Free-fatty acid receptor 4 inhibitory signaling in delta cells regulates islet hormone secretion in mice

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ABSTRACT

Objective

Maintenance of glucose homeostasis requires the precise regulation of hormone secretion from the endocrine pancreas. Free-fatty acid receptor 4 (FFAR4/Gpr120) is a G protein-coupled receptor whose activation in islets of Langerhans promotes insulin and glucagon secretion and inhibits somatostatin secretion. However, the contribution of individual islet cell types (α, β, and δ cells) to the insulinotropic and glucagonotropic effects of Gpr120 remains unclear. As Gpr120 mRNA is enriched in somatostatin-secreting δ cells, we hypothesized that Gpr120 activation stimulates insulin and glucagon secretion via inhibition of somatostatin release.

Methods

Glucose tolerance tests were performed in mice after administration of the selective Gpr120 agonist Cpd A. Gpr120 mRNA levels were assessed in pancreatic section by RNA in situ hybridization. Insulin, glucagon and somatostatin secretion were measured in static incubations of isolated mouse islets in response to endogenous (ω-3 polyunsaturated fatty acids) and pharmacological (Compound A and AZ-13581837) Gpr120 agonists. The effect of Compound A on hormone secretion was tested in islets isolated from mice with global or somatostatin cell-specific knockout (KO) of Gpr120. Calcium and cAMP dynamics in response to pharmacological Gpr120 agonists were measured in islets isolated from α, β and δ cell-specific GCaMP6 and CAMPER reporter mice, respectively.

Results

Acute exposure to Compound A increased glucose tolerance and circulating insulin and glucagon levels *in vivo*. Both pharmacological and endogenous Gpr120 agonists dose-dependently potentiated glucose-stimulated insulin secretion and arginine-stimulated glucagon secretion and concomitantly reduced somatostatin secretion in isolated islets. The effect of Compound A on hormone secretion was completely absent in islets from mice with either global or somatostatin cell-specific deletion of Gpr120, and was partially reduced upon blockade of somatostatin receptor signaling by cyclosomatostatin. Gpr120 agonists reduced forskolin-stimulated cAMP levels in δ cells, but did not evoke calcium signaling in islet cells.

Conclusions

This study supports a key contribution of inhibitory Gpr120-Gαi/o signaling in δ-cells in both insulin and glucagon secretion in part via mitigating somatostatin release.

Keywords: FFAR4, Gpr120, somatostatin, insulin, glucagon, islet of Langerhans

1. INTRODUCTION

G protein-coupled receptors are validated targets for the treatment of type 2 diabetes [1] and among these, the long-chain FA[1] receptor Gpr120/FFAR4 has been the subject of increasing interest in recent years as its activation has numerous beneficial effects on glucose and energy homeostasis in preclinical models. In rodents, Gpr120 activation alleviates obesity-induced chronic inflammation and associated insulin resistance [2; 3], promotes adipogenesis [4-6] and brown adipose tissue thermogenesis [7; 8], regulates food intake [9], and modulates enteroendocrine hormone secretion, including ghrelin [10-12], GLP-1 [13], GIP [14] and CCK [15; 16].

Gpr120 is also reportedly expressed in islet α, β and δ cells where its activation mitigates β-cell dysfunction [17] and apoptosis [18] and modulates islet hormone secretion. Gpr120 activation promotes GSIS [19-22]; potentiates arginine-induced glucagon secretion [23]; and inhibits GSSS [24].

Gpr120 signaling was reported to promote insulin secretion in insulin-secreting cell lines via intracellular calcium mobilization [19; 21]. Yet, transcriptomic profiling indicates that Gpr120 is primarily expressed in the δ cell with lower levels detected in α and β cells [18; 25-27]. Preferential expression of Gpr120 in islet δ cells was confirmed by knock-in of the LacZ reporter into the Gpr120 locus in mice [24]. However, the functional contribution of Gpr120 signaling in individual islet endocrine cell types to the net effect of its activation on insulin and glucagon secretion remains unknown. To clarify the role of Gpr120 in islet hormone secretion and define the precise contribution of δ-cell Gpr120 signaling in these processes, we measured insulin, glucagon and SST

[1] ALA: α-Linolenic acid; AZ: AZ-13581837; Cpd A: Compound A; cSST: cyclosomatostatin; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; FA: fatty-acids; GSIS: glucose-stimulated insulin secretion; GSSS: glucose-stimulated somatostatin secretion; KO: knock-out; SST: somatostatin; WT: wild-type

secretion in response to natural and synthetic Gpr120 agonists in isolated islets from WT, whole-body Gpr120 KO, and SST-cell specific Gpr120 KO mice.

