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ARTICLE

Serotonin (5-HT) receptor 2b activation augments glucose-stimulated insulin secretion in human and mouse islets of Langerhans

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Abstract

Aims/hypothesis The G_q-coupled 5-hydroxytryptamine 2B (5-HT_{2B}) receptor is known to regulate the proliferation of islet beta cells during pregnancy. However, the role of serotonin in the control of insulin release is still controversial. The aim of the present study was to explore the role of the 5-HT_{2B} receptor in the regulation of insulin secretion in mouse and human islets, as well as in clonal INS-1(832/13) cells.

Methods Expression of *HTR2B* mRNA and 5-HT_{2B} protein was examined with quantitative real-time PCR, RNA sequencing and immunohistochemistry. α-Methyl serotonin maleate salt (AMS), a serotonin receptor agonist, was employed for robust 5-HT_{2B} receptor activation. *Htr2b* was silenced with small interfering RNA in INS-1(832/13) cells. Insulin secretion, Ca^{2+} response and oxygen consumption rate were determined.

Results Immunohistochemistry revealed that 5-HT_{2B} is expressed in human and mouse islet beta cells. Activation of 5-HT_{2B} receptors by AMS enhanced glucose-stimulated insulin secretion (GSIS) in human and mouse islets as well as in INS-1(832/13) cells. Silencing *Htr2b* in INS-1(832/13) cells led to a 30% reduction in GSIS. 5-HT_{2B} receptor activation produced robust, regular and sustained Ca^{2+} oscillations in mouse islets with an increase in both peak distance (period) and time in the active phase as compared with control. Enhanced insulin secretion and Ca^{2+} changes induced by AMS coincided with an increase in oxygen consumption in INS-1(832/13) cells.

Conclusions/interpretation Activation of 5-HT_{2B} receptors stimulates GSIS in beta cells by triggering downstream changes in cellular Ca^{2+} flux that enhance mitochondrial metabolism. Our findings suggest that serotonin and the 5-HT_{2B} receptor stimulate insulin release.

Keywords Beta cell · Ca^{2+} oscillations · G protein-coupled receptor · Human islets of Langerhans · Insulin secretion · Mitochondrial respiration

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Abbreviations

AMS	α-Methyl serotonin maleate salt
DAG	Diacylglycerol
GLP-1	Glucagon-like peptide 1
GSIS	Glucose-stimulated insulin secretion
5-HT	5-Hydroxytryptamine; serotonin

IP3	Inositol triphosphate
OCR	Oxygen consumption rate
siRNA	Small interfering RNA

Introduction

Glucose-induced insulin secretion is modulated by G protein-coupled receptors and their endogenous ligands [1–3]. Therefore, pathways activated by such receptors are often evaluated as pharmaceutical targets in diabetes treatment. The introduction of incretin-based therapies (e.g. glucagon-like peptide 1 [GLP-1] analogues used for treating type 2 diabetes) is a successful example of this approach [4, 5].

Serotonin (5-hydroxytryptamine [5-HT]) receptors are expressed in rodent islets [6–8] and virtually all 5-HT receptors (5-$HT_{1–7}$) are G protein-coupled with the exception of 5-HT_3 receptors (ligand-gated K^+ and Na^+ ion channels) [9]. Recently, microarray and RNA sequencing analyses revealed transcripts of almost all 5-HT receptors in human islets [10, 11]. The natural ligand binding to these receptors, 5-HT, is synthesised from the amino acid tryptophan, is a well-established neurotransmitter in the central nervous system and is involved in regulation of mood and behaviour [9]. 5-HT has also been detected in islets of Langerhans in several mammalian species [12], including human islets [11], and is suggested to be co-released with insulin and ATP from rodent beta cells [13–16]. However, rodent islet studies examining the effects of 5-HT on insulin secretion have produced contradictory results; an inhibition [17, 18] or a stimulation [19] of glucose-stimulated insulin secretion (GSIS) has been observed. Others propose that 5-HT directly stimulates beta cell exocytosis by receptor-independent mechanisms [20], termed serotonylation, a process where serotonin covalently binds to small GTPases.

Recently, 5-HT was implicated in beta cell expansion and compensatory insulin secretion during pregnancy [6, 8, 14]. Both a lactogen-dependent increase in biosynthetic 5-HT enzymes (tryptophan hydroxylase [i.e. TPH1 and TPH2]), increasing intra-islet 5-HT [14] or increased 5-HT signalling via the 5-HT receptor 2b (5-HT_{2B} receptor) [6] mediates beta cell proliferation in pregnant rodents. Signalling through the 5-HT_{2B} receptor involves coupling via the G_q-protein and activation of the phospholipase C–inositol triphosphate (IP3)–diacylglycerol (DAG) pathway [9]. Muscarinic (M3) receptors are also known to activate this pathway in islet beta cells, thereby potentiating insulin secretion [2]. Consequently, we explored whether insulin secretion is modulated by targeting the 5-HT_{2B} receptors in human and mouse islets, as well as in INS-1(832/13) cells.

Methods

Human and mouse islets of Langerhans Human islets were obtained from the Nordic Network for Clinical Islet Transplantation in Uppsala, Sweden and islets were isolated and cultured as previously described [21].

Female NMRI mice (7–10 weeks old) (Taconic, Skensved, Denmark) were housed under a 12 h light–dark cycle and were fed standard chow and water ad libitum prior to islet isolation. Mouse islets were isolated by collagenase digestion and incubated overnight in RPMI1640 (10 mmol/l glucose) (Sigma Aldrich, Stockholm, Sweden) at 37°C and 5% (vol./vol.) CO_2 before experiments. The Ethics Committees in Uppsala and Lund/Malmö, Sweden approved the studies.

Cell culture INS-1(832/13) cells were grown in 10 cm tissue culture dishes (Sarstedt, Landskrona, Sweden) at 37°C and 5% CO_2. Cells were passaged every second day using 1 ml 0.05% (wt/vol.) trypsin-EDTA, and cultured in RPMI1640 (11.1 mmol/l D-glucose) (Sigma Aldrich) as previously described and received from H. Mulder (Lund University Diabetes Centre) [22]. The cells were tested mycoplasma free.

