibody Table

Peptide/ Protein Target	Antigen Sequence (if known)	Name of Antibody	Manufacturer, Catalog Number, and/of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
Ki67		Anti-Ki67; clone: SP6	Thermo Scientific; RM 9106-S1	Rabbit monoclonal antibody	1:250
Insulin	FVNQHLCGSHLVEALYLVC GERGFFYTPKTRREAEDL QVGQVELGGGPGAGSLQ PLALEGSLQKRGIVEQCC TSICSLYQLENYCN	Insulin antibody (H-86)	Santa Cruz Biotechnology, Inc; SC-9168	Rabbit polyclonal IgG	1:500
Insulin		Monoclonal antiinsulin	Sigma-Aldrich; I2018	Mouse monoclonal IgG1	1:500
Glucagon		Monoclonal antiglucagon	Sigma-Aldrich; G2654	Mouse monoclonal IgG1	1:500
Secondary Ab					
Mouse IgG		Alexa Fluor 488 antimouse	Jackson ImmunoResearch; 715-546-150	Donkey polyclonal	1:200
Rabbit IgG		Alexa Fluor 488 antirabbit	Jackson ImmunoResearch; 711-546-152	Donkey polyclonal	1:200
Mouse IgG		Alexa Fluor 647 antimouse	Invitrogen; A-21235	Goat polyclonal	1:200
Rabbit IgG		Alexa Fluor 647 antirabbit	Jackson ImmunoResearch; 711-606-152	Donkey polyclonal	1:200
Mouse IgG		Antimouse IgG	Sigma-Aldrich; B7264	Goat polyclonal	1:500

this problem. For this, a mouse *Pyy* cDNA was cloned into a vector downstream of a cassette that contains a CMV promoter followed by a loxP site flanked cassette of the Neo selection marker, the β-galactosidase gene, and a strong transcription termination sequence (Figure 1A). This whole unit was then flanked with sequences that allow for the targeting to the ROSA26 locus. Successful targeted ES cell clones were then injected to oocytes and chimeric mice were bred with C57BL/6 mice to generate germline transmitted heterozygous *Pyy* knock-in mice ($Pyy^{tg/+}$).

In order to investigate the specific function of PYY in pancreatic islets, we then crossed $Pyy^{tg/+}$knock-in mice with a transgenic line expressing the *Cre*-recombinase gene under the control of the rat *Ins2* gene promoter to generate $Pyy^{tg/+}$/*Rip-Cre* mice. To confirm successful Cre-induced recombination, genomic DNA from islets isolated from $Pyy^{tg/+}$/*Rip-Cre* and littermate *Rip-Cre* mice was extracted and PCR was performed using primers that will only generate a product when recombination and deletion of the stop cassette has occurred (420-bp amplicon). This was confirmed by a positive band for recombination only detected in DNA isolated from pancreatic islets of $Pyy^{tg/+}$/*Rip-Cre* mice but not in DNA from *Rip-Cre* mice islets (Figure 1B). To further confirm that the genomic rearrangement leads to the production of the PYY transgene in β-cells, immunofluorescent costaining for insulin and PYY was performed on the pancreas sections from $Pyy^{tg/+}$/*Rip-Cre* and control mice. Our results showed that PYY-positive cells in $Pyy^{tg/+}$/*Rip-Cre* mice were not only found at the islet periphery where PYY is normally expressed (as shown in control islet), but also found within the center of islets where β-cells reside (Figure 1C). More importantly, these PYY-positive cells located in the center of the islet were also positive to insulin (costaining in yellow). More representative images of islet positive to both insulin and PYY are shown in Supplemental Figure 1A. Coexpression of both insulin and PYY confirmed that PYY peptide was specifically up-regulated in β-cells of $Pyy^{tg/+}$/*Rip-Cre* but not *Rip-Cre* mice (Figure 1C), whereas endogenous PYY expression in peripheral α-cells of $Pyy^{tg/+}$/*Rip-Cre* mice was similar to that in control mice. Because previous reports (26, 27) have suggested that *Rip-Cre* activity seems to occur in certain brain regions but not other peripheral tissues, we tested the expression of PYY peptide in hypothalamic Arc and PVN, brain regions that are critical in the regulation of energy and glucose homeostasis. Importantly, no obvious production of PYY was detected in the hypothalamus (Figure 1D), and as a positive control, only a few cells in the brainstem where PYY is endogenously expressed were positive for PYY (Figure 1D), suggesting that the CMV promoter in the brain is not very efficient

