$G\alpha_{i/o}$-coupled receptor signaling restricts pancreatic β-cell expansion

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Gi-GPCRs, G protein-coupled receptors that signal via Gα proteins of the i/o class ($G\alpha_{i/o}$), acutely regulate cellular behaviors widely in mammalian tissues, but their impact on the development and growth of these tissues is less clear. For example, Gi-GPCRs acutely regulate insulin release from pancreatic β cells, and variants in genes encoding several Gi-GPCRs—including the α-2a adrenergic receptor, ADRA2A—increase the risk of type 2 diabetes mellitus. However, type 2 diabetes also is associated with reduced total β-cell mass, and the role of Gi-GPCRs in establishing β-cell mass is unknown. Therefore, we asked whether Gi-GPCR signaling regulates β-cell mass. Here we show that Gi-GPCRs limit the proliferation of the insulin-producing pancreatic β cells and especially their expansion during the critical perinatal period. Increased Gi-GPCR activity in perinatal β cells decreased β-cell proliferation, reduced adult β-cell mass, and impaired glucose homeostasis. In contrast, Gi-GPCR inhibition enhanced perinatal β-cell proliferation, increased adult β-cell mass, and improved glucose homeostasis. Transcriptome analysis detected the expression of multiple Gi-GPCRs in developing and adult β cells, and gene-deletion experiments identified ADRA2A as a key Gi-GPCR regulator of β-cell replication. These studies link Gi-GPCR signaling to β-cell mass and diabetes risk and identify it as a potential target for therapies to protect and increase β-cell mass in patients with diabetes.

islet | β cell mass | perinatal | G-protein coupled receptors | diabetes mellitus

The G protein-coupled receptors (GPCRs), including Gi-GPCRs (1), comprise the largest family of mammalian cell-surface receptors and the largest target group for Food and Drug Administration-approved drugs (2), including drugs used to treat diabetes (3). Gi-GPCR gene variants associated with human diseases are thought to influence disease risk by modifying acute cellular behaviors such as insulin release (3–9). However, diabetes also is associated with decreased pancreatic β-cell mass (10–13), and little is known about the role Gi-GPCR signaling plays in organ development or size or whether Gi-GPCR variants could impact disease risk by altering organ size.

Pancreatic β cells respond acutely to signaling through multiple GPCRs by altering insulin secretion. Examples include the gut incretins GIP and GLP1, which stimulate insulin secretion in a glucose-dependent manner through their cognate $G\alpha_s$-linked GPC receptors, GIPR and GLP1R; acetylcholine, which stimulates insulin secretion in a glucose-independent manner through the $G\alpha_q$-linked cholinergic receptor, muscarinic 3 (CHRM3); and catecholamines and somatostatin, which inhibit insulin secretion through the Gi-GPCRs α2A adrenergic receptor (ADRA2A) and SSTR3, respectively (3, 14). Variants in the genes encoding GIPR and ADRA2A alter the risk of type 2 diabetes (5, 6, 15).

Insulin secretory capacity depends on both the secretory capacity of individual β cells and total β-cell mass, which is reduced in both type 1 and type 2 diabetes (10–13). Two sources contribute to the pool of β cells in the pancreas: neogenesis from progenitor cells and proliferation of preexisting β cells. The β-cell population expands most dramatically during the perinatal and early postnatal period because of increased proliferation, which then falls markedly as adulthood approaches in both rodents and humans (16, 17).

Therefore, we asked whether Gi-GPCR signaling could modify diabetes risk by altering β-cell proliferation, especially during the perinatal expansion, when even modest changes in the high basal rates of proliferation potentially could have a large impact on final β-cell mass.

Results

To examine this question, we manipulated perinatal β-cell Gi-GPCR signaling and then measured glucose homeostasis in adult animals. First, we expressed a well-studied Gi-GPCR, serotonin receptor HTR1A, in developing islet cells under the control of the ePet1 enhancer from the *Fev* gene (Fig. S1*A*) (18, 19). The transgenic progeny of one ePet1-*Htr1a* transgenic founder displayed marked hyperglycemia (Fig. 1*A*) and failure to thrive and gain weight (Fig. 1*B*), together with marked reductions in β-cell numbers (*Htr1a*-H; Fig. 1*C*).