Quantitative real-time PCR mRNA was prepared from human islets and INS-1(832/13) cells (RNA Easy Plus mini kit; Qiagen, Hilden, Germany). cDNA was obtained by reverse transcription (Maxima First Strand cDNA Synthesis Kit; Fermentas, Thermo Scientific, Helsingborg, Sweden). Human *HTR2B* assay (Hs00265286) and reference genes encoding cyclophilin A (*PPIA*) (Hs0156700), polymerase 2 (*POL2A*, also known as *TIL1*) (Hs00172187) and hypoxanthine guanine phosphoribosyl transferase (*HPRT*) (Hs02800695) (Life Technologies, Stockholm, Sweden) were run on ABI PRISM 7900 (Applied Biosystems, Foster City, CA, USA). *Htr2b* expression in INS-1(832/13) cells was detected with fwd primer 69768: ATGAAGCAGACTGCCGAGAA and rev primer 692768: CACCGCCAAGGACATTAGAA using SYBR-Green (Invitrogen, Stockholm, Sweden) and normalised against *Ppia* (Rn03302269), *Pol2a* (Rn00585096) and *Hprt* (Rn01527840) (Invitrogen) on ABI PRISM 7900 (Applied Biosystems). Samples were run in triplicate, and the transcript quantity was normalised with the $2^{\Delta C_t}$ formula.

RNA sequencing Total RNA was extracted (as above) from human donor islets ($n = 50$). Islets from each donor were cultured in 5.5 mmol/l and 18.9 mmol/l glucose for 24 h. RNA-seq libraries were generated (TruSeq RNA sample preparation kit; Illumina, San Diego, CA, USA) and sequenced on an Illumina HiSeq 2000, using paired-end chemistry and 100 bp cycles to an average depth of 29 M read pairs per sample. Reads were aligned to the reference transcriptome (hg19) with Bowtie2 (www.genomebiology.

com/2009/10/3/R25, www.nature.com/nmeth/journal/v9/n4/full/nmeth.1923.html) and gene expression was estimated as fragments per kilobase of transcript per million mapped reads (FPKM) by the Rsem software (www.ncbi.nlm.nih.gov/pubmed/21816040).

Immunohistochemical analysis Sections (5 μm) of paraffin-embedded human ($n=5$) and mouse ($n=4$) pancreases were mounted on slides and air-dried overnight at 37°C. Slides were deparaffinised as previously described [23], and incubated with primary antibodies: goat anti-5-HT_{2B} (sc-15080, 1:50; Santa Cruz Biotech, Dallas, TX, USA), rabbit anti-glucagon (7811, 1:10,000; Euro Diagnostica, Malmö, Sweden) and guinea pig anti-proinsulin (9003, 1:2,500; Euro Diagnostica) overnight at 4°C in moisturising chambers. Slides were rinsed in PBS with Triton X-100 for 2 × 10 min. Secondary antibodies conjugated to Cy2, Texas Red or AMCA were applied [24]. Specificity of immunostaining was tested using primary antisera pre-absorbed with homologous antigen (5-HT_{2B}, sc-15080p; Santa Cruz Biotech, Dallas, TX, USA) (100 μg of peptide/ml antiserum). Immunofluorescence was examined under an epifluorescence microscope (Olympus BX60, Tokyo, Japan). Images were captured with a digital camera (Nikon DS-2Mv, Tokyo, Japan).

Insulin and 5-HT measurements Non-diabetic human islets (from 34 donors) and mouse islets (from 10 mice) were handpicked under a stereomicroscope. The islets were picked in a randomised fashion with large, small and medium sized islets.

For each individual donor (arriving sporadically), islets were divided into groups of five and for each individual mouse into groups of three in each well of a 96-well plate and pre-incubated for 30 min with secretion assay buffer (in mmol/l: 2.8 glucose, 114 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.16 $MgSO_4$, 25.5 $NaHCO_3$, 20 HEPES, 2.5 $CaCl_2$ and 0.2% (wt. vol) BSA [fatty acid free]). Incubations were performed using 2.8 and 16.7 mmol/l glucose with or without 5-HT_{2B} agonist α-methyl serotonin maleate salt (AMS) (Sigma Aldrich) or Xestospongin C (Tocris, Bristol, UK) in a 96-well plate at 37°C. Each experiment was performed with six to eight replicates for each condition. Insulin was determined after 1 h using Human and Mouse ELISA (Mercodia, Uppsala, Sweden).

INS-1(832/13) cells were seeded on 24-well plates, 24 h before the insulin secretion experiment resulting in ~ 100% confluence. Prior to the experiment, cells were washed in 1 ml secretion assay buffer (see above) followed by 2 h incubation in the same buffer. Cells were stimulated with 2.8 or 16.7 mmol/l glucose with or without 5-HT_{2B} receptor agonists AMS or Ro60-0175 (Sigma Aldrich). Concentrations of AMS and Ro60-0175 were determined from EC_{50} values [25–27] and based on previous in vitro studies [28, 29]. Insulin was measured with rat ELISA (Mercodia) after 1 h and normalised to total protein (BCA; Pierce Biotechnology, Rockford, IL, USA).

5-HT was measured with a 5-HT ELISA (LDN Labor Diagnostika Nord; Nordhorn, Germany) in INS-1(832/13) cells ($n=3$) and islets (islets from four mice) stimulated with 2.8 and 16.7 mmol/l glucose for 1 h (as above).

RNA interference Small interfering RNA (siRNA) sequences for *HTR2B* and negative control siRNA (sense: GAGACCCUAUCCGUGAUUATT, anti-sense: UAAUCACGGAUAGGGUCUCTT) (Invitrogen) were used. Cells were transfected with 60 nmol/l siRNA, using Lipofectamine 2000 (Invitrogen). Medium was changed after 24 h and cells were assayed for knock down after 48 h. Alternatively, cells were cultured for 72 h post-transfection and insulin secretion was measured in response to 2.8 and 16.7 mmol/l glucose with or without AMS.

Western blot analysis INS-1(832/13) cells were lysed (9 mol/l urea, 100 mmol/l HEPES, 1% (vol./vol.) Triton X-100, 0.25% (wt/vol.) IcoPAL-630, 0.25% dodecyl maltoside (wt/vol.), pH 7.2) and protein concentration was estimated (Pierce Biotechnology). Protein (10–30 μg) was loaded onto a 10% Tris-HEPES gel (Pierce Biotechnology) and electrophoresis was performed according to the manufacturer's protocol. Proteins were then transferred to an Immobilon-FL PVDF-membrane (Millipore, Billerica, MA, USA). Membranes were blocked in blocking buffer (LI-COR, Lincoln, NE, USA) and incubated overnight at 4°C with mouse pAb anti-HTR2b (556334, 1:500; Becton Dickinson, Franklin Lakes, NJ, USA) and β-actin (A5441, 1:5,000; Sigma Aldrich). Application of secondary antibodies and detection were performed as previously described [30].

