Urocortin3 mediates somatostatin-dependent negative feedback control of insulin secretion

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The peptide hormone urocortin3 (Ucn3) is abundantly expressed by mature beta cells, yet its physiological role is unknown. Here we demonstrate that Ucn3 is stored and co-released with insulin and potentiates glucose-stimulated somatostatin secretion via cognate receptors on delta cells. Further, we found that islets lacking endogenous Ucn3 have fewer delta cells, reduced somatostatin content, impaired somatostatin secretion, and exaggerated insulin release, and that these defects are rectified by treatment with synthetic Ucn3 in vitro. Our observations indicate that the paracrine actions of Ucn3 activate a negative feedback loop that promotes somatostatin release to ensure the timely reduction of insulin secretion upon normalization of plasma glucose. Moreover, Ucn3 is markedly depleted from beta cells in mouse and macaque models of diabetes and in human diabetic islets. This suggests that Ucn3 is a key contributor to stable glycemic control, whose reduction during diabetes aggravates glycemic volatility and contributes to the pathophysiology of this disease.

Insulin-secreting beta cells and glucagon-secreting alpha cells are organized in close proximity within the islets of Langerhans of the pancreas to facilitate the local coordination of the release of both hormones, which have diametrically opposing effects on the liver with respect to the regulation of hepatic glucose production1–7. Their response is critically dependent on ambient glucose, as high glucose levels stimulate insulin secretion and low glucose levels stimulate glucagon secretion as part of a counter-regulatory response to prevent hypoglycemia8. Somatostatin-secreting delta cells provide essential negative feedback to this process by inhibiting both insulin and glucagon release to ensure stable glycemic control over decades. Nevertheless, little is understood about the factors that promote somatostatin secretion to provide crucial negative feedback to beta and alpha cells6. Somatostatin release from delta cells is induced by glucose, sulfonylureas, amino acids, cholecystokinin and cyclic AMP and is inhibited by cholinergic stimulation11–18, although the effects of these compounds on somatostatin release have invariably been demonstrated in pancreas or islet perfusion experiments that complicate the distinction between delta cell–autonomous actions and paracrine effects that involve islet perfusion experiments that complicate the distinction between tostain release have invariably been demonstrated in pancreas or stimulation11–18, although the effects of these compounds on soma - acids, cholecystokinin and cyclic AMP and is inhibited by cholinergic

The peptide hormone urocortin3 (Ucn3) is abundantly expressed by mature beta cells, yet its physiological role is unknown. Here we demonstrate that Ucn3 is stored and co-released with insulin and potentiates glucose-stimulated somatostatin secretion via cognate receptors on delta cells. Further, we found that islets lacking endogenous Ucn3 have fewer delta cells, reduced somatostatin content, impaired somatostatin secretion, and exaggerated insulin release, and that these defects are rectified by treatment with synthetic Ucn3 in vitro. Our observations indicate that the paracrine actions of Ucn3 activate a negative feedback loop that promotes somatostatin release to ensure the timely reduction of insulin secretion upon normalization of plasma glucose. Moreover, Ucn3 is markedly depleted from beta cells in mouse and macaque models of diabetes and in human diabetic islets. This suggests that Ucn3 is a key contributor to stable glycemic control, whose reduction during diabetes aggravates glycemic volatility and contributes to the pathophysiology of this disease.

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Figure 1 Ucn3 is a paracrine factor expressed by mouse beta cells. (a) Quantification of Ucn3 expression relative to that of all genes that encode secreted factors in wild-type mouse islets. (b) Comparison of islet Ucn3 peptide content to that of other islet hormones. (c) Comparison of Ucn3 and insulin secretion from mouse islets in response to glucose (1,000 islets per well; Ucn3 molecular weight, 4,174 Da; insulin molecular weight, 5,785 Da). (d) Ucn3 and insulin colocalize in secretory granules of mouse beta cells by super-resolution structured illumination microscopy (SR-SIM) with thiolabeled Mander’s coefficients for insulin and Ucn3 of 62.7% and 59.6%, respectively. (e,f) Images of an Ins1-H2b-mCherry reporter line crossed to either an Sst-Cre (e) or a Gcg-Cre (f) allele and a floxed YFP reporter. (g,h) FACS purification of mCherry+ beta cells and delta (g) or alpha (h) cells. (i) Gene expression by qPCR of Crhr2 and a panel of established alpha, beta and delta cell markers. AU, arbitrary units. Scale bars, 10 μm (d) and 50 μm (e,f). Error bars show mean ± s.e.m.

As Ucn3 is released at a concentration that is approximately four orders of magnitude lower than that of insulin and would quickly dilute further in the systemic circulation, it probably has a paracrine role. We therefore sought to identify the islet cell type that expresses Crhr2 and responds to Ucn3 directly. We have previously demonstrated the presence of mRNA for the alpha isoform of Crhr2 (hereafter referred to as Crhr2α) in both mouse and human islets.28 The lack of reliable antibodies for this receptor combined with its relatively low abundance28 have to date precluded the identification of the cell type(s) within islets that express(es) Crhr2α and can respond to Ucn3. We therefore crossed Ins1-H2b-mCherry reporter mice21 to delta (Sst-Cre; Fig. 1e) or alpha (Gcg-Cre; Fig. 1f) cell reporter mice. Expression analysis by quantitative PCR (qPCR) on FACS-purified mCherry+ beta and yellow fluorescent protein (YFP)+ delta (Fig. 1g) and alpha (Fig. 1h) cells confirms that Crhr2 is specifically expressed by delta cells (Fig. 1i). We also developed a novel Crhr2α-Cre transgenic reporter mouse, which confirmed lineage expression of Crhr2α in somatostatin-positive cells (Supplementary Fig. 2).