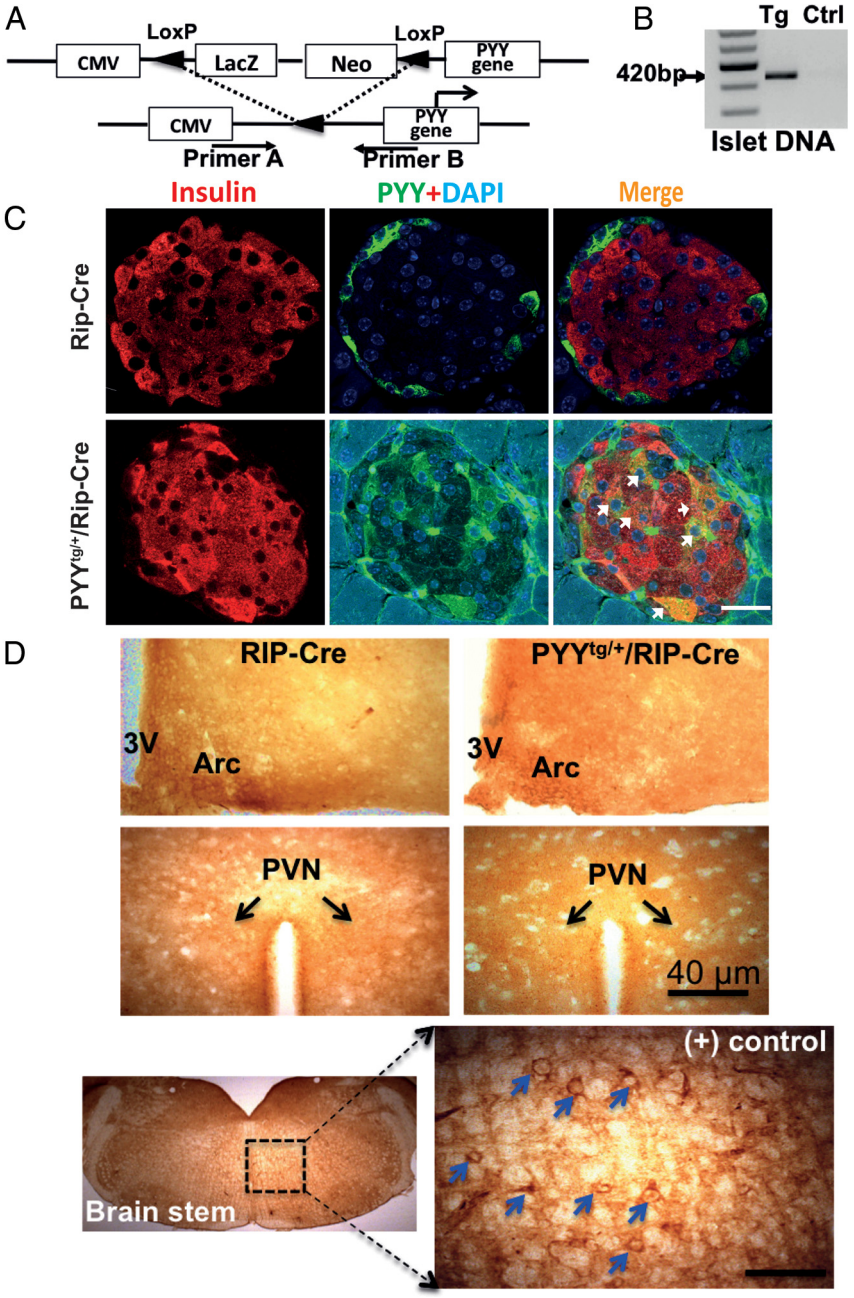

Figure 1. Evaluation of PYY overexpression in the pancreas and brain. A schematic diagram of the *Pyy* transgene construct is shown in A, and specific primers used in PCR for confirmation are labeled as primer A and B. PCR using gDNA extracted from isolated islets from $Pyy^{tg/+}$/*Rip-Cre* (Tg) and control (Ctrl) mice confirms successful Cre-induced recombination (B). Immunofluorescent costaining of insulin (red) and PYY (green) in the islets of Langerhans in the pancreas of $Pyy^{tg/+}$/*Rip-Cre* mice and controls confirmed that PYY is overexpressed in β-cells in the center of the islet (indicated by white arrow), in contrast to distinct peripheral location in control mice (C). Scale bar, 25 μm. Immunohistochemical staining of PYY (D) in the hypothalamic Arc (top panel) and PVN (middle panel) of $Pyy^{tg/+}$/*Rip-Cre* mice was compared with that in control mice, showing no difference, whereas PYY staining at the brainstem was used as a positive control (bottom panel, indicated by blue arrow). The images are representative of staining observed in tissues obtained from 15-week-old mice (3 mice per group). Scale bar, 5 μm.

to produce PYY. Therefore, the lack of detectable PYY production in the hypothalamus avoids any potential confounding effects that might be induced by the use of the *Rip-Cre* line.

Metabolic characteristics of pancreatic β-cell-specific *Pyy*-overexpressing mice

$Pyy^{tg/+}$/*Rip-Cre* mice were born in the expected Mendelian ratio and no significant differences in appearance or body weight were observed at birth between these mice and their control littermates (data not shown). $Pyy^{tg/+}$/*Rip-Cre* and control mice were fed a chow diet and body weight was monitored weekly from weaning onwards. $Pyy^{tg/+}$/*Rip-Cre* mice gained similar body weight to control mice when expressed in absolute weight (Figure 2A) or as a percent of initial body weight (Figure 2B). Similarly, spontaneous food intake (Figure 2C) and fasting-induced food intake (Figure 2D) of $Pyy^{tg/+}$/*Rip-Cre* mice were similar between $Pyy^{tg/+}$/*Rip-Cre* and control mice. In contrast to the anorexigenic effects of peripheral PYY, central PYY administration stimulates food intake (28, 29). However, no change in food intake in $Pyy^{tg/+}$/*Rip-Cre* mice was noted, consistent with the lack of PYY expression in the hypothalamus induced by the *Rip-Cre* activation.

Interestingly, dissected WAT weights from 4 depots (inguinal, epididymal, mesenteric, and retroperitoneal), and the sum of all adipose depots of $Pyy^{tg/+}$/*Rip-Cre* mice was significantly lower than that of *Rip-Cre* mice (Figure 2E), indicating that overexpression of PYY in islets can reduce fat mass, particularly epididymal fat. In addition, there was a marked increase in whole-body lean mass determined by dual-energy x-ray absorptiometry in $Pyy^{tg/+}$/*Rip-Cre* mice compared with their controls (Figure 2F). In order to investigate whether $Pyy^{tg/+}$/*Rip-Cre* mice displayed differences in energy expenditure, $Pyy^{tg/+}$/*Rip-Cre* and control mice were tested by indirect calorimetry. Interestingly, $Pyy^{tg/+}$/*Rip-Cre* mice displayed significantly higher VO_2 (Figure 2G) and energy expenditure than controls during both the dark phase and the light phase (Figure 2H). Similarly, RER was markedly higher in $Pyy^{tg/+}$/*Rip-Cre* mice compared with respective controls particularly during the dark phase (Figure 2I), indicating