Calcium imaging Islets ($n=19$ islets from five control mice vs $n=27$ from five AMS-treated mice) were attached to CellTak (Corning, New York, NY, USA)-coated glass bottom dishes (MatTek, Ashland, MA, USA) overnight. Fura 2-AM (4 μmol/l) (TEFLabs, Austin, TX, USA) were applied for 40 min followed by 30 min of de-esterification in imaging buffer. Imaging was performed as previously described [31]. One frame per second was recorded at 37°C under perfusion at 0.7 ml/min for 5 min at 2.8 mmol/l glucose, followed by 20 min at 16.7 mmol/l glucose, then 20 min at 2.8 mmol/l glucose with or without AMS. A marked region around each islet and the light intensity was recorded to acquire the mean light intensity at 340 nm (exposure 150 ms) and 380 nm (exposure 100 ms). Intensity measurements were used to calculate the ratio of bound Fura-2 (340 nm) and unbound (380 nm) Ca^{2+}.

Mitochondrial respiration INS-1(832/13) cells were seeded in 24-well culture microplates (XF24) (Seahorse

Bioscience, Billerica, MA, USA) (100,000 cells/well) in 500 μl of RPMI1640 medium. Plates were incubated for 48 h at 37°C in 5% CO_2. Prior to assay, the medium was removed and replaced by 750 μl assay medium (in mmol/l: 114 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.16 $MgSO_4$, 20 HEPES, 2.5 $CaCl_2$ and 0.2% (wt/vol.) BSA, pH 7.2) supplemented with 2.8 mmol/l glucose. Oxygen consumption rate (OCR) was determined in 7 μl volume above the plated cells. Respiration was measured in 2.8 mmol/l glucose for 60 min followed by transition to 16.7 mmol/l glucose. Respiration driving ATP synthesis and proton leak were determined by the addition of oligomycin (4 μg/ml). After 30 min, 4 μmol/l of the mitochondrial uncoupler FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone) was added to determine maximal respiratory capacity. After an additional 10 min, 1 μmol/l rotenone was added to block transfer of electrons from complex I to ubiquinone.

Statistical analysis All data are presented as means ± SEM. Differences in expression levels and insulin release were analysed with Mann–Whitney *U* test. RNA sequencing data are presented as relative logarithmic values normalised to total number of reads; *p* values were determined by a paired *t* test. The AUC for Ca^{2+} measurements was analysed using the trapezoidal rule and *p* values were calculated with Student's *t* test. The proportion of islets displaying different Ca^{2+} profiles was analysed with one-way ANOVA and Tukey's multiple comparisons test. Statistical testing was performed using Statistical Package for the Social Sciences (SPSS) version 19.0 software (SPSS, Chicago, IL, USA) or GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). Significance for all tests was considered as $p<0.05$. Outliers from each experiment were determined by using a Grubb's outlier test in GraphPad Prism.

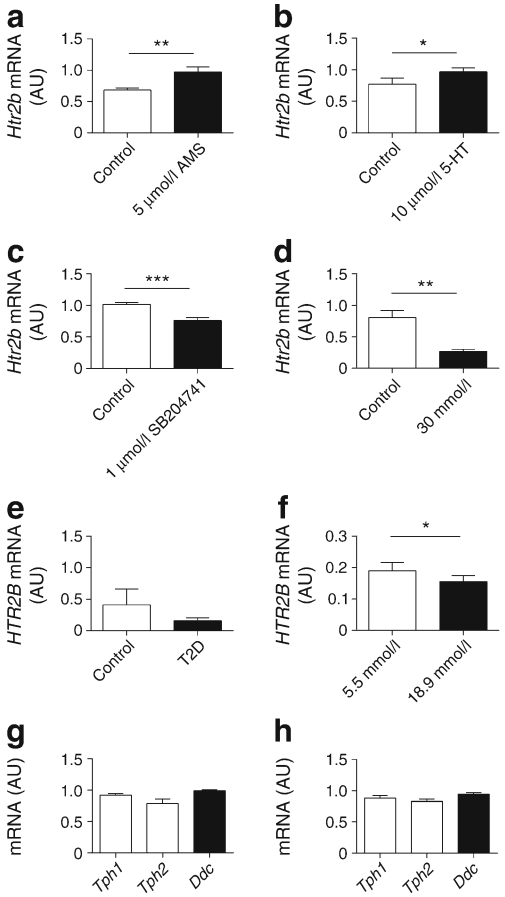

Fig. 1 (**a**–**d**) *Htr2b* expression in INS-1(832/13) cells with or without AMS (**a**), with or without 5-HT (**b**), with or without SB204741 (**c**) and incubated in 11.1 mmol/l glucose (control) and 30 mmol/l glucose (**d**). (**e**) *HTR2B* expression in islets from non-diabetic donors (control) and donors with type 2 diabetes (T2D). (**f**) RNA sequencing in non-diabetic human islets incubated in 5.5 mmol/l and 18.9 mmol/l glucose. (**g**, **h**) *Tph1*, *Tph2* and *Ddc* expression in INS-1 (832/13) cells (**g**) and in mouse islets of Langerhans (**h**). Data are shown as means ± SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ for indicated comparisons

Results

***HTR2B* RNA expression in INS-1(832/13) cells and human islets** Expression levels of *Htr2b* in INS-1(832/13) cells increased significantly after 36 h incubation with the 5-HT_{2B} receptor agonist AMS (5 μmol/l) ($n=5$, $p=0.002$) (Fig. 1a) and the natural ligand 5-HT (10 μmol/l) ($n=3$, $p=0.034$) (Fig. 1b) compared with untreated cells. Incubation of cells with the 5-HT_{2B} receptor antagonist SB204741 [32] (1 μmol/l) decreased expression of the receptor when compared with untreated cells (Fig. 1c; $n=3$, $p=0.0003$). Interestingly, in INS-1(832/13) cells incubated in 30 mmol/l glucose, a condition used to examine glucotoxicity, expression of *Htr2b* was reduced compared with cells grown in cell culture medium ($n=7$, $p=0.006$) (Fig. 1d). This suggests that expression of *Htr2b* in INS-1(832/13) cells is regulated by its own activation and inhibition. In addition, expression of *HTR2B* is negatively regulated by a glucotoxic challenge. In human islets, the expression of *HTR2B* was not altered in islets from donors with type 2 diabetes compared with those from controls (Fig. 1e [$n=6$ non-diabetic and 4 type 2 diabetes islet donors]). However, RNA sequencing in islets ($n=50$) collected from non-diabetic humans and incubated for 24 h in 18.9 mmol/l glucose revealed decreased expression of *HTR2B* when compared with islets cultured in 5.5 mmol/l glucose ($p=0.04$). Thus human islets displayed a regulation in response to glucose similar to that shown by the INS-1(832/13) cells.