Endogenous Ucn3 promotes somatostatin secretion

We next turned to Ucn3-null mice,27 in which transcriptome analysis confirmed the absence of Ucn3 expression (Fig. 2a) and revealed marked reductions in the expression of known delta cell markers including Sst, Rbp4, and Hhex (refs. 29,30) as compared to wild-type littermates (Fig. 2b–d and Supplementary Table 1). These observations suggest that the absence of Ucn3 precipitated relative delta cell deficiency. Indeed, Ucn3-null islets displayed selective reductions in somatostatin content (Fig. 2e) and relative delta cell numbers (Fig. 2f) compared to control islets. Ucn3-null islets demonstrated impaired basal and glucose-stimulated somatostatin release compared to wild-type islets, which we fully rescued by...
Figure 2 Ucn3-null islets are deficient in delta cell number and somatostatin secretion. (a–d) Transcriptome analysis of Ucn3-null islets compared to wild-type littermates for Ucn3 (a) Sst (b), Hhex (c) and Rbp4 (d) (n = 3); data normalized to 1 × 10^7 reads. (e) Somatostatin, insulin and glucagon content of Ucn3-null and control islets (n = 4). (f) Relative delta, beta and alpha cell numbers of Ucn3-null and control islets (n = 3 for control, 4 for Ucn3-null, 5–7 islets per animal). (g) Somatostatin secretion from Ucn3-null islets compared to control islets (n = 4, 95 islets per well); (h,i) Insulin secretion from Ucn3-null islets compared to control islets in static incubation (n = 4, 7 islets per well) and in islet perfusion (i) (n = 2, 150 islets per chamber, representative of two experiments). Significance determined by Student’s t-test (e,f), two-way analysis of variance (ANOVA) for treatment and genotype followed by Holm–Sidak’s multiple comparison test (h,i), or two-way ANOVA for genotype and its interaction with time for each block (i). Error bars show mean ± s.e.m.; *P < 0.05, **P < 0.01, ***P < 0.001.

c-o-stimulation with synthetic Ucn3 (Fig. 2g). Ucn3-null islets hypersecreted insulin during the first and second phases of secretion, compared to controls, which normalized upon the addition of synthetic Ucn3 peptide (Fig. 2h,i). Islets from Chr2r2-null mice showed comparable defects in somatostatin content and release compared to wild-type littermate controls (Supplementary Fig. 3).

Next we tested the contribution of endogenous Ucn3 to somatostatin secretion. Ucn3 promoted the release of somatostatin under basal- and high-glucose conditions (Fig. 3a). Co-stimulation with Ucn3 and the Chr2r2-selective antagonist astressin 2B (Ast2B)22 prevented this. Notably, Ast2B by itself fully prevented the somatostatin secretion induced by high-glucose conditions and returned somatostatin secretion to levels not different from those observed under basal-glucose conditions in vitro (Fig. 3a).

We determined that glucose-stimulated somatostatin secretion is inhibited by the ATP-sensitive potassium channel (K_ATP) agonist diazoxide and the L-type calcium channel blocker isradipine, as is well established for insulin secretion (Fig. 3b). We then tested whether glucose acts primarily on beta cells to induce the release of Ucn3 (Fig. 1c), which then triggers somatostatin release, or whether Ucn3 amplifies somatostatin release triggered by delta cell–autonomous glucose sensing. Co-stimulation with Ucn3 under high-glucose conditions potentiates somatostatin release and inhibits insulin secretion (Fig. 3b). Similarly, Ucn3 potentiates the somatostatin release triggered by sulfonylureas (Fig. 3c); however, exogenous Ucn3 cannot overcome the inhibition of somatostatin secretion imposed by either diazoxide or isradipine (Fig. 3b).

We further explored the contribution of endogenous Ucn3 to insulin output in perfusion experiments. Acute inhibition of endogenous Ucn3 by Ast2B during the second phase of insulin secretion caused an immediate elevation in insulin secretion compared to control islets not treated with Ast2B (Fig. 3d), secondary to alleviated Ucn3-dependent somatostatin tone (Fig. 3e). Furthermore, Ast2B enhanced the potentiation of glucose-stimulated insulin secretion induced by a sub-maximal dose of the insulinotropic peptide exendin-4 (Fig. 3g).

We next assessed the contributions of Ucn3-mediated somatostatin repression on glucose homeostasis in vivo. Pre-treatment with Ucn3 caused a marked delay in glucose clearance (Fig. 3g), accompanied by lower plasma insulin 10 min after glucose challenge (Fig. 3h) compared to saline controls, in agreement with the Ucn3-stimulated somatostatin repression of insulin demonstrated in vitro (Fig. 3a–e). Somatostatin antagonists fully prevented the robust reduction in glucose tolerance caused by acute Ucn3 administration (Fig. 3i).

Mindful of the conspicuous differences between rodent islets, in which Ucn3 is exclusively expressed in beta cells, and primate islets, in which Ucn3 is expressed by both beta and alpha cells,20,25,26, we measured the ability of Ucn3 to promote somatostatin release from human islets. Ucn3 stimulated somatostatin release from human islets under both basal and hyperglycemic conditions (Fig. 3j). We found that Ast2B blocked the actions of Ucn3 and tended to inhibit glucose-stimulated somatostatin secretion from human islets (Fig. 3j), which was similar to our observations in mouse islets (Fig. 3a). Under the hypoglycemic conditions associated with alpha cell activity, Ast2B inhibited somatostatin secretion (Fig. 3j).