We could not study this transgenic line further because of premature mortality. Animals from a second ePet1-*Htr1a* transgenic line with a lower transgene copy number (*Htr1a*-L; Fig. S1*B*) bred normally and displayed normal weight gain (Fig. S2*A*) but also exhibited impaired glucose tolerance (Fig. 1*D*) and glucose-stimulated insulin release (Fig. 1*E*). Blood glucose decreased

Significance

This paper shows that a class of receptors known to modulate insulin release by pancreatic β cells also regulates the proliferation of these cells and restrains the perinatal β-cell expansion that establishes adult β-cell mass, suggesting that alterations in signaling by these receptors could contribute to the decreased β-cell numbers seen in patients with type 2 diabetes. Further, inhibition of signaling through these receptors potentially could be used to generate more β cells for people with diabetes.

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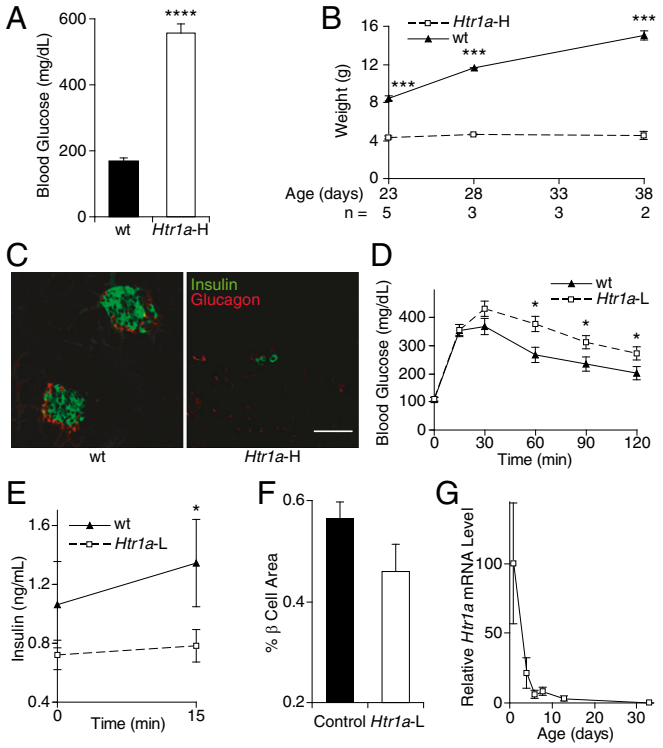

Fig. 1. Glucose metabolism in *ePet1-Htr1a* mice. (*A*) Blood glucose levels were measured in nonfasting *Htr1a*-H mice ($n = 3$) and nontransgenic control littermates ($n = 4$). (*B*) Weights of *Htr1a*-H mice and control littermates are shown. (*C*) Representative pancreatic sections from an *Htr1a*-H mouse and a control littermate at age P7 were stained for insulin (green) and glucagon (red). (Scale bar, 50 μm.) (*D* and *E*) Blood glucose ($n = 19$ *Htr1a*-L and 12 wild-type mice) (*D*) and plasma insulin ($n = 19$ *Htr1a*-L and 13 wild-type mice) (*E*) levels were measured at the indicated time points following i.p. glucose injection in adult mice. (*F*) β-Cell area was measured as a percent of total pancreatic area in adult control ($n = 17$) and *Htr1a-L* ($n = 12$) mice. (*G*) *Htr1a* mRNA levels were measured in the pancreata of *Htr1a*-L mice at the ages shown ($n = 5$–10 mice at each age). All data points represent the mean ± SEM. $^*P < 0.05$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs. wild-type animals by two-tailed Student's *t* test.

normally in these mice after insulin injection (Fig. S2*B*), making impaired insulin sensitivity an unlikely cause of their impaired glucose tolerance. The *Htr1a*-L adults also had ∼30% decreased β-cell mass (Fig. 1*F*). After high perinatal expression, *Htr1a* expression shut off within 2 wk after birth in *Htr1a*-L animals (Fig. 1*G*), suggesting that HTR1A signaling during perinatal β-cell expansion led to persistent decreased β-cell mass and impaired glucose homeostasis in the adults.