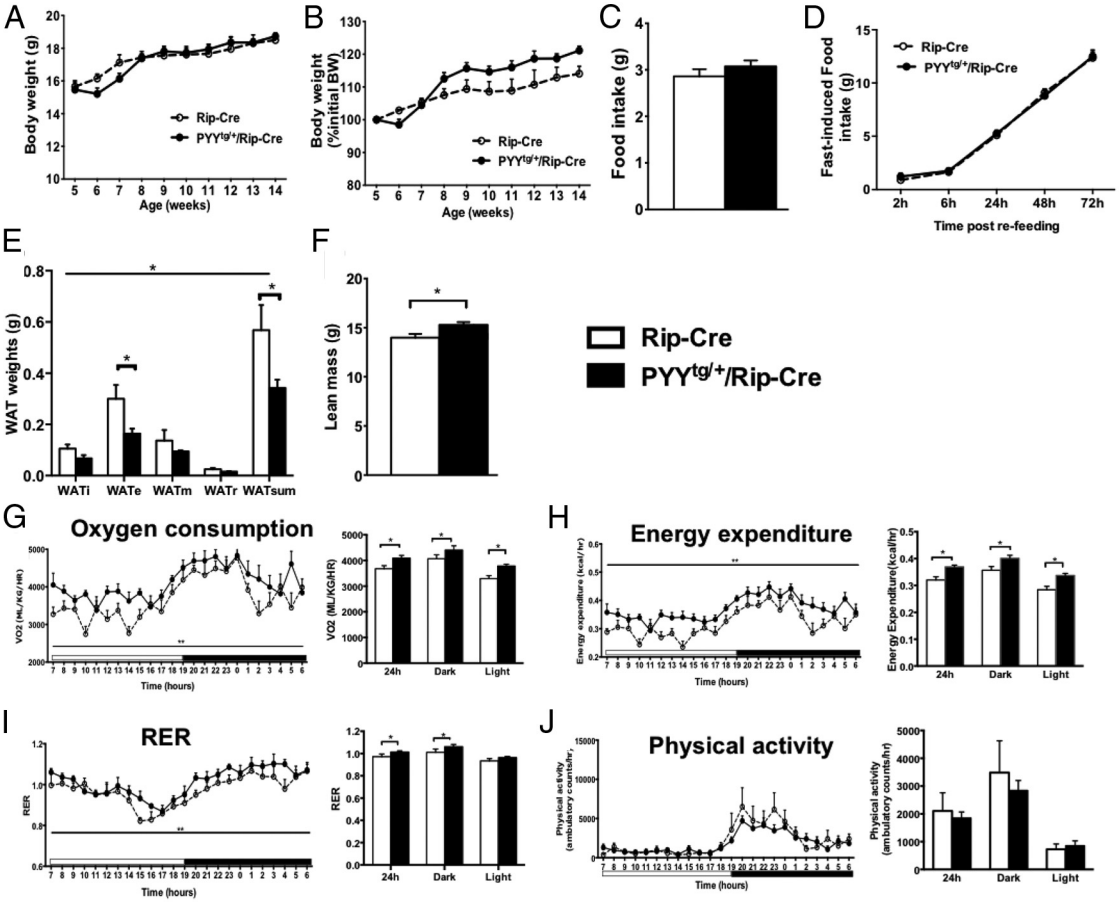

Figure 2. Metabolic characteristics of *Pyy*$^{tg/+}$*/Rip-Cre* vs littermate *Rip-Cre* mice. Body weights of *Pyy*$^{tg/+}$*/Rip-Cre* and *Rip-Cre* mice were expressed as absolute values (A) or as a percent of initial body weight (B). No significant difference in spontaneous food intake (C) or fasting-induced food intake (D) was observed compared with control mice. Dissected WAT weights at inguinal, epididymal, mesenteric, retroperitoneal WAT, and summed WAT weight (E) were markedly reduced in *Pyy*$^{tg/+}$*/Rip-Cre* mice, compared with their respective controls, whereas lean mass (F) was significantly increased *Pyy*$^{tg/+}$*/Rip-Cre* mice. VO_2 (G), energy expenditure (H), RER (I), and physical activity (J) are shown over 24-hour period, with open and filled horizontal bars indicating the light and dark phases, respectively, in *Pyy*$^{tg/+}$*/Rip-Cre* and *Rip-Cre* mice at 14 weeks of age. The bar graphs next to each parameter represents changes over 24 hours, separating dark phase and light phase. For comparison of energy expenditure by analysis of covariance (ANCOVA), the common lean mass was 13.98 g. Data are means ± SEM of 8–12 mice per group. *, $P < .05$ vs controls.

that these mice prefer to use carbohydrate as a fuel. On the other hand, ambulatory activity was not affected by overexpression of islet PYY (Figure 2J), suggesting that enhanced energy expenditure in these mice is not due to the alteration in physical activity.

Overexpression of pancreatic islet PYY improves glucose homeostasis and enhances insulin action

Having established alterations in fat mass and energy expenditure in mice overproducing PYY in the islets, we next examined the impact of elevated islet PYY levels on whole-body glucose homeostasis. Interestingly, basal serum insulin levels both at fed and fasted conditions were significantly increased (Figure 3, A and B) in *Pyy*$^{tg/+}$*/Rip-Cre* mice, whereas no differences in fed and fasted glucose levels were noted (Figure 3, C and D). To investigate the impact of β-cell-specific PYY overexpression on whole-body glucose clearance, we conducted ip GTT in 13-week-old *Pyy*$^{tg/+}$*/Rip-Cre* and control mice. Compared with age-matched control mice, *Pyy*$^{tg/+}$*/Rip-Cre* mice showed improved glucose tolerance (Figure 3E) as well as reflected in significantly decreased area under the curve (Figure 3F). Although fasting insulin level at the start of the GTT was elevated in *Pyy*$^{tg/+}$*/Rip-Cre* mice, the relative increase during GTT was less than that in control mice (Figure 3G). More importantly, *Rip-Cre* mice were reported to show defective first phase GSIS on some genetic background (30) but not others (31). Notwithstanding this, islet-specific PYY overexpression leads to elevated insulin levels relative to *Rip-Cre* controls (Figure 3G). On the other hand, when insulin responsiveness test was performed on these mice, a trend towards enhanced insulin responsiveness in *Pyy*$^{tg/+}$*/Rip-Cre* mice compared with control mice was observed (Figure 3H).

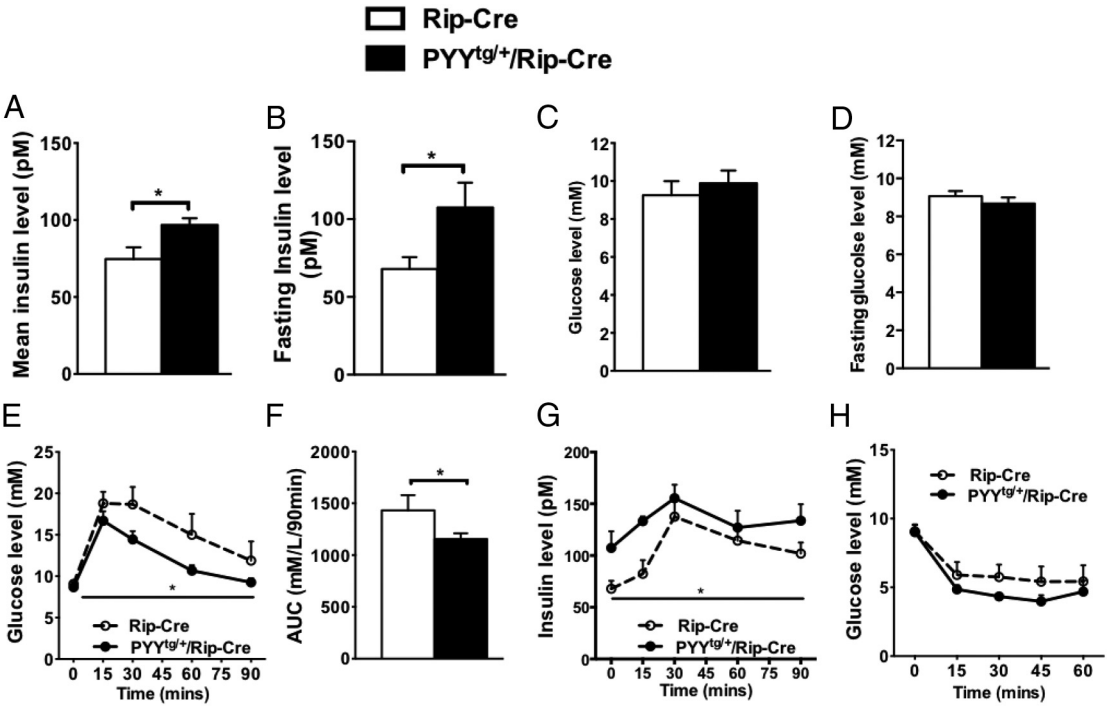

Figure 3. Overexpression of PYY in the islets improves glucose homeostasis in mice. Nonfasted serum insulin level (A) and fasted insulin level (B) in *Pyy*$^{tg/+}$*/Rip-Cre* and *Rip-Cre* mice at 15 weeks of age. Serum glucose level under nonfasted (C) and fasted (D) conditions, the change in blood glucose levels (E), area under curve (AUC) (F), and insulin levels (G) during a GTT in *Pyy*$^{tg/+}$*/Rip-Cre* and *Rip-Cre* mice at 12 weeks of age. Glucose levels during an ITT are shown in H. Data are means ± SEM of 8–12 mice per group. *, $P < .05$ vs controls.