Enzymes required for 5-HT synthesis, tryptophan hydroxylase 1 and 2 (encoded by *Tph1*, *Tph2*), as well as dopa decarboxylase (encoded by *Ddc*), were expressed at the mRNA level in both INS-1(832/13) cells (Fig. 1g; $n=3$) and mouse islets (Fig. 1h; $n=3$ mice). Expression of *Htr2a* transcripts was found in mRNA preparations from both species, while *Htr2c* receptor transcripts were not (data not shown).

5-HT$_{2B}$ protein is present in human and mouse beta cells Next, we investigated the tissue distribution of 5-HT$_{2B}$ receptors in human ($n=5$) and mouse ($n=4$) pancreas (Fig. 2). 5-HT$_{2B}$ receptors (Fig. 2a) were primarily present in human beta cells (Fig. 2d). In contrast, no 5-HT$_{2B}$ immunofluorescence was observed in alpha cells (Fig. 2d). In mouse islets, a similar pattern was observed, revealing 5-HT$_{2B}$ immunofluorescence in insulin-positive cells (Fig. 2e, f, g). Antibody specificity was verified by lack of staining in human pancreas (Fig. 2h, i) after pre-absorption with homologous antigen.

Activation of the 5-HT$_{2B}$ receptor augments GSIS The compound AMS has previously been tested in vitro, ex vivo [29] and in vivo [33] and possesses a 100-fold higher binding selectivity for 5-HT$_{2B}$ receptors compared with 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors [34]. We exposed INS-1(832/13) cells and human and mouse islets to AMS at 2.8, 8.3 or 16.7 mmol/l glucose. In human islets, AMS increased insulin secretion at 8.3 mmol/l glucose (10 μmol/l AMS; $n=13$, $p=0.04$) and 16.7 mmol/l glucose (5 μmol/l AMS; $n=11$, $p=0.03$) compared with controls stimulated with glucose alone ($n=34$; Fig. 3a). Similarly, a robust stimulation of insulin secretion was observed when mouse islets ($n=10$) were treated with 16.7 mmol/l glucose and AMS (10 μmol/l) as compared with glucose alone ($p=0.002$) (Fig. 3b). Last, AMS (10 μmol/l) potentiated GSIS in INS-1(832/13) cells ($n=18$, $p=0.002$) (Fig. 3c). Addition of other secretagogues, such as carbachol (muscarinic activation) or forskolin (cAMP stimulation), in combination with AMS had no additional effect on GSIS from INS-1(832/13) cells (data not shown). To further evaluate the potentiation of GSIS by 5-HT$_{2B}$ receptor activation, we used Ro60-0175 (10 μmol/l), another 5-HT$_{2B}$ receptor agonist. Importantly, this agonist also potentiated GSIS in INS-1 (832/13) cells ($n=6$, $p=0.02$) (Fig. 3d). 5-HT$_{2B}$ mainly acts on the G_q, phospholipase C, IP3–DAG pathway, so we investigated whether the effects of AMS could be abolished using

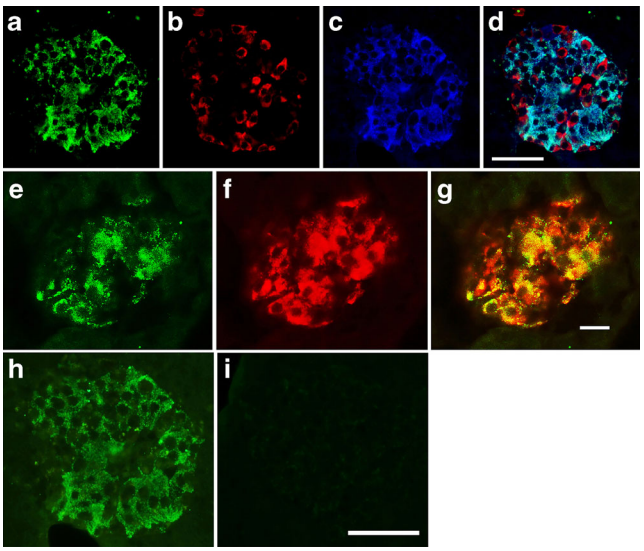

Fig. 2 (**a**–**d**) Immunostaining of 5-HT$_{2B}$ (**a**), glucagon (**b**) and insulin (**c**), and overlay (**d**), in human pancreas. (**e**–**g**) Immunostaining of 5-HT$_{2B}$ (**e**) and insulin (**f**), and overlay (**g**), in mouse pancreas. (**h**, **i**) Human pancreas with immunostaining of 5-HT$_{2B}$ (**h**) and 5-HT$_{2B}$ antibody pre-absorbed with blocking peptide (**i**). Scale bars, 50 μm