Next we examined whether these adult metabolic effects of expressing a Gi-GPCR were specific to HTR1A or could be generalized to other Gi-GPCRs when expressed on perinatal β cells. We used the reverse tetracycline transactivator under control of the *rat insulin 2* gene promoter (RIP-rtTA) (20) to drive expression of the synthetic $G\alpha_{i/o}$-coupled receptor activated solely by synthetic ligand (RASSL) Ro1 (21, 22) on β cells specifically during perinatal development (βTet-Ro1 mice). Ro1 can be activated by the synthetic ligand spiradoline but has basal $G\alpha_{i/o}$ signaling activity in the absence of ligand (23). Perinatal expression of Ro1 on β cells even without spiradoline administration led to worsened adult glucose homeostasis (Fig. 2*A*), with reduced insulin release after glucose challenge despite the higher glucose levels (Fig. 2*B*).

Because perinatal transgenic expression of Gi-GPCRs on β cells restricted β-cell expansion and impaired adult insulin secretion and glucose homeostasis, we asked whether endogenous Gi-GPCRs regulate perinatal β-cell development and subsequent adult glucose homeostasis. To answer this question, we expressed the $G\alpha_{i/o}$ signaling inhibitor pertussis toxin (PTX) in β cells by crossing ROSA-PTX knockin mice to ePet1-cre transgenic mice (islet-PTX mice) (18, 24). These animals responded to a glucose-tolerance test with lower blood glucose elevations and markedly increased glucose-stimulated insulin secretion (Fig. 3 *A* and *B*), consistent with previous data (24).

Although we hypothesize that increased β-cell mass contributed to the improved glucose tolerance and insulin secretion observed in the islet-PTX mice, these improvements could result from decreased Gi-GPCR inhibition of insulin secretion alone, because PTX expression persists in adult β cells in this model. Therefore we blocked $G\alpha_{i/o}$ signaling selectively in developing β cells (Fig. S3) by treating mothers of RIP-rtTA/tetO-PTX double transgenic mice (βTet-PTX mice) with doxycycline until postnatal day 7. As adults, the βTet-PTX mice treated perinatally with doxycycline had improved glucose homeostasis (Fig. 3*C*), increased insulin release (Fig. 3*D*), and increased β-cell mass (Fig. 3*E*).

To determine how $G\alpha_{i/o}$ signaling modulates β-cell mass, we compared β-cell replication in FACS-purified β cells from MIP-GFP/βTet-PTX triple transgenic mice versus controls. We found an increased fraction of β cells in the G2/M phase of the cell cycle in neonates expressing PTX versus controls (Fig. 3*F* and Fig. S4). This result suggests that Gi-GPCRs suppress β-cell proliferation in a cell-autonomous manner during perinatal development, and this suppression in turn impacts adult β-cell mass and glucose homeostasis.

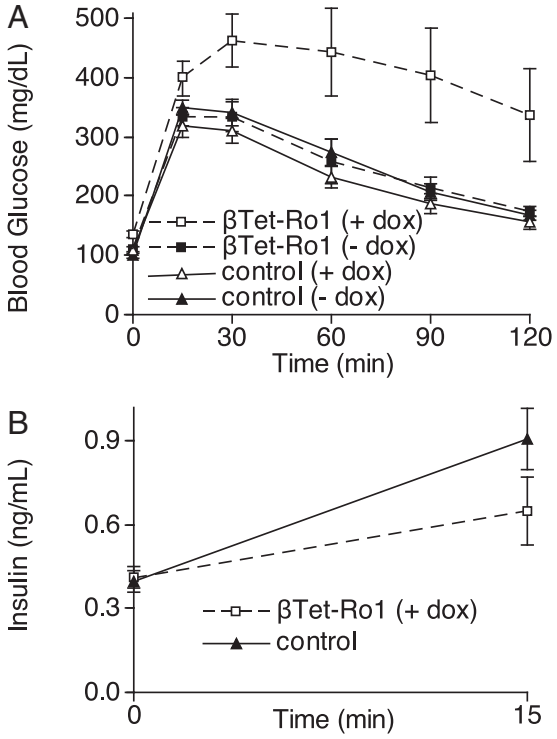

Fig. 2. Glucose metabolism in βTet-Ro1 mice. (*A*) Glucose-tolerance tests were performed in adult βTet-Ro1 mice and control littermates whose mothers were treated with or without doxycycline from conception until day P7. (*B*) Plasma insulin levels were measured before and 15 min after i.p. glucose injection in βTet-Ro1 mice ($n = 4$) and controls ($n = 17$). All data points represent the mean ± SEM. See Table S1 for weights and Tables S2 and S3 for statistical analysis of *A*.