To understand the possible mechanism underlying the increased insulin level and improved glucose clearance, we examined whether β-cell-specific PYY overexpression had any effects on islets themselves. Immunofluorescent costaining for insulin (green) and glucagon (red) on pancreas sections did not reveal any gross abnormalities in islet morphology and architecture (Figure 4A). Interestingly, compared with control islets, there was less insulin staining in *Pyy*$^{tg/+}$*/Rip-Cre* islets (Figure 4A), whereas glucagon staining was similar between the groups. This implies a possible decrease in insulin content in *Pyy*$^{tg/+}$*/Rip-Cre* islets, which is associated with increased serum insulin levels (Figure 3A). Interestingly, islet number (Figure 4B) and average islet size (Figure 4C) were significantly increased in *Pyy*$^{tg/+}$*/Rip-Cre* mice compared with controls. Moreover, the distribution of islet size between control and *Pyy*$^{tg/+}$*/Rip-Cre* mice revealed a significant increase in the average size of individual islets (Figure 4D). β-cell mass was also significantly increased in *Pyy*$^{tg/+}$*/Rip-Cre* mice (Figure 4E), whereas pancreas weight was not affected (Figure 4F). These results raise the possibility that the enhanced β-cell proliferation may contribute, at least partially, to the increased insulin levels in islet-specific PYY-overexpressing mice. To test this, immunofluorescent costaining of insulin and Ki67, a cellular marker of cell proliferation, was performed. In line with increased β-cell mass, there were more Ki67-positive β-cells in *Pyy*$^{tg/+}$*/Rip-Cre* mice as compared with control islets (Figure 5A), as evidenced by nuclei staining in β-cells (Figure 5B). Quantification of Ki67-positive β-cells confirmed the suggestion of an increased rate of β-cell proliferation in *Pyy*$^{tg/+}$*/Rip-Cre* mice (Figure 5C). To examine whether islet composition was also altered in *Pyy*$^{tg/+}$*/Rip-Cre* mice, we quantified the areas of insulin-stained cells and total islet cells. The proportion of β-cells within islets was similar in *Pyy*$^{tg/+}$*/Rip-Cre* and control mice (Supplemental Figure 1B). β-cell function was then assessed using ex vivo GSIS assay on islets isolated from *Pyy*$^{tg/+}$*/Rip-Cre* and control *Rip-Cre* mice. At basal glucose level (2mM glucose), islets from *Pyy*$^{tg/+}$*/Rip-Cre* mice released significantly more insulin compared with control islets (Figure 5D). This enhanced basal insulin secretion correlates with the elevated insulin level in the serum of *Pyy*$^{tg/+}$*/Rip-Cre* mice (Figure 3A). At high-glucose concentration (20mM), *Pyy*$^{tg/+}$*/Rip-Cre* islets displayed a tendency for increased insulin release compared with control islets, but the difference was not statistically significant.

Next, to explore the underlying mechanisms that led to increased β-cell mass and insulin release associated with islet-specific PYY overexpression, we first measured changes in the mRNA levels of islet-associated transcription factors as well as genes involved in β-cell function in islets from *Pyy*$^{tg/+}$*/Rip-Cre* and control *Rip-Cre* mice. Interestingly, expression levels of several transcription fac-