Loss of Ucn3 expression is a hallmark of diabetes

Ucn3 expression appears relatively late in pancreas development and coincides with the acquisition of functional maturity by human embryonic stem cell–derived beta cells, suggesting that Ucn3 is a hallmark of maturity.20,25,26 We therefore assessed Ucn3 expression during diabetic conditions associated with beta cell dysfunction. Ucn3
peptide content and mRNA are markedly lower in beta cells from both leptin-deficient (ob/ob) mice (Fig. 4a,b) and leptin receptor-deficient (db/db) mice (Fig. 4c), as compared to lean, age-matched controls. This is not the result of congenital deficiencies in leptin signaling, as Ucn3 expression in islets from ob/ob mice was not lower compared to lean littermates until the onset of obesity and frank diabetes around 4 weeks of age (Supplementary Fig. 4). Glucose-stimulated somatostatin release from ob/ob islets was impaired compared to lean controls and no longer inhibited by Ast2B (Fig. 4d), which is in line with the loss of endogenous Ucn3. We hypothesized that the resulting loss of negative feedback would cause increasingly volatile plasma glucose levels, and we adapted a method for continuous glucose monitoring in mice to assess this (Fig. 4e and Supplementary Video 1). Continuous measurement of glucose at 5-min intervals over 72 h in freely moving,

**Figure 3** Endogenous Ucn3 promotes somatostatin-mediated negative feedback. (a) Somatostatin secretion from wild-type mouse islets in response to Ucn3 or its antagonist Ast2B (n values in each bar, 100 islets per well). (b) Interactions between Ucn3 and diazoxide or irsapidine on somatostatin (top, n values in each bar, 50 or 100 islets per well) or insulin (bottom, n = 7, 12 islets per well) secretion. (c) Ucn3 amplifies somatostatin secretion induced by tolbutamide (n values in each bar, 80 or 50 islets per well). (d,e) Inhibition of endogenous Ucn3 by Ast2B acutely de-represses insulin secretion (d) via reduced somatostatin release (e) (n = 3, 150 (d) or 270 (e) islets per chamber). (f) Ast2B enhances exendin-4 (Ex4)-induced insulin secretion (n = 6, 12 islets per well). (g,h) Ucn3 impairs glucose tolerance (g) and suppresses glucose-stimulated plasma insulin (h) in vivo, whereas Ast2B has no effect (n = 7 for saline and Ucn3 groups, 6 for Ast2B). (i) Ucn3-mediated glucose intolerance is prevented by somatostatin antagonists (n = 5). (j) Effects of Ucn3 and Ast2B on insulin secretion from human islets (n values in each bar, 50 islets/well; normalized secretion across two (left) or three (right) individual donors. Significance determined by one-way ANOVA followed by Student's t-test with Welch's correction for unequal variance as necessary (a-c,f,h,i), two-way ANOVA for the interaction of treatment and time for each block followed by the comparison of individual time points by Student’s t-test (d,e) or two-way ANOVA for treatment and its interaction with time followed by Holm–Sidak’s multiple comparison test (g,i). NS, not significant; *P < 0.05, **P < 0.01, ***P < 0.001. Error bars show mean ± s.e.m.
untethered animals in their home cages indicated the accuracy of plasma glucose control in lean control mice (Fig. 4c). In sharp contrast, plasma glucose profiles of diabetic ob/ob littersmates acquired simultaneously with the lean controls (Fig. 4c) not only showed the robustly elevated average plasma glucose values that define diabetestes, but revealed a lengthened period of the glucose excursions and increased variance across the average plasma glucose values compared to lean controls (Supplementary Table 2).

To specifically interrogate the role of islet Ucn3 in glucose homeostasis, we generated a novel transgenic mouse model that overexpresses Ucn3 selectively in beta cells upon doxycycline administration, contingent on the presence of two distinct transgenes (Supplementary Fig. 5), and crossed this onto the ob/ob background. We induced transgenic Ucn3 expression in bitransgenic ob/ob mice at 6.5 weeks, when moderate diabetes and obesity had set in and when endogenous Ucn3 expression had disappeared (Fig. 4 and Supplementary Fig. 4). Restoration of Ucn3 in ob/ob mice acutely elevated plasma glucose levels compared to pre-induction, and these levels remained higher than in non-inducible ob/ob controls for the remainder of the experiment, independent of body weight (Fig. 4g). Induction of transgenic Ucn3 expression in lean mice, which maintain endogenous Ucn3, had no effect (Fig. 4g). Induction of transgenic Ucn3 similarly led to an exacerbation of hyperglycemia in mice rendered diabetic by the beta cell toxin streptozotocin (STZ), which markedly reduces beta cell mass and, in our experiments, depleted residual beta cells of Ucn3 (Supplementary Fig. 6). In a third experiment, we built on previous observations that full expression of endogenous Ucn3 does not occur until 2 weeks postpartum20 and coincides with the elevation in plasma glucose and drop in plasma insulin that occur around this age.26,34 Administration of doxycycline to pregnant dams unable to express transgenic Ucn3 from embryonic day (E)10.5 onward resulted in the premature expression of Ucn3 in bitransgenic offspring (Fig. 4h), causing premature elevations in plasma glucose at postnatal day (P)2 and P10 compared to control littersmates (Fig. 4i). Onwards from 3 weeks post-parturition, at which point endogenous Ucn3 is fully expressed (Fig. 4j), we found that transgenic Ucn3 no longer affected plasma glucose (Fig. 4i), as was previously observed in nondiabetic bitransgenic controls (Fig. 4g and Supplementary Fig. 6).