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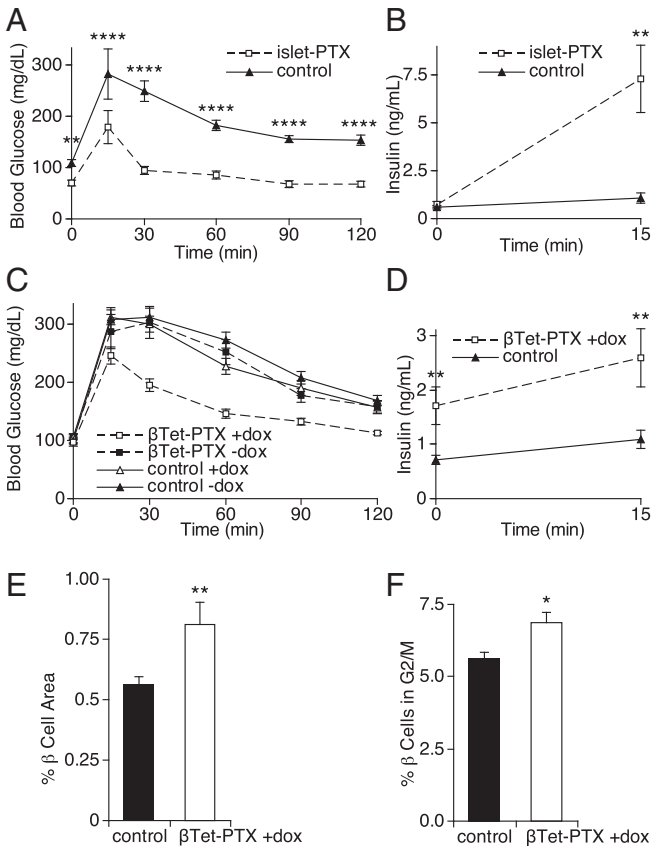

Fig. 3. Blocking $G\alpha_{i/o}$ signaling with PTX in β-cells. (*A*) Glucose-tolerance tests were performed in adult islet-PTX ($n = 7$) and control ($n = 7$) mice. (*B*) Plasma insulin levels were measured at the indicated times after i.p. glucose injection in islet-PTX ($n = 5$) and control ($n = 5$) mice. (*C*) Glucose-tolerance tests were performed in adult βTet-PTX mice and control littermates whose mothers were treated with or without doxycycline from conception until day P7. (*D*) Plasma insulin levels were measured at the indicated times after i.p. glucose injection in βTet-PTX ($n = 8$) and control ($n = 10$) mice. (*E*) β-Cell area was measured as a percent of total pancreatic area in adult βTet-PTX ($n = 7$) and control ($n = 17$) mice. (*F*) Replication rate in β cells from βTet-PTX ($n = 6$) and control ($n = 10$) mice at age P1 was measured by determining the percentage of β cells in the G2 and M phases of the cell cycle by flow cytometry. All data points represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$ vs. control animals by two-tailed Student's *t* test. See Tables S4 and S5 for weights for animals in *A* and *C*, respectively, and Tables S6 and S7 for statistical analysis of *C*.

If $G\alpha_{i/o}$ signaling constrains β-cell replication, which endogenous Gi-GPCRs mediate this effect? To examine this question, we quantified the expression of all nonolfactory GPCRs in β cells isolated from mice at embryonic day 18.5 (E18.5) and postpartum day 4 (P4) by RT-PCR (Fig. 4*A* and Tables S8 and S9). We found multiple mRNAs encoding Gi-GPCRs and G protein subunits in these cells, and many also were expressed robustly in adult β cells (Fig. 4*A* and Tables S9 and S10). Among the most highly expressed Gi-GPCR mRNAs in both perinatal and adult β cells was *Adra2a* mRNA, which has been implicated in type 2 diabetes (5, 6). To examine whether ADRA2A might mediate part of the $G\alpha_{i/o}$-mediated suppression of β-cell replication we observed in Fig. 3*F*, we measured β-cell replication in neonates lacking ADRA2A receptors. We found a 17% increase in neonatal β-cell replication in *Adra2a*-null animals, demonstrating that ADRA2A is an endogenous Gi-GPCR suppressor of neonatal β-cell replication (Fig. 4*B* and Fig. S5). Additionally, treatment with the ADRA2A agonist guanfacine reduced β-cell replication in isolated adult islets, and the ADRA2A antagonist rauwolscine blocked this effect (Fig. 4*C* and Fig. S6).