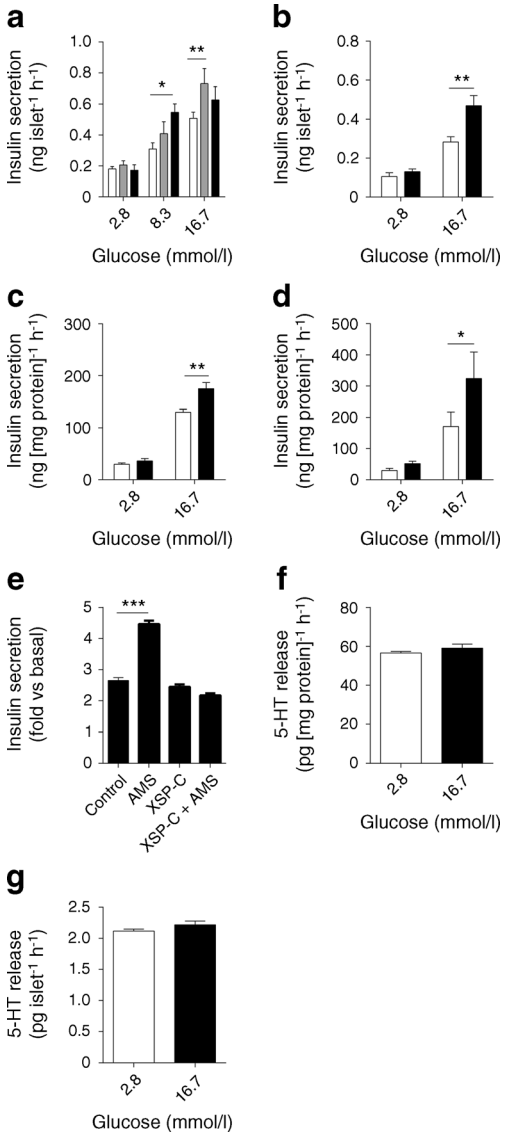

Fig. 3 (**a**) Human islets stimulated with glucose (2.8, 8.3 or 16.7 mmol/l) (white bars) with addition of 5 μmol/l (grey bars) or 10 μmol/l AMS (black bars). (**b**) Mouse islets stimulated with 2.8 or 16.7 mmol/l glucose (white bars) with addition of 10 μmol/l AMS (black bars). (**c**) INS-1(832/13) cells stimulated with 2.8 or 16.7 mmol/l glucose (white bars), with addition of 10 μmol/l AMS (black bars). (**d**) INS-1(832/13) cells stimulated with 2.8 or 16.7 mmol/l glucose (white bars) with addition of 10 μmol/l Ro60-0175 (black bars). (**e**) Mouse islets incubated with 10 mmol/l glucose (control) with addition of 10 μmol/l AMS, 0.4 μmol/l Xestospongin C (XSP-C) or 10 μmol/l AMS plus 0.4 μmol/l Xestospongin C; all measurements are fold vs basal (2.8 mmol/l glucose). (**f**, **g**) 5-HT release from INS-1 (832/13) cells (**f**) and in mouse islets (**g**) stimulated with 2.8 or 16.7 mmol/l glucose. Data are means ± SEM. *$p<0.05$, **$p<0.01$ and ***$p<0.001$ for indicated comparisons

the IP3 receptor inhibitor Xestospongin C. Indeed, addition of 0.4 μmol/l Xestospongin C reduced the potentiating effect of AMS on GSIS in mouse islets ($n=4$, Fig. 3e).

5-HT is released from INS-1(832/13) cells and mouse islets 5-HT was released from both INS-1(832/13) cells ($n=3$) and mouse ($n=4$) islets at 2.8 mmol/l glucose (Fig. 3f, g). No additional increase in 5-HT concentration was observed when subjecting cells/islets to an increased glucose concentration (16.7 and 10 mmol/l, respectively). This suggests that glucose does not regulate release of 5-HT from rodent beta cells; rather, 5-HT leaks from the secretory vesicles as previously described [15].

Silencing 5-HT$_{2B}$ receptors inhibits GSIS An alternative approach to study the function of the receptor is to reduce its expression in beta cells. RNA interference targeting *Htr2b* resulted in a 70% knockdown of the receptor at the mRNA level ($n=3$, $p=0.001$) (Fig. 4a) and a 30% reduction at the protein level ($n=4$, $p=0.002$) (Fig. 4b, c). GSIS was significantly reduced as compared with controls ($n=10$, $p=0.001$) (Fig. 4d) after exposing siRNA-treated cells to 2.8 vs 16.7 mmol/l glucose. Next, we stimulated *Htr2b* siRNA-treated cells with AMS at 16.7 mmol/l glucose. In contrast to control cells, no potentiation of GSIS was observed (Fig. 4d). This implies that the agonist has high selectivity for the 5-HT$_{2B}$ receptor and stimulates insulin secretion via this receptor.

5-HT$_{2B}$ receptor activation alters islet Ca^{2+} profiles Intracellular Ca^{2+} levels in mouse islets generally display distinct

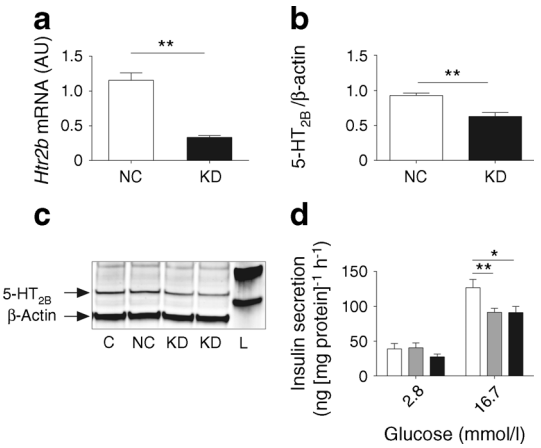

Fig. 4 (**a**, **b**) Expression of *Htr2b* (**a**) and ratio of 5-HT$_{2B}$ protein (**b**) in INS-1(832/13) negative control (NC) cells and after knockdown (KD). (**c**) Representative western blot with 5-HT$_{2B}$ protein in untreated control INS-1(832/13) cells (C) and in NC cells and after KD, with protein ladder (L) shown. (**d**) INS-1(832/13) cells stimulated with 2.8 mmol/l and 16.7 mmol/l glucose (white bars), post KD (grey bars) and stimulated with AMS (10 μmol/l) (black bars). Data are means ± SEM. *$p<0.05$ and **$p<0.01$ for indicated comparisons

first-phase oscillations in response to elevated glucose concentrations. This is followed by a second-phase train of oscillations of lower amplitude and intensity (Fig. 5a). A striking increase (threefold, $p=6.02\times10^{-6}$) in peak duration (time in s that the peak is in a plateau phase or active phase) was observed in mouse islets treated with AMS (10 μmol/l) as compared with control islets treated with glucose alone (Fig. 6a). Moreover, a significant increase in the distance between Ca^{2+} peaks or period (peak-to-peak distance in ms) was observed in AMS-treated islets compared with controls ($p=0.015$) (Fig. 6b). This suggests that 5-HT$_{2B}$ activation amplifies the effects of glucose on intracellular Ca^{2+}. In fact, 11% of the AMS-treated islets displayed this distinct profile of strong regular Ca^{2+} bursts; this was never observed in control experiments (11% vs 0%) (Fig. 5b and Table 1). As a consequence of increased distance between peaks and peak duration the frequency of oscillations in the second phase was reduced by 43% in AMS-treated islets (2.95 vs 1.69 oscillations/min, $p=0.027$; Fig. 6c), as well as the difference in the ratio between first-phase and second-phase oscillations (see variable C_{3B}, ratio C_1/C_{4M}) (Fig. 6d and Table 2; $p=0.03$).