**Figure 4.** Ucn3 marks mature beta cells and aggravates hyperglycemia. (a–c) Ucn3 immunoreactivity (a) and gene expression (b) (n = 9 controls, 8 ob/ob) in islets from ob/ob and db/db mice (c). (d) Somatostatin release from ob/ob islets compared to lean controls in response to Ast2B. (e) Loss of Ucn3 correlates with increased glycemic volatility and extended period length in ob/ob animals compared to lean controls. n values reflect the number of data points. P values reflect differences in variance between animals, see Supplementary Table 2. (f) Doxycycline-inducible Ucn3-overexpressing mice (Supplementary Fig. 5) crossed on the ob/ob background facilitate the restoration of Ucn3 expression by beta cells. (g) Effect of Ucn3 induction on plasma glucose and body weight in doxycycline-inducible Ucn3-overexpressing mice. (h) Doxycycline administration to pregnant dams from E10.5 onward induces Ucn3 prematurely in bitransgenic but not control offspring at P2. (i,j) Effect of premature Ucn3 induction on plasma glucose (i) before and after endogenous Ucn3 is expressed in all beta cells at 3 weeks of age (j). Scale bars, 50 μm in all panels except the detail in f, lower right (10 μm). Significance determined by Student’s t-test (b), one-way ANOVA followed by Student’s t-test with Welch’s correction as necessary (d), and linear regression between groups before and after induction with Student’s t-test to compare the glucose values immediately before and after induction (g). Significance of Ucn3 induction in f determined by Student’s t-test for P2 and P10 groups and by two-way ANOVA for treatment and time for older ages. Error bars show mean ± s.d. in e and mean ± s.e.m. in all other panels; *P < 0.05, **P < 0.01, ***P < 0.001.

**Loss of UCN3 in beta cells from diabetic humans and macaques.** Shortly after birth, human UCN3 is present in both alpha and beta cells,20,25 a pattern that persisted in islets from lean, nondiabetic donors (Fig. 5a and Supplementary Fig. 7a). In contrast, islets from donors with type 2 diabetes across a range of body mass indices (BMIs) demonstrated selective depletion of UCN3 from beta cells, whereas alpha cells retained UCN3 expression (Fig. 5b and Supplementary Fig. 7b).
Figure 5  UCN3 is lost from the beta cells of individuals with type 2 diabetes and prediabetic macaques. (a,b) UCN3 in the islets of a 20-year-old nondiabetic donor with a healthy BMI (a), and in a 37-year-old morbidly obese type 2 diabetic donor (b). We applied masks based on insulin (red) and glucagon (white) to isolate UCN3 staining in beta and alpha cells. Data for 16 additional human donors with and without diagnosed type 2 diabetes are presented in Supplementary Figure 7 and support these observations. (c–e) UCN3 staining in macaques on a control diet (c) and on a high-fat diet and classified as either ‘diet-resistant’ (d) or ‘diet-sensitive’ (e). Similar data for 14 additional macaques across these cohorts are presented in Supplementary Figure 8 and support these observations. Scale bars, 50 µm.

To further corroborate that UCN3 in beta cells is preferentially downregulated compared to UCN3 in alpha cells upon metabolic challenge, we assessed UCN3 staining in a cohort of macaques chronically maintained on a high-fat diet, stratified into ‘diet-resistant’ and ‘diet-sensitive’ cohorts (Supplementary Table 3). Islets from control macaques on a normal diet demonstrated intense UCN3 immunoreactivity in both beta and alpha cells (Fig. 5c). In contrast, islets from macaques on the high-fat diet displayed selectively reduced UCN3 immunoreactivity in beta cells, which was particularly apparent in the diet-sensitive, insulin-resistant cohort (Fig. 5d,e and Supplementary Fig. 8).

DISCUSSION

Here we demonstrate that Ucn3 constitutes a local signal that, under hyperglycemic circumstances, promotes a negative feedback loop that attenuates insulin release via somatostatin (Fig. 6a). Previously it was proposed that Ucn3 engages in autocrine feedback to promote insulin release,19,27 but the discovery here that Chrhr2 is absent on primary beta cells and is expressed by delta cells makes this model untenable. Moreover, Ucn3 administration increases plasma glucose28 and reduces glucose tolerance dependent on uninterrupted feedback by endogenous somatostatin, whereas Ucn3 deletion increases glucose tolerance27; these observations are difficult to reconcile with an insulinotropic role for Ucn3. Instead, these observations are fully compatible with the key role for Ucn3 in somatostatin-mediated negative feedback that we propose here. Furthermore, the de-repressed insulin release that acutely normalizes upon application of exogenous peptide phenocopies the hyperinsulinemia associated with islets null for Sst29 or the delta cell-specific transcription factor Hhex30, which offers strong support for the participation of Ucn3 and somatostatin in the same negative feedback loop. Reduced delta cell number and somatostatin content may contribute to the impaired glucose-stimulated somatostatin release in Ucn3-null mice compared to controls. Nevertheless, inhibition of endogenous Ucn3 with Ast2B acutely affects somatostatin and thereby insulin secretion, establishing that endogenous Ucn3 participates in the physiological control of somatostatin release. Mechanistically, the co-dependence of somatostatin secretion on the class B G protein–coupled receptor (GPCR) ligand Ucn3 and delta cell–autonomous stimulus secretion coupling mediated by KATP and L-type calcium channels resembles the well-known glucose-dependent insulinotropic actions of incretins on beta cells (Fig. 6b,c). Blocking the actions of endogenous Ucn3 and somatostatin with Ast2B is additive to the potentiating effects of incretins on glucose-stimulated insulin secretion, offering further support for the notion that Ast2B and incretins increase insulin secretion by distinct mechanisms.