To determine the source of ligand for the ADRA2A receptor in perinatal β cells, we stained for catecholamine-producing sympathetic neurons. We detected tyrosine hydroxylase-expressing sympathetic neurons innervating pancreatic islets as early as day E17.5 (Fig. 4*D*). Interestingly, β cells also robustly express *Galr1* (Fig. 4*A*), which encodes a Gi-GPCR for galanin, another sympathetic neurotransmitter; and we found that galanin also inhibits β-cell replication (Fig. 4*E*).

Discussion

These studies shed light on a classic problem: How does a growing organ know when it has enough cells and should stop cell proliferation? Signals that restrain proliferation counter the more familiar forces driving proliferation and thereby set limits on organ growth. As in many biologic processes, this negative regulation is at least as important as positive regulation, and defects in negative regulation can have dire pathologic consequences. Limiting cell numbers may be particularly important for the β cell, as evidenced by the contraction in β-cell mass seen in settings such as starvation, insulinoma, or in maternal islets in the postpartum period (25–27). Our data demonstrate that $G\alpha_{i/o}$-coupled signaling restricts β-cell replication, titrating cell number to provide for the organism's metabolic needs.

Modulating β-cell Gi-GPCR signaling during the perinatal period, even just until 1 wk postpartum, had a lasting impact on adult β-cell mass and insulin secretory capacity. This perinatal sensitivity results from the high proliferation rates during the perinatal period. During fetal development, newly differentiated β cells initially are quiescent but enter a phase of rapid proliferation shortly before birth that lasts through the perinatal/early childhood period in both rodents and humans (16, 17, 28). Because the population size grows as an exponential function of the cellular replication rate, small changes in the replication rate have large effects on population size when the underlying replication rate is high. This sensitivity of adult β-cell mass to small perinatal changes in β-cell replication may explain how insults during the perinatal period, such as prematurity, intrauterine growth retardation, or nutrient restriction, increase the risk of diabetes as an adult in both animal models and humans (29–33).

Our results also suggest that the loss of sympathetic ligands working through their cognate Gi-GPCRs on β cells could explain the increased perinatal β-cell replication in mice lacking neurons in the pancreas (34) and may explain how activating variants in *ADRA2A* and stress contribute to diabetes risk in humans (5, 6, 35). Although important, sympathetic ligands alone likely do not explain all the PTX-sensitive inhibition of β-cell proliferation, and other Gi-GPCRs, such as Cnr1, along with non–cell-autonomous effects of $G\alpha_{i/o}$ signaling, surely contribute as well (24, 36–38).

Of course, Gi-GPCR inhibition is balanced by stimulators of β-cell proliferation, including $G\alpha_s$- and $G\alpha_q$-coupled GPCRs (39–41) such as the parasympathetic signaling target on β cells, CHRM3 (39, 42). Parasympathetic signaling through the vagus nerve also regulates β-cell mass (43–46) and has been implicated in the regulation of β-cell mass by the liver (47), although liver-specific secreted factors (48, 49) also may regulate β-cell proliferation directly (49).

Together, these data establish Gi-GPCR signaling in general and ADRA2A signaling in particular and implicate neural signaling and the CNS working through the autonomic nervous system as important determinants of β-cell proliferation and ultimately of β-cell population size, insulin production capacity, and metabolic control in the adult. These results have implications for understanding pancreatic islet development and the pathophysiology and pharmacotherapy of diabetes and could contribute to regenerative therapies for people with diabetes (50).