The area of the Ca^{2+} dip prior to the initial Ca^{2+} burst, which likely represents Ca^{2+} uptake into endoplasmic reticulum stores, was also measured (see variable A_d Fig. 5a, Table 2). Although a 23% reduction in Ca^{2+} was observed in AMS-treated islets, reflecting increased cytoplasmic Ca^{2+}, this did not reach statistical significance (Table 2).

Roughly half of control (58%) and AMS-treated islets (44%) displayed an extended first phase (Fig. 5c and Table 1), while 20% of all islets in the experimental set-up displayed no clear first phase (Fig. 5d and Table 1). This suggests a heterogeneity in Ca^{2+} responses that typically is observed in islets. The number of imaged islets was: $n=19$ control islets from 5 mice vs $n=27$ AMS-treated islets from 5 mice.

5-HT$_{2B}$ receptor activation enhances mitochondrial metabolism As AMS potentiated GSIS in islets/cells and since alterations in Ca^{2+} flux are suggested to influence activation of mitochondrial enzymes [35], we investigated whether these events were associated with changes in mitochondrial metabolism. INS-1(832/13) cells were stimulated with glucose with or without the addition of AMS and oxygen consumption was determined. OCR increased under both conditions, but cells treated with AMS exhibited a greater glucose-induced increase in respiration (Fig. 7a, b, $p=0.05$). Maximum mitochondrial respiratory rate increased in AMS-treated cells (Fig. 7c, $p=0.007$), and this was further confirmed when analysing the apex of maximum respiratory capacity (Fig. 7d, $p=0.017$). A significant increase in spare respiratory capacity was observed in AMS-stimulated cells compared with controls (Fig. 7e, $p=0.04$). These data suggest that AMS treatment enhances beta cell respiratory capacity.

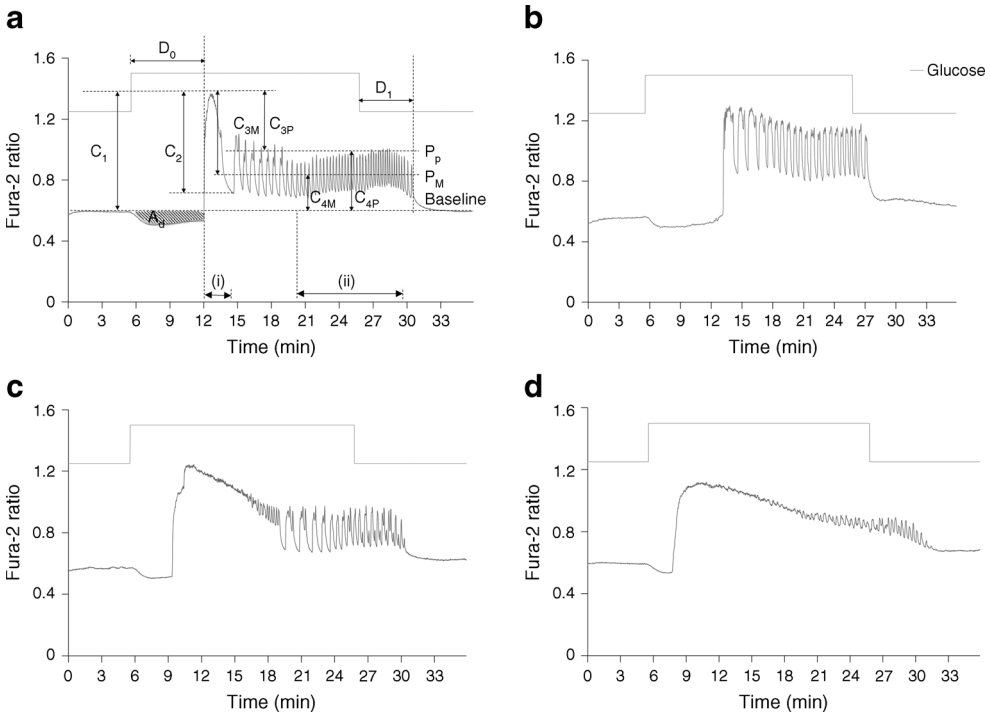

Fig. 5 (**a**) First-phase Ca^{2+} oscillation in response to 16.7 mmol/l glucose followed by second-phase oscillations in untreated mouse islets. Explanations for the letters C_1, C_2 etc. are presented in Table 2. (**b**) When treated with 10 μmol/l AMS, 11% of the islets had a distinct Ca^{2+} profile in response to 16.7 mmol/l glucose. (**c**) AMS-treated islets with an extended first phase, followed by a distinct Ca^{2+} profile. (**d**) In the total experimental set-up 20% of islets displayed no clear first phase. (i), First-phase area under graph during first 2 min; (ii), second-phase frequency of oscillations and area under graph measured during last 10 min interval

Neither difference in proton leak nor in coupling efficiency was observed between the two treatments (data not shown).

Discussion

In the present study, we explored the regulatory role of 5-HT_{2B} receptor activation on GSIS. We show that pharmacological activation of 5-HT_{2B} receptors, with two different agonists (AMS and Ro60-0175), potentiates GSIS in human and mouse islets and in INS-1 (832/13) cells. Moreover, a reduction in *Htr2b* mRNA expression in INS-1(832/13) reduced GSIS by nearly 30%. Together, these findings support the functional importance of this receptor in control of GSIS. This function can be further understood in light of the cellular expression of 5-HT_{2B} receptors, which was localised to islet beta cells in human and mouse islets.