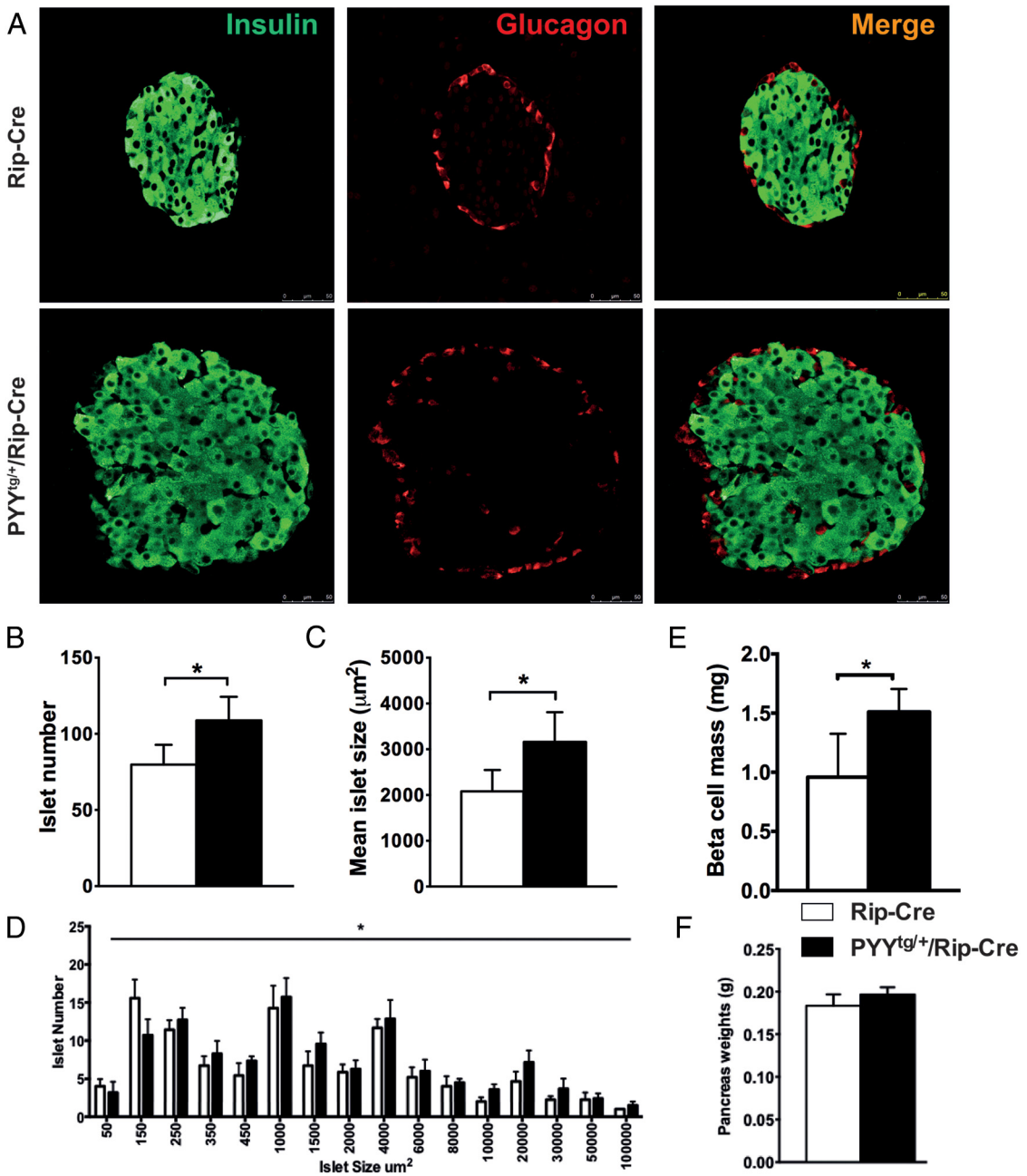

Figure 4. Effects of overexpression of PYY in the islets on islet morphology, islet number, islet size, β-cell mass, and the size of pancreas. Immunofluorescent costaining for insulin and glucagon of islets (A) from *Pyy*$^{tg/+}$/*Rip-Cre* mice and *Rip-Cre* mice. Average islet number and size (B and C) and distribution of islets (D) in 15-week *Pyy*$^{tg/+}$/*Rip-Cre* and *Rip-Cre* mice. β-cell mass is shown in E and pancreas weight (F) in *Pyy*$^{tg/+}$/*Rip-Cre* and *Rip-Cre* mice was measured at 15 weeks of age. Data are means ± SEM of 8–12 mice per group. *, $P < .05$ vs controls. The images were representatives of 3–4 mice per group. Scale bar, 50 μm.

tors important for islet development and the maintenance of the β-cell phenotype were up-regulated in *Pyy*$^{tg/+}$/*Rip-Cre* islets; *NeuroD1* (Figure 6A) and *MafA* (Figure 6B) mRNA levels were significantly increased, whereas *Pdx1* (Figure 6C) mRNA levels tended to be increased. The up-regulation of β-cell-specific transcription factors *NeuroD1* and *MafA* is in line with increased insulin levels under fed and fasting states. On the other hand, mRNA expression of peroxisome proliferator-activated receptor-γ (*Pparγ*) was markedly down-regulated in *Pyy*$^{tg/+}$/*Rip*$^{Cre/+}$ islets as compared with control islets (Figure 6D).

To trigger insulin release, glucose is transported into β-cells via the glucose transporter *Glut2*, phosphorylated by the glucose sensor glucokinase (*Gk*), and metabolized by glycolysis and the Krebs cycle resulting in an increase in the ATP to ADP ratio. The latter leads to the closure of ATP-sensitive K^+ (K_{ATP}) channels, membrane depolarization, subsequent opening of voltage-dependent Ca^{2+} channels (*Vdccs*), Ca^{2+} influx, and ul-

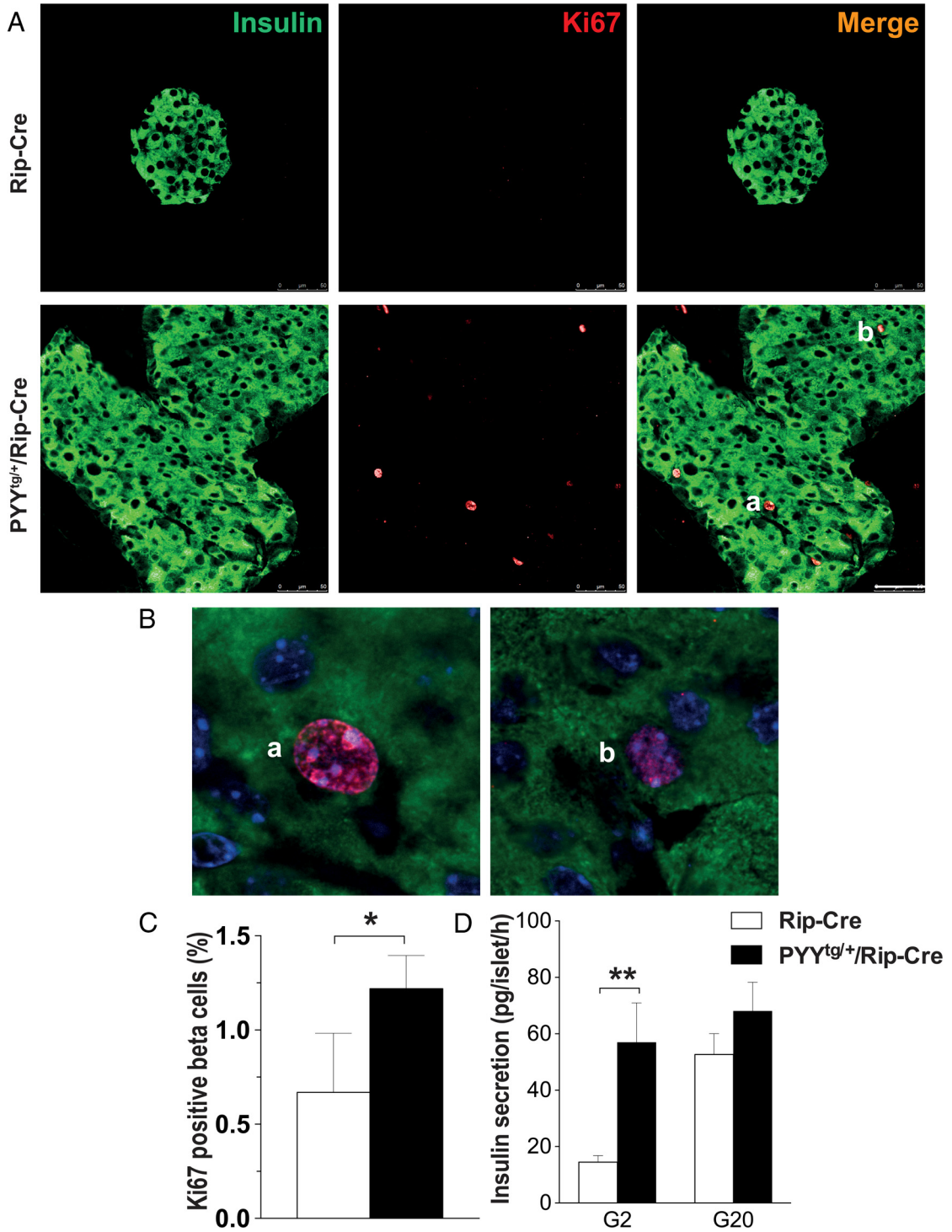

Figure 5. Effect of islet-specific PYY overexpression on β-cell proliferation and ex vivo analysis of glucose induced insulin secretion. Immunofluorescent costaining of insulin and Ki67 is shown in A with higher magnification of Ki67-positive cells in B. Quantification of Ki67-positive β-cells is shown in C. Ex vivo analysis of GSIS of islets from *Pyy*$^{tg/+}$*/Rip-Cre* mice and *Rip-Cre* mice is represented in D. The images were representatives of 3–4 mice per group. Scale bar, 50 μm. Data are means ± SEM of 8–12 mice per group. *, $P < .05$ vs controls.