A

Gαi/o-linked GPCR Genes in β Cells			
Gene	E18.5 by RT-PCR		Adult RPKM
	Mean	SEM	
Cxcr6	63.17	21.52	0.20
Adra2a	22.67	4.80	58.67
Gabbr2	10.17	2.94	15.98
Aplnr	6.29	0.74	0.12
Galr1	4.91	1.55	16.51
Ccr9	4.60	1.65	0.030
Adora1	3.53	0.43	1.27
Sstr3	2.78	0.28	44.56
Gpr19	2.74	0.88	0.60
S1pr1	2.12	0.33	0.15
P2ry2	2.03	0.96	0.015
Niacr1	1.90	1.06	0.78
Gpr183	1.83	0.26	0.067
Gabbr1	1.66	0.45	4.06
Ednra	1.61	0.46	0.031
Ffar3	1.50	0.49	6.22
Ptger3	1.18	0.24	6.35
Casr	1.07	0.18	23.25

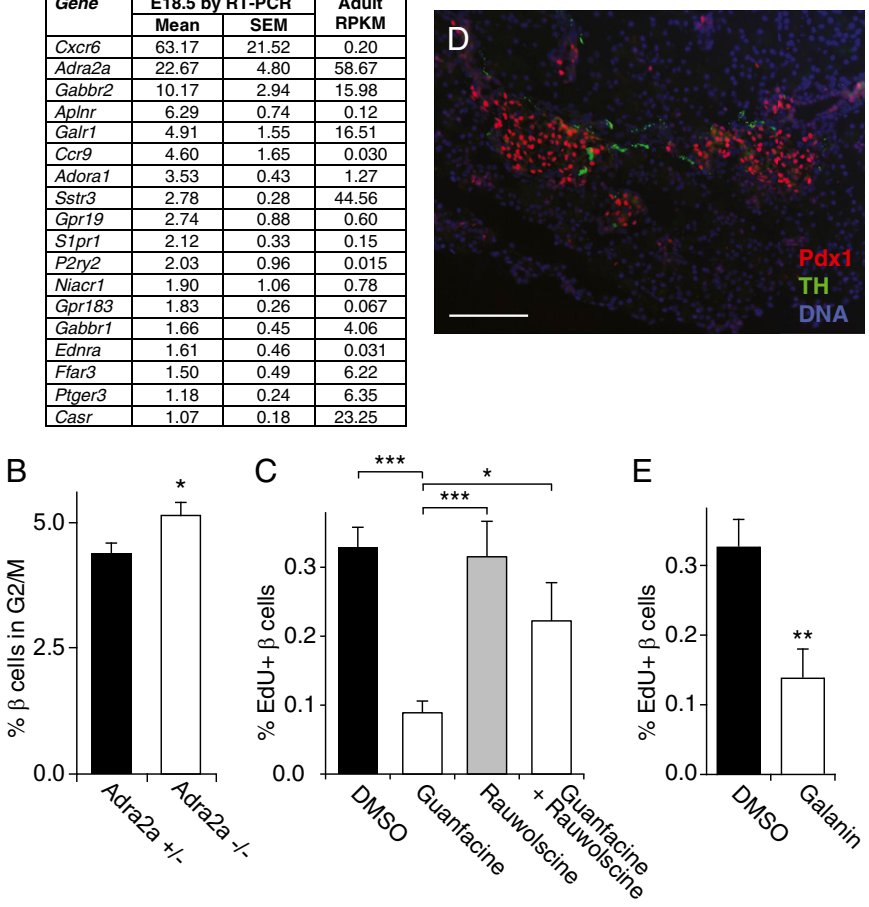

Fig. 4. Inhibition of β-cell proliferation by ADRA2A. (*A*) mRNA encoding Gi-GPCRs expressed by mouse β cells at E18.5 are listed in order of expression level measured by RT-PCR relative to *Actb*/1000. The last column shows the expression level in adult mouse β cells as determined by sequencing expressed in RPKM (62). For complete data on expression of GPCR and related mRNAs, see Tables S8, S9, and S10. (*B*) The replication rate in β cells at day P1 from Adra2a$^{+/-}$ ($n = 6$) and Adra$^{-/-}$ ($n = 6$) mice was measured by determining the percentage of β cells in the G2 and M phases of the cell cycle by flow cytometry. (*C* and *E*) Adult mouse islets were cultured with the drugs shown and labeled with EdU before harvesting. The replication rate in β cells was calculated as the percent of cells costaining for the β-cell nuclear marker. (*D*) A pancreatic section from a mouse embryo at E17.5 was stained for tyrosine hydroxylase (TH, green), the β-cell nuclear marker Pdx1 (red), and the nuclear DNA stain DAPI (blue). (Scale bar, 50 μm.) *$P < 0.05$ and ***$P < 0.001$ vs. control *Adra2a*$^{+/-}$ animals (*B*) or vs. cells treated with guanfacine alone (*C*) by two-tailed Student's *t* test.