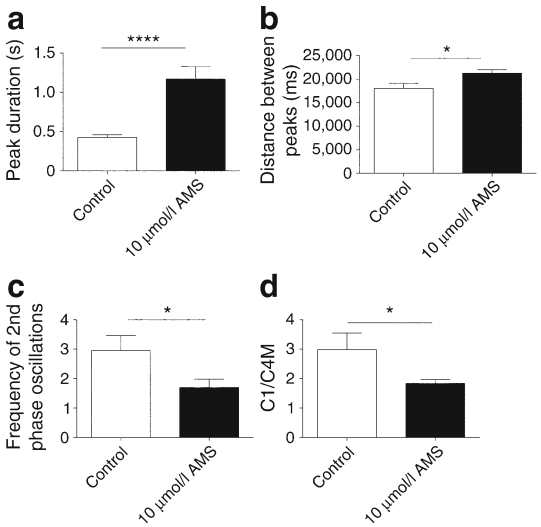

Fig. 6 (**a**) Peak duration of Ca^{2+} traces in control and AMS-treated mouse islets. (**b**) Distance between Ca^{2+} peaks in control and AMS-treated mouse islets. (**c**) Frequency of second-phase oscillations in control and AMS-treated mouse islets. (**d**) First-phase and second-phase ratio (C1/C4M) in control and AMS-treated mouse islets. Data are means ± SEM. *$p < 0.05$ and ****$p < 0.0001$ for indicated comparisons

Limited information exists regarding the effects of 5-HT_{2B} signalling on beta cell function. A previous report shows that tamoxifen-induced beta cell-specific knockout of *5htr2b* results in mice remaining normoglycaemic during an intraperitoneal glucose tolerance test when challenged by a high-fat diet in vivo [36]. Such results infer that 5-HT_{2B} is unimportant for islet function. We performed acute manipulations of 5-HT_{2B} signalling, using agonists and gene silencing, which could be considered a weakness when opposed to in vivo studies. However, in a chronic experimental situation the known redundancy of 5-HT receptors can become more prominent in an in vivo situation. To this end, no studies have explored in vitro beta cell function in islets isolated from *Htr2b* beta cell-specific knockout mice. This enhances the novelty of the present findings.

A recent study suggests that an additional member of the 5-HT2 family (5-HT_{2C}) is increased in islets from diabetic db/db mice and in palmitate-treated MIN-6 cells [37], and that this results in reduced GSIS. Here, we were unable to detect the transcript of 5-HT_{2C} in either human or mouse islets.

Table 1 Proportion of islets displaying the different Ca^{2+} profiles

Treatment	Short distinct first phase (see Fig. 5a)	Regular strong oscillations (see Fig. 5b)	Extended first phase (see Fig. 5c)	No clear first phase (see Fig. 5d)	*p* value
Control	21 (4/19)	0 (0/19)	58 (11/19)	21 (4/19)	0.025
AMS	30 (8/27)	11 (3/27)	44 (12/27)	19 (5/27)	NS

Data shows the percentage of islets displaying different Ca^{2+} profiles with real values in parenthesis

Statistics were calculated in each group after normalisation of data with one-way ANOVA and Tukey's multiple comparison test. $p < 0.05$ was considered significant

Most studies suggest that 5-HT inhibits insulin secretion [11, 15, 17, 18]. Clinically, 5-HT systems in the central nervous system are targeted by antidepressants and long-term use of antidepressants is associated with an increased type 2 diabetes risk [38, 39]. Since beta cells express multiple 5-HT receptors [10, 11] and the serotonin transporter (SERT) [40], direct effects on insulin secretion are likely. Indeed, selective serotonin re-uptake inhibitors (SSRIs) inhibit insulin secretion in clonal beta cells [41, 42], possibly through an increased 5-HT concentration in the extracellular space. We observed positive effects of acute exposure of 5-HT_{2B} receptor agonists on GSIS. Notably, we show that IP3 receptor inhibition reversed the effects of AMS on insulin secretion, suggesting that the effect on GSIS is partly mediated by an increase in intracellular Ca^{2+}. This is to some extent corroborated by our findings since we observed a 23% reduction in Ca^{2+} uptake in the endoplasmic reticulum in AMS-treated islets. We also show a significant increase in peak duration (plateau of peak) and distance between peaks (period), which suggest that islets spend more time in an active phase and the period between peaks are prolonged. Similar Ca^{2+} patterns are observed when glucose is raised from an intermediary concentration to a high concentration that causes a regimen change in Ca^{2+} oscillation from fast to slow oscillations with greater amplitude and increased plateau phase [43]. Thus, 5-HT_{2B} receptor activation appears to amplify the effects of glucose on intracellular Ca^{2+}. In agreement with previous studies [44, 45] we also found that islet beta cells represent a heterogeneous population. In this case, this could be accounted for by findings indicating that 5-HT, and possibly 5-HT receptor expression, is confined to a subset of beta cells [14].

Changes in intracellular Ca^{2+} may also affect mitochondrial metabolism via activation of dehydrogenases operating in the tricarboxylic acid cycle [35]. Alterations in Ca^{2+} oscillations, caused by 5-HT_{2B} receptor activation, may amplify the production of metabolic coupling factors during GSIS [46]. In fact, INS-1(832/13) cells stimulated with glucose and AMS displayed an increase in glucose-stimulated oxygen consumption, indicating enhanced mitochondrial activity. We also observed an increased spare respiratory capacity, suggesting that

Table 2 Summary of Ca^{2+} imaging of AMS-treated and untreated mouse islets

Variable	Mean Control	Mean AMS	Difference (%)	*p* value	SEM Control	SEM AMS
A_t (total area under graph [time in ms × Fura-2 ratio])	298,892	327,472	10	0.58	32,780	36,087
A_1 (first-phase area [initial 2 min] [time in ms × Fura-2 ratio])	49,836	41,736	−16	0.24	4,210	4,848
A_2 (second-phase area [during 10 min interval before low-glucose response] [time in ms × Fura2 ratio])	124,172	135,590	9	0.46	10,801	10,211
A_d (dip area)	19,908	15,351	−23	0.12	2,926	1,280
C_1 (difference in 340/380 ratio between baseline and highest point of first oscillation)	0.56	0.48	−13	0.28	0.04	0.05
C_2 (difference in 340/380 ratio between highest point of first oscillation and lowest point before second-phase oscillations)	0.35	0.26	−26	0.16	0.05	0.04
C_{3M} (difference in 340/380 ratio between highest point of first oscillation and mean mid-point ratio of second-phase oscillations)	0.31	0.22	−31	0.07	0.03	0.04
C_{3P} (difference in 340/380 ratio between highest point of first oscillation and mean peak ratio of second-phase oscillations)	0.24	0.13	−46	0.030	0.03	0.03
C_{4M} (difference in 340/380 ratio between baseline and mean mid-point ratio of second-phase oscillations)	0.24	0.27	11	0.39	0.02	0.02
C_{4P} (difference in 340/380 ratio between baseline and mean peak ratio of second-phase oscillations)	0.40	0.36	−10	0.58	0.07	0.03
D_0 (delay in response to high glucose), min	6.12	5.91	−3	0.73	0.47	0.39
D_1 (delay in response to low glucose), min	4.97	5.75	16	0.43	0.34	0.79