timately insulin granule exocytosis (32). We then evaluated whether these genes were impacted by PYY overexpression in β-cells. Although there was no significant change in *Glut2* mRNA levels between the 2 groups (Figure 6E), the mRNA levels of sulfonylurea receptor 1 (*Sur1*), the regulatory subunit of the K_{ATP} channel, were increased by 2-fold in *Pyy*$^{tg/+}$*/Rip-Cre* islets (Figure 6F). There was also a tendency for increased mRNA levels of *Kir6.2* ($P = .068$) (Figure 6G), the pore-forming subunit of the K_{ATP} channel. Additionally, mRNA expression of the subunit of the Ca^{2+} channel *Vdcca1d* was signifi-

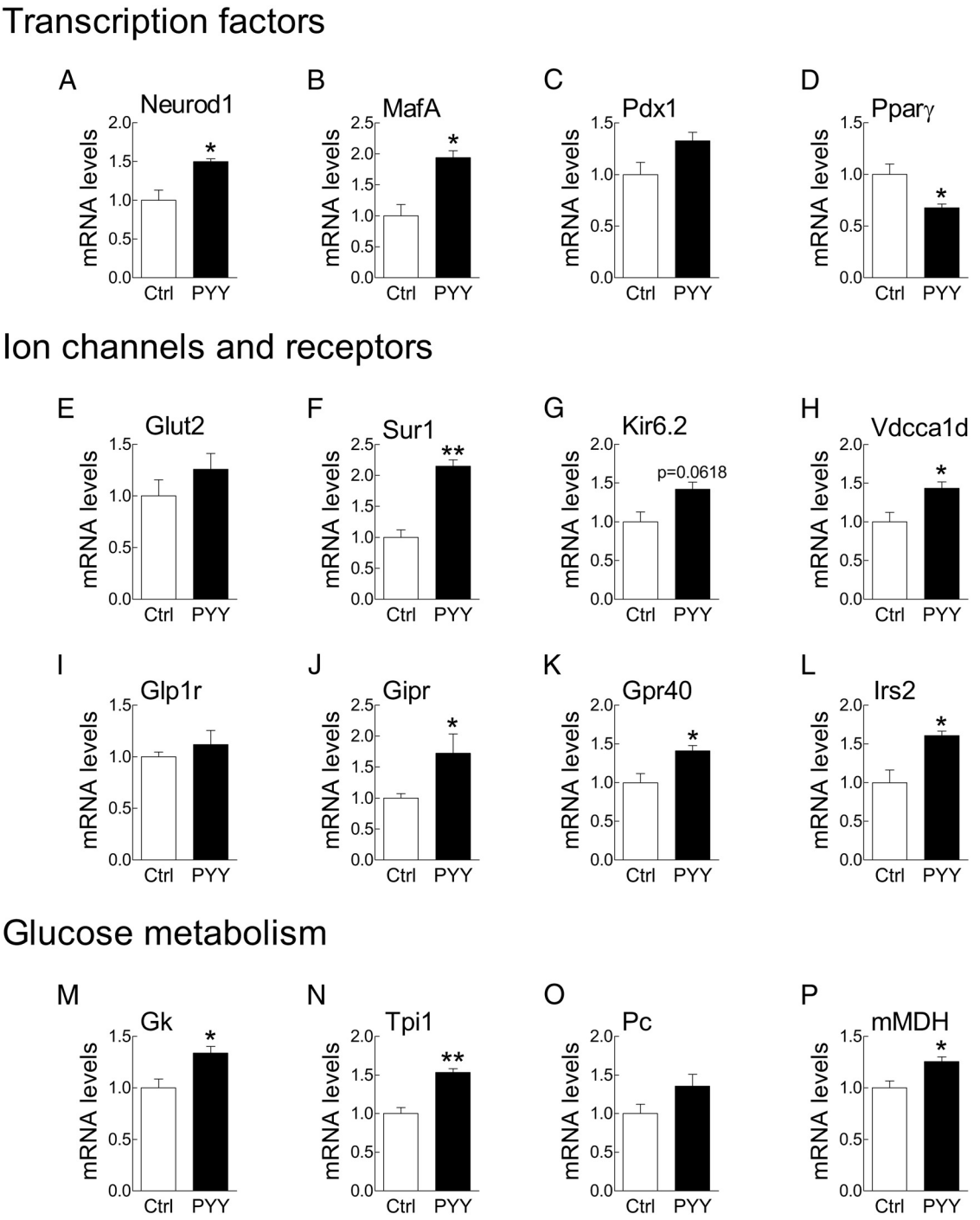

Figure 6. Effects of overexpression of islet-specific PYY on expression of islet-associated genes important in islet development and maintenance of the β-cell phenotype, insulin secretion, and glucose metabolism. Changes in the mRNA levels of islet-associated transcriptional factors (A–D) and genes that are involved in regulating insulin secretion (I–L) and glucose metabolism (M–P). Data are means ± SEM of 8–12 mice per group. *, $P < .05$ vs controls.

cantly increased (Figure 6H). Besides these increases in expression of components of the insulin secretion pathway (32), PYY overexpression in β-cells also increased the expression of genes involved in amplifying the pathway of insulin secretion (32). Thus, the mRNA levels of gastric inhibitory polypeptide receptor (*Gipr*) (Figure 6J) and G protein-coupled receptor 40 (*Gpr40*) (Figure 6K) were up-regulated in *Pyy*$^{tg/+}$*/Rip-Cre* islets, whereas there was no alteration in glucagon-like peptide 1 receptor (*Glp1r*) mRNA levels (Figure 6I). Importantly, *Pyy*$^{tg/+}$*/Rip-Cre* islets also displayed increased expression of insulin receptor substrate 2 (*Irs2*) (Figure 6L) in association with the increase in β-cell proliferation (Figure 5, A–C).