Experimental Procedures

Animals. Mice were housed on a 12-h/12-h light/dark cycle in controlled-climate rooms (21.5–22.5 °C). All mouse lines were maintained on a C57BL/6J genetic background (Jackson Laboratories-West). All procedures were approved by the University of California, San Francisco (UCSF) Institutional Animal Care and Use Committee and were conducted in accordance with UCSF regulations. The RIP-rtTA (51), MIP-GFP (52), ePet1-cre (18), Ro1-RASSL (21), ROSA-PTX (24), and *Adra2a*$^{+/-}$ (53) mouse lines are described elsewhere. RIP-rtTA and tetO transgenic mice were given food containing 200 mg/kg doxycycline (BioServ) at the indicated times. Animals identified as sick by veterinary staff were not used for experiments.

Note that the expression pattern of ePet transgenics including ePet-cre (18, 19, 54) compare favorably with many other commonly used pancreas-, islet-, and β-cell–specific transgenic mouse lines, which, like ePet, also express in serotonergic brainstem nuclei as well as in the pancreas. However, unlike ePet, when used as cre drivers other pancreatic promoters frequently cause recombination in other CNS locations including the hypothalamus (Fig. S7) (55–57). (For a thorough discussion of the advantages and limitations of pancreatic cre driver lines, see ref. 58). Further, many of the most commonly used pancreas-specific transgenic lines were generated with a human growth hormone gene cassette that expresses along with the transgene, impacting pancreatic gene expression and islet function (59). The ePet transgenics do not include this cassette.

Generation of ePet1-*Htr1a* and tetO-PTX Transgenic Mice. The plasmid pFLAG-*Htr1a*+iresGFP was generated by linking signal and FLAG epitope sequences to the 5′ end of the mouse HTR1A coding sequence followed by the IRES-eGFP sequence from pIRES-EGFP2 (Clontech Laboratories). The 4-kb pFLAG-5-*Htr1a*+iresGFP insert was inserted downstream of the mouse *Pet1* enhancer and minimal β-globin promoter in pBAC-ePet (18) to generate the ePet1-*Htr1a* transgene (Fig. S1*A*). The plasmid ptetO-S1-PTX was generated by inserting the S1-PTX cDNA [provided by Eitan Reuveny, Weizmann Institute of Science, Rehovot, Israel (60)] downstream of the β-globin intron in the tTA-inducible pUHG 10-3 plasmid. Transgenes were injected into mouse oocyte pronuclei, and transgenic founders were identified by PCR analysis of genomic DNA. Transgene copy number was estimated by real-time PCR with genomic DNA. tetO-PTX mice are available through the Mutant Mouse Regional Resource Centers (https://www.mmrrc.org/catalog/sds.php?mmrrc_id=14241).

Glucose- and Insulin-Tolerance Tests. After overnight fast, mice received 2 mg/kg of glucose for glucose-tolerance tests or 0.75 units/kg of insulin for insulin-tolerance tests via i.p. injection. Glucose was measured from tail blood at the indicated times with a OneTouch Ultra Glucometer and test strips (LifeScan, Inc.). Insulin was assayed in plasma from the tail vein by ELISA kit (Apco Diagnostics) at 0 and 15 min after glucose injection.