The clustering of alpha, beta and delta cells enables cross-talk, either via the islet microcirculation or through communication by an assortment of paracrine signals36–39. It is debated whether the circulation in the rodent islet favors mantle-to-core communication40–42 or vice versa43–45. The Ucn3-dependent, somatostatin-mediated negative feedback loop that we discovered depends on both. Several factors can resolve the discrepancy between the suggested unidirectionality of the communication between different cell types of the islets that is based on classical anatomical studies and the reality of bi-directional cross-talk that we demonstrate here. First, recent work elegantly visualizes prior suggestions of dynamically regulated local blood flow within the islet microcirculation that can locally reroute blood flow. Second, delta cells have well-developed projections that receive and release paracrine signals at some distance from the cell body8. Third, beta cells are well connected through gap junctions that synchronize insulin release across all beta cells in an islet47–49, suggesting that it may not be necessary for each individual beta cell to receive direct somatostatin-mediated feedback to ensure effective inhibition of the entire pool. Of course, the architecture of adult human islets features a much greater degree of intermingling of alpha and delta cells with beta cells, facilitating the multi-directional cross-talk we discovered (Fig. 6).
ATP secretion, the actions of Ucn3 on the δ cell mechanistically resemble the actions of incretins on the β cell as both cells respond to a class B GPCR peptide ligand to potentiate exocytosis under elevated ambient glucose conditions. ∆Ψ↓, membrane depolarization. cAMP, cyclic adenosine monophosphate; Gip, glucose-dependent insulinotrophic peptide; GipR, Gip receptor; Glp1, glucagon-like peptide 1; Glp1R, Glp1 receptor; Prkac, protein kinase cAMP-dependent catalytic; Rapgef4, Rap guanine nucleotide exchange factor 4; Slc2a2, solute carrier family 2 member 2.

The late onset of Ucn3 expression in development agrees with its role as the major paracrine signal for promoting somatostatin-mediated feedback; until E15.5, when delta cells appear in substantial numbers of the pancreas, a negative feedback is evident under diabetic conditions, in which the loss of Ucn3 not only correlates with hyperglycemia but is associated with increased glycemic volatility. Paradoxically, restoration of Ucn3 by β cells in several paradigms of relative β cell insufficiency aggravated hyperglycemia, consistent with Ucn3 imposing negative feedback on insulin release. This also explains why Ucn3-null mice maintain glucose tolerance after being fed a high-fat diet. Perhaps the downregulation of Ucn3 in the beta cells of human subjects with diabetes is not a passive response to the adverse consequences of glucotoxicity and lipotoxicity, but instead reflects an innate ability by the beta cell to actively adapt to changes in glucose demand by releasing the ‘brake’ on insulin release. However, as a paracrine action of beta cell-derived factors inhibit alpha cell activity under normal circumstances, downregulation of Ucn3 in the beta cells of individuals with diabetes may also disrupt somatostatin-mediated negative feedback on alpha cells. The therapeutic potential of this UCN3-mediated feedback loop is determined by its net effect on the combined inhibition of insulin and glucagon. Restoring Ucn3 in a setting of relative insulin deficiency, such as obesity or STZ-induced diabetes, may be used to generate the Crhr2α-α cell line to model the feedback loop. We acquired images of both somatostatin (in 1973) and urocortin 3 (in 2001) and who would have been delighted by their participation in the same feedback loop. We acknowledge J. Vaughan for iodination of tracers and H. Park, K. Tigyi and S. Dölleman for their excellent assistance with various aspects of these studies. We thank R. Yang and K. Nakamura at Dexcom Inc. for their assistance in unblinding CGM data at the conclusion of the CGM experiment. We performed this research with the support of the Network for Pancreatic Organ Donors with Diabetes (nPOD), a collaborative Type 1 diabetes research project sponsored by the Juvenile Diabetes Research Foundation (JDRF). We obtained human islets through the integrated islet distribution program (IIDP). M.O.H. is the recipient of a Career Development Award of the JDRF and the Clayton Medical Research Foundation, Inc. (to M.O.H. and W.W.V.) and the Clayton Medical Research Foundation, Inc. (to M.O.H. and W.W.V.) for generating the BAC construct used to generate the Crhr2α–mCherry–Cre reporter line. We acknowledge N. Justice (University of Texas Health Science Center, Houston, Texas, USA) for generating the BAC construct used to generate the Crhr2α–mCherry–Cre reporter line. We acknowledge N. Justice (University of Texas Health Science Center, Houston, Texas, USA) for generating the BAC construct used to generate the Crhr2α–mCherry–Cre reporter line.
ONLINE METHODS

General experimental approaches. No samples, mice or data points were excluded from the reported analysis with the following two exceptions: (i) for the STZ experiment, we excluded STZ-treated animals that failed to develop hyperglycemia (defined as plasma glucose >200 mg/dl; one wild-type and two transgenic animals) at the start of induction at 6 weeks, and (ii) for hormone secretion experiments, on rare occasions, individual samples yielded very high values in both technical replicates that we attributed to the carry-over of an islet during supernatant collection. Such samples were excluded from the analysis as were samples with hormone concentrations below the minimum detectable dose. Insofar as distribution of animals across experimental groups was not determined by their genotype, we randomized to experimental groups unless noted otherwise. For glucose tolerance tests (GTTs), we organized treatments in random order and assigned animals by picking animals at random from their home cages in the morning of the experiment. To avoid bias in islet hormone secretion experiments (static and perfusion), we hand-picked the required number of islets per well or chamber from a pool of all islets and assigned each batch as the next replicate to each subsequent treatment. Experiments were performed in a blinded fashion as noted below. Animal experiments were generally monitored by an investigator with no in-depth knowledge of the expected outcome. During GTTs, an assistant recorded glucose values and evaluated these only after the conclusion of the test. During the continuous glucose monitoring experiment, receivers were blinded and did not display any glucose data to the investigator performing calibration measurements. The measurements of plasma glucose in the premature Ucn3 induction experiment at P2 and P10 were done on full litters before their genotypes were determined. All hormone concentrations were measured in duplicate by a different investigator from the one carrying out the secretion assay, who had no in-depth knowledge of the expected outcome of an experiment.