We also assessed changes in the expression of several genes involved in β-cell glucose metabolism. Interest-

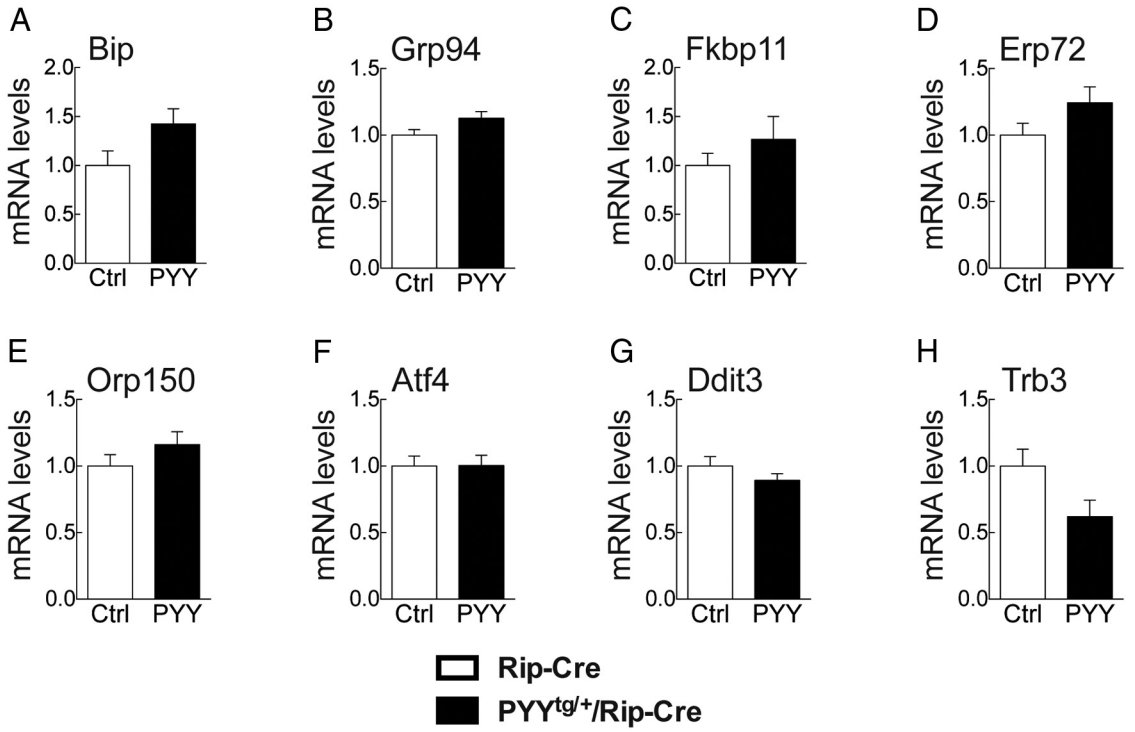

Figure 7. Effect of islet-specific PYY overexpression on ER stress in β-cells isolated from *Pyy*$^{tg/+}$*/Rip-Cre* mice and *Rip-Cre* mice. Changes in the mRNA levels of unfolded protein response and ER stress genes in the islets of *Pyy*$^{tg/+}$*/Rip-Cre* and *Rip-Cre* mice were shown in A–H. Data are means ± SEM of 8–12 mice per group. *, $P < .05$ vs controls.

ingly, expression levels of the glycolytic genes *Gk* (Figure 6M) and triosephosphate isomerase 1 (*Tpi1*) (Figure 6N) were significantly increased in *Pyy*$^{tg/+}$*/Rip-Cre* islets. The expression of the anaplerotic enzyme pyruvate carboxylase (*Pc*) (Figure 6O) tended to be increased and the mRNA levels of mitochondrial malate dehydrogenase were significantly increased in *Pyy*$^{tg/+}$*/Rip-Cre* islets (Figure 6P). These results are all in line with an elevated insulin secretion. In addition, we examined whether islet-specific PYY overexpression altered endoplasmic reticulum (ER) homeostasis by analyzing the expression of a panel of unfolded protein response genes. The expression ER stress-inducible chaperones, folding enzymes, and proapoptosis transcription factors was similar in *Pyy*$^{tg/+}$*/Rip-Cre* and control islets (Figure 7, A–H), suggesting the absence of ER stress with PYY overexpression.

Taken together, the data suggest that PYY overexpression in islets affects multiple pathways involving β-cell proliferation, glucose metabolism, and insulin release, thereby contributing to improved glucose tolerance.

Discussion

In this study, we demonstrate a critical role for pancreas-derived PYY in β-cell function as well as general glucose homeostasis regulation. Using a novel pancreatic islet-specific PYY overexpression mouse model, we found that elevated PYY levels only in the pancreatic β-cells significantly improved glucose tolerance. Moreover, this ameliorated glucose response is likely due to increased β-cell mass as well as an enhanced insulin release. Importantly, these changes occur via alterations in several key regulatory transcription factors and genes playing a critical role in β-cell proliferation, maintenance of β-cell phenotype, glucose metabolism, and insulin secretion. Importantly, in addition to the influence on whole-body glucose metabolism, islet-specific PYY overproduction leads to decreased fat weights and increased lean mass. These phenomena were associated with increased energy expenditure and RER, but in the absence of any change in body weight and food intake. Together, findings from this study reveal for the first time a critical dual role of pancreas-derived PYY in the control of both glucose and energy homeostasis.

Pancreatic β-cell PYY has a crucial impact on the β-cell phenotype

Insulin synthesis and release from β-cells are controlled by multiple signals in a concerted manner. Although previous studies have shown that PYY is expressed in all cell types at the development stage of pancreas (10, 11), in-

cluding β-cells, PYY can only be found in α-cells in adult pancreas. The presence of PYY in the pancreatic α-cells indicates a potential role of PYY in the local regulation of islet physiological function. It has been postulated that PYY1–36, which is believed to be the active form of PYY in islet α-cells (33), could be released locally and act on the Y1 receptors on the β-cells to inhibit insulin secretion in a paracrine fashion. However, we observed elevated serum insulin level in the nonfasted and fasted condition as well as enhanced insulin secretion at basal glucose conditions ex vivo in isolated islets. The increased serum insulin levels could be in part explained by increased β-cell mass. Interestingly, in ex vivo islets, the marked increase in basal insulin secretion suggests β-cell hypersensitivity. The latter may explain the elevated basal plasma insulin levels in fed and fasted *Pyy*$^{tg/+}$*/Rip-Cre* mice. However, the absence of an equivalent enhancement at high glucose (20mM) may indicate a loss of glucose-responsive insulin secretion with PYY overexpression. Therefore, other regulatory factors may contribute to the glucose-induced changes in plasma insulin levels in *Pyy*$^{tg/+}$*/Rip-Cre* mice. Such factors, which are absent ex vivo, may include amino acids, neurotransmitters, fatty acids, and incretin hormones. Indeed, we observed a significant increase in the expression of *Gipr* and *Gpr40*, both involved in the amplification of GSIS (34–36). Interestingly, gastric inhibitory polypeptide has also been proposed to stimulate β-cell proliferation and inhibit apoptosis (37).