Control ($n = 19$ islets from 5 mice) vs AMS-treated ($n = 27$ islets from 5 mice); *p* values calculated with Student's *t* test

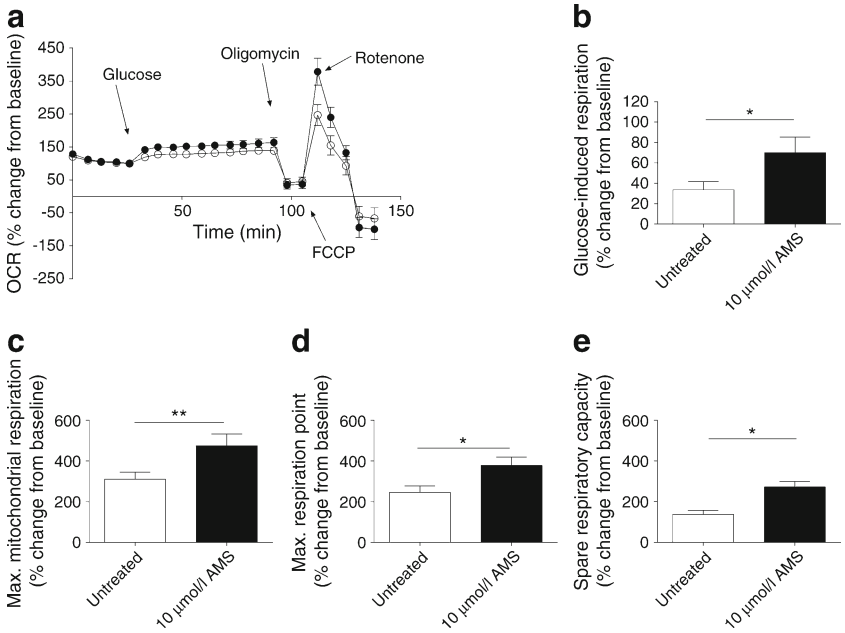

Fig. 7 (**a**) INS-1(832/13) cells stimulated with 2.8 (at baseline) and 16.7 mmol/l glucose either with (black circles) or without (white circles) AMS. AMS was added at the same time as additional glucose (indicated by the arrow); oligomycin, FCCP and rotenone were added to both groups as shown. (**b**) INS-1(832/13) cells stimulated with 16.7 mmol/l glucose without (untreated) or with addition of AMS. (**c**) Maximal respiration point in cells without (untreated) or with AMS. (**d**) Maximal respiration rate without (untreated) or with AMS. (**e**) Spare respiratory capacity in cells stimulated without (untreated) or with AMS. Data are means ± SEM ($n = 8$). *$p < 0.05$ and **$p < 0.01$ for indicated comparisons

5-HT_{2B} receptor activation is involved in priming cells to meet an increased metabolic demand. This agrees with previous data, suggesting that signalling through 5-HT_{2B} receptors in pregnant rodent beta cells is involved in proliferation, an energy-demanding process [6]. Interestingly, treatment with the selective serotonin re-uptake inhibitor fluoxetine disturbs mitochondrial respiration by increasing the formation of reactive oxygen species [42]. Thus, 5-HT overload may have detrimental effects on beta cell function, while physiological 5-HT levels or activation of specific 5-HT receptors may serve an enhancing role.

Information on individual effects of 5-HT receptors on GSIS in vitro is limited. We recently showed that *HTR1D* and *HTR2A* are overexpressed in islets from individuals with type 2 diabetes as compared with islets from non-diabetic individuals. The amine 5-HT and activation of 5-HT_{1D} receptors in non-diabetic human islets inhibits GSIS while activation of 5-HT_{2A} receptors potentiates GSIS. Interestingly, islets from donors with type 2 diabetes lose the inhibitory effect of 5-HT on GSIS, suggesting that alterations in 5-HT production or 5-HT receptor expression may contribute to beta cell dysfunction [11].

5-HT_3 receptors are implicated in compensatory increase in insulin secretion during pregnancy [8] and global *Htr3a* knockout mice on a high-fat diet display a defective first-phase insulin release in vitro [36]. Additionally, beta cell-specific *Tph1*-knockout mice fed a high-fat diet become increasingly glucose intolerant in vivo, exhibiting an insulin secretory defect in vitro, suggesting that basal 5-HT production in beta cells is essential in GSIS. Importantly, we show that mouse islets and INS-1(832/13) cells harbour the enzymes necessary for 5-HT synthesis and that low amounts of 5-HT are released from INS-1(832/13) cells and mouse islets.

Currently, the incidence of type 2 diabetes is increasing worldwide and there is an urgent need to develop novel therapeutic strategies that improve beta cell function. In sum, we provide novel data on localisation, function and signalling events caused by activation of 5-HT_{2B} receptors in beta cells in three different species. This information could be further explored to provide a drug target for type 2 diabetes treatment.

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Duality of interest The authors declare that there is no conflict of interest associated with this manuscript.

Contribution statement The study was designed by MF. Data acquisition and analysis and RNA isolation for RNA sequencing of human islets incubated in high vs low glucose was performed by EOL, PV and JF. Data acquisition and analysis and interpretation of Ca^{2+} measurements were performed by IGM, HB, MF and LE. Experimental set-up, analysis and interpretation of mitochondrial respiration were performed by HB, ABagge and MF. All other data analysis and experiments were performed by HB, AM, CN, MDN, ABalhuizen, NW, IA and MF. The manuscript was drafted by HB, IA and MF and all authors were involved in critical revision and approved the final version of the manuscript before submission. MF is the guarantor of this work.

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