Pancreatic Histology. Mice were anesthetized with Avertin and transcardiac perfused with 10 cm^3 of 4 °C PBS, followed by 10 cm^3 of 4% paraformaldehyde (PFA). The pancreas was isolated, fixed in 4% (wt/vol) PFA at

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4 °C for 4 h, taken through an ethanol dehydration series, mounted in paraffin, cut into 6-μm-thick sections by a microtome at 4 °C, and mounted on glass slides. Sections were incubated with primary antibodies against insulin (rabbit, 1:1,000; EMD Millipore Corporation), glucagon (guinea pig, 1:2,000; EMD Millipore Corporation), pdx1 [guinea pig, 1:2,000 (61)], and tyrosine hydroxylase (rabbit, 1:500; EMD Millipore Corporation) in PBS with 5% (vol/vol) normal goat serum overnight at 4 °C. After washes in 4 °C PBS, slides were stained with secondary antibodies: Cy3-conjugated goat anti-guinea pig or anti-rabbit and FITC-conjugated goat anti-rabbit or anti-guinea pig (1:500; Jackson ImmunoResearch Laboratories) in PBS with 5% normal goat serum for 30 min. After washes in 4 °C PBS, stained slides were coverslipped with Vectastain mounting medium containing DAPI and were sealed with clear nail polish. Images were obtained with a Zeiss Axio Scope widefield fluorescence microscope and AxioVision software.

For measurement of the β-cell area, every 30th pancreatic section through the entire pancreas was imaged using a 10× objective. The area of fluorescent staining in β cells was quantified by circling stained cells using AxioVision. The total pancreatic section area was measured similarly using a 1.25× microscope objective. The percent β-cell area was defined as the β-cell area divided by pancreatic section area, multiplied by 100. Controls in Figs. 1*F* and 3*E* were combined for statistical power.

Pancreatic Gene-Expression Analysis. Mice at the indicated ages were anesthetized with 4% (wt/vol) Avertin, and the pancreas was removed, minced immediately in RNAlater (Ambion), and incubated at 4 °C overnight. Pancreatic fragments were pelleted for 2 min at 400 × *g* at 4 °C in a microcentrifuge and then were resuspended in 5 mL of TRIzol and were homogenized with a pellet pestle motor using an RNase-free pestle (Fisher Scientific). GFP^+ β cells were separated by FACS from digested pancreata, and RNA was purified as previously described (62).

cDNA was obtained by reverse transcription from these RNA samples, and TaqMan real-time PCR was performed with an Applied Biosystems 7300 Real-Time PCR System using 50 ng of cDNA per reaction in 96-well plates or 384-sample microfluidic plates (Applied Biosystems). Results were normalized to levels of *Gapdh* mRNA for *Htr1a* expression, to *Gusb* in Fig. 4, and to the average of *Actb*, *Gapdh*, and *Ppia* in Tables S8, S9, and S10. Data from four independent isolations were used for Fig. 4*A*. Primers and probe sequences are available on request.

Adult islet and β-cell RNA-sequencing data were derived from published data from massively parallel sequencing of cDNA purified from isolated 4-mo-old adult mouse islets and sorted β cells (62) and were expressed as reads per kilobase of exon model per million mapped reads (RPKM).

β-Cell Replication Rates. β-Cell replication in MIP-GFP mice was measured by flow cytometry with gating and parameters as previously described (63).

For in vitro proliferation experiments, islets were isolated and cultured for 5 d as previously described (64), followed by 6 d of treatment with the drugs shown at 1 μm with 0.1% DMSO. On day 6 of treatment, islets were treated with 10 mM 5-ethynyl-2-deoxyuridine (EdU) for 3 h and then were fixed immediately in 4% PFA/10 mM PBS solution for 25 min. Fixed islets were washed three times with 10 mM PBS for 20 min, permeabilized with 0.3% Triton X-100 in 10 mM PBS for 3 h, blocked overnight at 4 °C in 5% goat serum/0.15% Triton-X 100/10 mM PBS, and then washed twice with antibody dilution buffer for 15 min at room temperature. Islets were stained with primary antibody, rabbit anti-human NKX6.1 (1:500; Sigma-Aldrich), and secondary antibody, Cy3-conjugated goat anti-rabbit (1:500; Sigma-Aldrich), diluted in 1% BSA/0.2% Triton X-100/10 mM PBS for 24 h at 4 °C. After immunostaining, EdU was labeled with the Click-iT EdU Alexa Fluor Imaging Kit (Invitrogen). Islets were imaged using a Leica SP5 confocal laser scanning microscope (Leica). The Volocity software (PerkinElmer) colocalization macro was used to count nuclei costaining for EdU and the unique β-cell nuclear marker Nkx6.1 (Fig. S3) (28). The percent of proliferating β cells was calculated by dividing the number of costaining nuclei by the total number of $Nkx6.1^+$ nuclei and multiplying by 100.

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