Biological materials and ethics statements. All in vivo experiments were carried out on male mice, with the exception of the induction of Ucn3 on an ob/ob background, which we conducted on females as our crosses did not yield Ucn3-inducible ob/ob males at the anticipated 1:32 ratio. We obtained commercial male C57BL/6NHSd mice from Harlan (Indianapolis, IN) at 6 weeks of age, which were used between 8 and 16 weeks of age. We maintained mice in group housing on a 12-h light/12-h dark cycle with free access to water and standard rodent chow. We isolated islets as previously described. Mice null for Ucn3 (Ucn3<sup>tm1Pbl</sup>) or Chrh2 (Chrh2<sup>tm1Kflee</sup>) have previously been described. We monitored blood glucose levels by glucose stick from the tail (One-touch Ultra2, LifeScan, Chesterbrook, PA) except for early postnatal measurements, which were terminal. We obtained approval for all mouse procedures from the Salk Institute for Biological Studies Institutional Animal Care and Use Committee and performed experiments in compliance with the Animal Welfare Act and the Institute for Laboratory Animal Research (ILAR) Guide to the Care and Use of Laboratory Animals. We obtained human islets via the NIDDK–supported Integrated Islet Distribution Program (IIDP). We obtained human islets from 18 donors (11 female, 7 male), ranging in age from newborn to 72 years old via the Network of Pancreatic Islet Donors with Diabetes (nPOD). The Salk Institute for Biological Studies Institutional Review Board declared the human islet material and the human pancreas histological specimens used in this study exempt from IRB review under 45 CFR 46.101(b) Category (4) on April 16, 2008 and August 6, 2013, respectively. We obtained macaque pancreas samples from the Oregon National Primate Research Center (ONPRC) Obese Resource, from 17 male rhesus macaques (Macaca mulatta) (15 adults from 6.5 to 14 years of age and two 1-year-old juveniles) that were euthanized for other studies and are described in detail elsewhere. Briefly, control animals were maintained on a 14.6% fat calories diet (Test Diet, Richmond, IN, USA), high-fat diet animals were maintained on a 36% fat calories diet, supplemented with 500 ml of Kool-Aid + 20% fructose three times a week. Food intake was recorded daily, water was provided ad libitum and lights were on from 7 a.m. to 7 p.m. Macaque care and procedures were done according to the ONPRC Institutional Animal Care and Use Committee at the Oregon Health and Science University. The data in Supplementary Table 3 was originally published elsewhere and are included here to enable the reader to assess the metabolic parameters of the macaques used in this study. Cell lines used (HEK 293T (ref. 55) and Ins1 (ref. 56)) were not formally authenticated, but they displayed characteristics consistent with their phenotype, including the expression of insulin and secretion of Ucn3 for Ins1 cells. We did not screen for mycoplasma infection.

Generation of transgenic mice. We generated inducible Ucn3-transgenic mice by inserting the full mouse Ucn3 coding region downstream of a doxycycline inducible promoter consisting of seven tetO-responsive elements followed by a minimal CMV promoter. We inserted the full mouse Ucn3 coding region downstream of a doxycycline-inducible promoter (Supplementary Fig. 5a). To validate that the expression of Ucn3 from the transgenic cassette is doxycycline-dependent and yields processed, bioactive Ucn3 peptide, we transiently transfected the cassette into Ins1 rat insulinoma cells along with a helper vector encoding reverse tetracycline transactivator protein. Conditioned medium from Ins1 cells stimulated with doxycyclone dose-dependently stimulated Chrh2, as measured by CAMP response element–dependent luciferase activity in HEK 293T cells (Supplementary Fig. 5b). Conditioned media from non-induced Ins1 cells has residual activity on Chrh2, probably attributable to endogenous rat Ucn3 that is secreted by Ins1 cells. Application of the same conditioned media to cells carrying Chrh1 did not induce luciferase activity (Supplementary Fig. 5c), demonstrating that transgenic Ucn3 produced by Ins1 cells upon doxycyclone stimulation is processed into a bioactive form that recapitulates the strict receptor preference of synthetic Ucn3 for Chrh2. Conditioned media from HEK 293T cells transiently transfected with the pTRE-tight–Ucn3 construct and the appropriate helper vector and stimulated with doxycycline did not promote Chrh2-dependent luciferase production, indicating that post-translational processing by the beta cell is required for the production of bioactive Ucn3 (data not shown). We linearized this construct and used it to generate a transgenic mouse line. We obtained five founder lines, of which one founder did not pass on the transgene to its offspring. We crossed the remaining four founder lines to rip-tTA mice to generate doxycycline-inducible, beta cell–specific over-expression of Ucn3. We evaluated each and maintained the line that combined the highest levels of transgenic Ucn3 message with no detectable ectopic expression at non-pancreatic sites. In brief, using primers that distinguish between endogenous and transgenic Ucn3 transcripts and a third primer set that detects total Ucn3 message levels (Supplementary Fig. 5a), we detected robust induction of transgenic Ucn3 <i>in vivo</i>, effectively doubling total Ucn3 message levels, but only in islets of an animal that carried both the pTRE-tight–Ucn3 and rip–tTA transgenes and received doxycycline (Supplementary Fig. 5d). Backcrossing of pTRE-tight–Ucn3 × rip–tTA transgenic animals on a Ucn3-null background (Supplementary Fig. 5e) revealed that the doxycycline-mediated induction of Ucn3 is mosaic and restricted to a relatively small subset of beta cells (Supplementary Fig. 5f). Transgenic animals, when naive to doxycycline, showed normal intraperitoneal (i.p.) glucose tolerance (Supplementary Fig. 5g), but after one week of doxycyclone exposure developed notable glucose intolerance in line with a mechanism of Ucn3-induced somatostatin-mediated repression of insulin release (Supplementary Fig. 5h). Comparison of the susceptibility of rip-tTA and pTRE-tight–Ucn3 single transgenic animals to the diabetogenic effects of STZ reveals that rip-tTA single transgenic animals are innately protected from STZ-induced diabetes, whereas pTRE-tight–Ucn3 transgenic animals are not (Supplementary Fig. 5i) This suggests that the partial protection against STZ-mediated hyperglycemia we observed in transgenic animals (Supplementary Fig. 5i) is probably attributable to the presence of the rip-tTA transgenic cassette. We administered doxycycline by custom diet (Harlan Teklad, Madison, WI) at 1,000 mg/kg.