It is possible that PYY in β-cells could directly interact with the machinery of β-cell proliferation, and/or insulin secretion, thereby increasing insulin release and improving glucose tolerance. However, we cannot rule out the possibility of β-cell-released PYY binding to Y1 receptors on the β-cells to regulate β-cell function in an autocrine manner. This could explain, at least partially, the discrepancy between the stimulatory role of β-cell-derived PYY and the inhibitory role of PYY in the α-cells in the regulation of insulin secretion. Interestingly, an earlier study has also shown that the mice with adult onset, pancreas-specific ablation of PYY expressing cells display disrupted islet morphology and significantly reduced insulin secretion (13), which does not appear to be in line with the inhibitory role of α-cell-derived PYY. More work is warranted to delineate the specific role of PYY from different sources. Nonetheless, these data are clearly suggestive of a critical role of islet PYY in β-cell function and proliferation, as well as supporting the possibility that expression of PYY in different cells could have differential effects on β-cell function.

In this study, we also demonstrate that similar to gut-derived PYY, locally produced PYY within the β-cells was able to alter glucose homeostasis, as evidenced by improved whole-body glucose tolerance, consistent with the phenotypes observed in global PYY transgenic mice (38). This suggests that in addition to gut-released PYY, islet-specific PYY may also contribute to the beneficial phenotypes induced by global PYY overexpression (38). Furthermore, the beneficial effects elicited by β-cell PYY on glucose metabolism were achieved, at least in part, by increasing β-cell mass.

In keeping with the significant increase in β-cell mass and insulin release, mechanistically, we observed a range of changes in β-cell-specific transcription factors as well as genes involved in glucose metabolism and β-cell function. Thus, in association with increased insulin secretion and β-cell proliferation, increased mRNA levels of *NeuroD1* and *MafA*, and to a lesser extent *Pdx1*, 3 key β-cell transcription factors that play major roles in β-cell development and function (39–47), clearly indicates the important impact of islet PYY in maintaining the β-cell phenotype. This is also in line with the notion that PYY is coexpressed in all islet cell types, including β-cells at the early stage of pancreas development (11). On the other hand, the reduced expression of *Pparγ* may also contribute to the up-regulation of insulin secretion and increased β-cell proliferation. Indeed, although *Pparγ* ablation showed its limited role in normal β-cell physiology (48), its overexpression under condition of increased insulin demand and secretion revealed a negative impact on β-cell mass in association with altered glucose tolerance and increased islet cell apoptosis (49). Together, reduced *Pparγ* mRNA levels and increased β-cell-specific genes mRNA levels in the islets of *Pyy*$^{tg/+}$*/Rip-Cre* mice could explain the increased nonfasted and fasted insulin level and ameliorated whole-body glucose tolerance.

PYY overexpression in β-cells also leads to increased expression of key components of the stimulation-secretion coupling pathway of insulin secretion in β-cells, namely *Sur1*, *Vdcca1d*, and to a lesser extent *Kir6.2*. A link between the loss of *Sur1* in islets and attenuated GSIS is well established (50). Besides, *Sur1* and *Vdcca1d* expression has been shown to be down-regulated in β-cells of human type 2 diabetic subjects and animal models of diabetes (51). Reduced *Kir6.2* expression is also associated with β-cell dysfunction (52–54). These results indicate an increased secretory function of β-cells in PYY overexpression mice, which may explain in part the hypersecretion observed in transgenic islets under basal glucose conditions. Interestingly, these changes in K_{ATP} channel protein expression were also accompanied by up-regulation of genes involved in glucose metabolism, including *Gk*, *Tpi1*, mitochondrial malate dehydrogenase, and to a lesser extent *Pc*. These changes may also contribute to the observed enhanced insulin secretion in transgenic animals. Indeed, hypersensitivity to glucose in rat islet correlated with increased expression and activity of *Gk* (55).

Moreover, because increased *Irs2* expression promotes β-cell growth and especially survival, whereas decreased *Irs2* level leads to reduced proliferation (23) and apoptosis (3, 56), increased islet *Irs2* mRNA expression together with increased Ki67-positive β-cells in islets from islet-specific PYY-overexpressing mice strongly suggests improved β-cell proliferation and survival, which could subsequently result in increased β-cell mass. Cells that are stimulated to secrete large amounts of protein over a short period of time are highly dependent on a functional unfolded protein response (57), interestingly, PYY overexpression in the β-cells enhances insulin release without inducing ER stress.

Thus, together with changes in several key transcription factors that are important for β-cell development, and the maintenance of the β-cell phenotype, these data strongly support a critical role of locally produced pancreatic PYY on the regulation of β-cell mass as well as insulin release via the modulation of genes involved in multiple signaling and metabolic processes in β-cells.

Pancreatic islet PYY is important in whole-body energy metabolism

Our study also revealed that elevated PYY level in the pancreatic islets elicits marked changes in adiposity, lean mass, energy expenditure, and/or RER in $Pyy^{tg/+}$/*Rip-Cre* mice, however, does not influence body weight and food intake. Increased lean mass in $Pyy^{tg/+}$/*Rip-Cre* mice could contribute to increased energy expenditure therefore influencing RER. Decreased fat mass and increased lean mass could also contribute to improved whole-body glucose metabolism, suggesting dual benefits of islet-derived PYY in both glucose and energy metabolism. Furthermore, the effect of islet-specific PYY overexpression on glucose metabolism is most likely to be a direct and local response, whereas its action on energy homeostasis is secondary to the changes in islets. Additionally, locally produced PYY could modulate β-cell function and secretion of insulin that can release into the circulation to impact other peripheral tissues, such as adipose tissue, and elicit whole-body changes. Because pancreatic polypeptide, which is exclusively produced in specific cells of the islet, gets released into the circulation to have effects on target receptors in the brain to induce satiety, it is plausible for islet PYY to also get into the circulation, and act in an endocrine fashion, thereby directly influencing insulin action on target tissues for example.

It is important to mention that when interpreting the results, we were aware of the fact that *Rip-Cre* mice have been reported to have impaired glucose tolerance due to the failure of insulin release during the first phase in response to glucose stimulation (30). However, other colonies do not show this phenotype (31). In the present study, our islet-overproducing PYY mice were able to normalize the impairment of glucose tolerance relative to *Rip-Cre* control mice, further supporting a beneficial role of islet PYY in the control of glucose homeostasis and also highlighting the importance of using appropriate controls in this type of study.

In summary, this study shows for the first time the critical role of pancreatic PYY in the regulation of energy and glucose homeostasis as well as β-cell function. The results of this study suggest that PYY released from different sources could work together to control energy and glucose metabolism. More importantly, understanding the mechanisms by which islet PYY regulates insulin release could aid the identification of potential therapeutic targets to modulate insulin secretion. Hence, in combination with current antiobesity drugs, therapeutics modulating pancreatic PYY activity could offer augmented efficacy and effectiveness, not only through modulating insulin secretion locally but also influencing whole-body energy balance. This would potentially lead to reductions in adiposity and subsequently improving the clinical outcome of obese and diabetic patients.

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