The generation of the Ins1–H2b-mCherry reporter mouse that faithfully and selectively marks all beta cells by nuclear expression of a histone2B-mCherry fusion protein under control of the 10 kb mouse Ins1 promoter is described in detail elsewhere. Crossed to either Sirt-Cre<sup>26</sup> or Gec-Cre<sup>26</sup> and Rosa-lsl-YFP<sup>29</sup> transgenic lines, these mice facilitate FACS-mediated collection of highly pure beta, delta and alpha cells from dissociated mouse islets. Each sample consists of a pool of dissociated islets from a dozen triple transgenic animals hemizygous for each transgene.

To generate Chrh2<sup>Δ2.0 Cre–Cre</sup> reporter mice, we manipulated a 140-kb BAC clone (RP24–351G5; http://bacpac.chori.org/) by homologous recombination to replace the start codon of the alpha isoform of the Chrh2 gene in exon 3,
which is skipped in the beta isoform of Crhr2, with a reporter cassette containing mCherry fused to Cre recombinase by a constitutively proteolytic I2A processing site. We used a neomycin selection marker flanked by FRT sites for positive selection, which we later removed by crossing our founder to an actin-FLP1ing site. We used a neomycin selection marker flanked by the Salk transgenic core and under the supervision of Y. Dayn, and backcrossed to the C57BL/6 background.

Glucose tolerance test and insulin release. We weighed animals in the morning after an overnight fast (Supplementary Table 4). For a GTT or insulin release experiment, male mice received a single 2 g/kg i.p. injection of glucose (dextrose; Sigma, St. Louis, MO, D9559) at time 0. We determined plasma glucose levels using tail vein blood by glucometer (OneTouch Ultra2; LifeScan, Milpitas, CA). We collected blood by retro-orbital bleed to determine plasma insulin levels using a Radioimmunoassay (RIA) kit (EMD Millipore, Billerica, MA; catalog: #SRI-13K). Where indicated, we pretreated male mice at t = −5 min with mouse Ucn3 peptide, the Crhr2 antagonist Astrinsin2β for an equimolar mixture of the somatostatin receptor antagonists Sst3-ODN-8 (carbamoyl−des−AA1,2,4,5,12,13[d−Cys3,Tyr7,d−AgI8 (Me,2−napthoyl)]−SS; #315−260−15) (ref. 60) and Sst2 ant. (H2N−[dCys3−Tyr7−dAph(Cbm)8−Lys9−Thr10−Cy514−1]2Nal15−NH2; #406−028−15) (ref. 61), all at 100 nmol/kg i.p. in saline. All peptides were synthesized in-house using a tert-buty1-oxyl-carbonyl strategy, characterized by mass spectrometry and purified to >98% purity by reverse-phase high-pressure liquid chromatography.

Continuous glucose measurements. We anesthetized mice by an i.p. injection of a cocktail of ketamine (80 mg/kg) and xylazine (8 mg/kg) and shaved and sterilized the flat of their backs. We gently introduced Dexcom Seven Plus continuous glucose sensors (Dexcom Inc., San Diego, CA) subcutaneously in two ob/ob males and two lean age-matched males at 10 weeks of age by means of a modified technique with a guiding needle for sensor insertion. We attached and affixed the transmitters to the back of the animal using acrylamide-based veterinary glue. We anesthetized mice by an i.p. injection of commercially available RIA kits (EMD Millipore, Billerica, MA; catalog numbers #HI-14, #SRI-13K and #GL-32K) for the manufacture's instructions. We measured somatostatin by the same RIA that was developed in-house following the discovery of somatostatin67 using an anti-Sst14 antisera developed in-house (S201; diluted 1:50,000)68. We previously described general RIA buffers and procedures69. We radioabeled Tyr3-Sst14 with 125I by the chloramine T oxidation method and a 1:1:10 molar ratio of peptide:Na125I:chloramine T. We purified tracer by HPLC using a 0.1% trifluoroacetic acid-acetonitrile solvent system and a diphenyl column. We used synthetic Sst14 as a standard, EC50 and minimal detectable dose are 20 pg/tube and 1 pg/tube, respectively. We measured Ucn3 by a RIA we developed in-house using an anti-mUcn3 antisera (#7255; diluted 1:300,000) from approximately 1,000 pooled islets in a 100 µl of elution buffer, we verified RNA quality by BioAnalyzer (Agilent, Santa Clara, CA). We generated Indexed sequencing libraries using the TrueSeq RNA sample Prep Kit v2 (Illumina Inc., San Diego, CA) and sequenced at 50 cycles, single read on an Illumina HiSeq 2000 platform. We mapped sequencing reads from Ucn3-null and wild-type islets (n = 3 individuals each) to mouse genome version mm9 (NCBI build 37) using STAR62. We sequenced at least 42 million reads for each library: We used bedtools63 to create count tables of the sorted bam files using reads aligning to RefSeq defined exons and used DESeq64 for statistical comparison. Non-islet transcriptome data in (Supplementary Fig. 1) were obtained from the Encode project65 (GEO accession: GSE30567). We prepared normalized genome browser tracks using HOMER (http://homer.salk.edu) and uploaded into the University of California Santa Cruz genome browser to generate browser plots.

Quantitative PCR. We determined gene expression from cDNA of FACS-purified delta, beta and alpha cells and normalized to hypoxanthine guanine phosphoribosyl transferase (Hprt) as previously described70 on a Lightcycler 480 (Roche, Indianapolis, IN) using validated primers designed using the online design feature (http://universalprobelibrary.com; navigate to ‘Assay Design Center’) and listed in Supplementary Table 5.

Peptide hormone assays. We isolated islets for secretion experiments by injecting collagenase up the ductal tree through the common bile duct and cultured overnight in RPMI (11 mM glucose, 10% FBS, penicillin (100 U/ml)/streptomycin (100 µg/ml)) as previously described71. We conducted all secretion experiments in Krebs-Ringer Buffer. To avoid bias in static secretion experiments, we hand-picked the required number of islets per well from a pool of all islets and assigned as the next replicate to each subsequent treatment. We carried out islet perfusions using a custom-built perfusion setup with six parallel chambers. We measured human and mouse insulin and glucagon secretion and peptide content with commercially available RIA kits (EMD Millipore, Billerica, MA; catalog numbers #HI-14, #SRI-13K and #GL-32K) for the manufacture’s instructions. We measured somatostatin by the same RIA that was developed in-house following the discovery of somatostatin67 using an anti-Sst14 antisera developed in-house (S201; diluted 1:50,000)68. We previously described general RIA buffers and procedures69. We radioabeled Tyr3-Sst14 with 125I by the chloramine T oxidation method and a 1:1:10 molar ratio of peptide:Na125I:chloramine T. We purified tracer by HPLC using a 0.1% trifluoroacetic acid-acetonitrile solvent system and a diphenyl column. We used synthetic Sst14 as a standard, EC50 and minimal detectable dose are 20 pg/tube and 1 pg/tube, respectively. We measured Ucn3 by a RIA we developed in-house using an anti-mUcn3 antisera (#7255; diluted 1:300,000) from approximately 1,000 pooled islets in a 100 µl volume over the course of 4 h. We iodinated Tyr3Ne12−mUcn3 as above for use as a tracer and used synthetic mUcn3 as a standard. EC50 and minimal detectable dose are 20 pg/tube and 1 pg/tube respectively. Cross-reactivity to rUcn1 and r/hCRH was <0.01% and no message for Crh, Ucn1 and Ucn2 can be detected in mouse islets21.

Immunohistochemistry. We conducted immunohistochemistry as previously described72 using primary antibodies against Ucn3 (rabbit anti-human Ucn3; generated in-house; #7218, 1:2,000), insulin (guinea pig anti-insulin; Dako Cytomation Inc., Carpenteria, CA; #A0564, 1:500) or (mouse anti-insulin; Abcam, Cambridge, MA; #ab6995, 1:1,000), glucagon (guinea pig anti-glucagon; EMD Millipore, Billerica, MA; #4031-01F, 1:7,000), somatostatin (sheep anti-somatostatin; American Research Products Inc., Waltham, MA; #13-2366, 1:1,000), or LacZ (chicken anti-LacZ; Abcam, Cambridge, MA; #ab9361, 1:1,000). We colocalized insulin and Ucn3 in mouse beta cells at sub-cellular resolution on 2-µm-thick paraffin slices of mouse pancreas by SR-SIM using a Zeiss Elyra PS.1 Super-Resolution Microscope. We used an automated iterative maximum correlation threshold (MCT) algorithm73 to determine thresholds for the insulin and Ucn3 signal before the determination of the thresholded Manders’ coefficients for insulin with Ucn3 and Ucn3 with insulin. We generated the colocalization pixel map in Imaris. We applied masks to human islet images using the same MCT method based on insulin and glucagon staining to isolate and compare Ucn3 intensity in beta and alpha cells of the same human
All confocal images were acquired at 63× using a 1.4-numerical aperture (NA) oil-immersion lens at 1024 × 1024 resolution (pixel dimension, 0.22 × 0.22 μm) applying 2× line average and acquiring each channel sequentially. We acquired images at the Waitt Advanced Biophotonics Center Core Facility at the Salk Institute and at the Veterinary Medicine Advanced Imaging Facility and Plant Biology core facilities at the University of California, Davis.

Statistical analyses. We conducted statistics in Prism 6.0e for Mac (Prism, GraphPad Software, Inc.). We reported all values as mean values across biological replicates and assumed normality, unless otherwise noted. We evaluated experiments with more than two treatments by ANOVA followed by Student’s t-test to determine which means differed statistically, and we applied Welch’s correction for unequal variance when necessary. Experiments with multiple treatments across genotypes were evaluated by two-way ANOVA, followed by Holm–Sidak’s multiple comparison test. We evaluated perfusion data by two-way ANOVA for treatment and the interaction of treatment and time for each block.

We assessed the effects of in vivo Ucn3 induction on plasma glucose by linear regression for differences in slope and intercept between groups before and after induction. We tested all data two-tailed, with exception of the perfusion data, the human islet somatostatin secretion data and the Ucn3 induction data, which we evaluated one-tailed under the hypothesis, formulated by prior mouse static secretion experiments, that Ucn3 stimulates somatostatin release. We evaluated variation in deep sequencing data by DESeq. We evaluated differences in mean glucose values and continuous glucose monitoring by the Brown–Forsythe test for the equality of variances based on median glucose values as summarized in Supplementary Table 2. We subsequently evaluated differences in mean glucose values and period length by Student’s t-test with adjustment for unequal variances. We based the desired minimal number of animals per group (n) for GTTs on prior experience in comparable experiments54 and on common practice in the field for these experiments and adopted an n value that sufficed to detect a 50% change in the mean (assuming a s.d. of 40%, a significance level P of 0.05, and a power of 0.8). The number of replicates per group for experiments on Ucn3-inducible animals was limited by the number of experimental animals we could reasonably generate (for example, only 1 in 16 offspring of either sex of the cross to generate Ucn3-inducible ob/ob animals carried the required combination of transgenes and alleles). Continuous glucose monitoring was limited to four animals by the number of available sensors. We based the number of replicates in static secretion experiments on prior experience with these assays although the total number of islets required per well typically limited the number of replicates in somatostatin and Ucn3 secretion assays. The number of replicates in islet perfusion experiments was limited to the number of parallel slots available in the fraction collector.

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