2. MATERIALS AND METHODS

2.1 Reagents and solutions

RPMI-1640 and FBS were from Life Technologies Inc. (Burlington, ON, Canada). Penicillin/Streptomycin was from Multicell Wisent Inc (Saint-Jean-Baptiste, QC, Canada). FA-free BSA was from Equitech-Bio (Kerrville, TX, USA). Cpd A was from Cayman Chemical (Ann Arbor, MI, USA). AZ was generously provided by AstraZeneca (Gothenburg, Sweden). Insulin and glucagon RIA kits were from MilliporeSigma (Billerica, MA, USA). SST RIA kits were from Eurodiagnostica (Malmö, Sweden). Insulin and glucagon ELISA kits were from Alpco Diagnostics (Salem, NH, USA) and Mercodia (Uppsala, Sweden), respectively. cSST was from Tocris bioscience (Minneapolis, MN, USA). Aprotinin was from Roche Diagnostics (Rotkreuz, Switzerland). All other reagents were from MilliporeSigma unless otherwise specified.

2.2 Animals

All procedures involving animals were approved by the Institutional Committee for the Protection of Animals (IACUC) at the Centre Hospitalier de l'Université de Montréal, with the exception of GCaMP6 or CAMPer reporter mice, which were maintained for islet collection under the supervision of the IACUC of UC Davis. Animals at each institution were handled in accordance with the National Institutes of Health guide for the care and use of Laboratory animals. All mice were housed under controlled temperature on a 12h light/dark cycle with unrestricted access to water and standard laboratory chow and were sacrificed at 10-12 weeks of age for islet isolation.

C57BL/6N male mice were purchased from Charles River (Saint-Constant, QC, Canada).*Whole-body Gpr120 KO mice.* Mice carrying LoxP sites flanking exon 1 of Gpr120 ($Gpr120_{floxNeofrt}$) were obtained from Ingenious Targeting Laboratory, Ronkonkoma, NY, U.S.A. The neo cassette was removed by crossing the mice to ROSA26:FLPe mice (129S4/SvJaeSor-Gt(ROSA)26Sortm1(FLP1)Dym/J, The Jackson Laboratory, Bar Harbor, ME, USA), and the resulting mice were back-crossed onto a C57BL/6N background for more than 9 generations. Unexpectedly, homozygous $Gpr120_{flox}$ (prev-Flox) mice displayed an important reduction in Gpr120 gene expression and function in islets (**Supplementary Fig. 1A & B**) and other organs (data not shown), suggesting abnormal transcription of Gpr120 resulting from insertion of the LoxP sites and making them unsuitable for conditional KO studies. These $Gpr120_{flox}$ mice were crossed with transgenic E2A-Cre mice (B6.FVB-Tg(EIIa-cre)C5379Lmgd/J, The Jackson Laboratory) to remove exon 1 ($Gpr120_{\Delta}$) to generate whole-body Gpr120 KO animals (Gpr120KO). As the E2A-Cre mouse was of a mixed B/6N and B/6J background carrying the NNT mutation ($Nnt_{C57BL/6J}$), only $Gpr120_{\Delta}$ mice lacking E2A-Cre and $Nnt_{C57BL/6J}$ were kept for subsequent crossings. Male WT and Gpr120KO experimental animals were generated by crossing heterozygous $Gpr120_{\Delta}$ mice. Genotyping primers are listed in **Supplementary Table 1**. Animals were born at the expected Mendelian ratio and expression of Gpr120 was completely eliminated in KO islets (**Supplementary Fig. 1C**).

Whole-body Gpr40 KO mice. Male WT and Gpr40 KO mice were generated and genotyped as previously described [28].

SST cell-specific Gpr120 KO mice. Mice carrying LoxP sites flanking exon 1 and approximately 1.5 kb of sequence upstream of exon 1 (promoter region) of *Ffar4* on a C57BL/6N background

(C57BL/6-Ffar4$_{tm1.1Mrl}$) were purchased from Taconic Biosciences (hereafter designated Gpr120$_{+/fl}$ or Gpr120$_{fl/fl}$) and crossed with heterozygous SST-Cre mice (B6N.Cg-Sst$_{tm2.1(cre)Zjh}$/J, Jackson Laboratory) also on the C57BL/6N background. Gpr120$_{+/fl}$ and SST-Cre;Gpr120$_{+/fl}$ mice were crossed to generate experimental groups. Male WT, Gpr120$_{fl/fl}$ (Flox), SST-Cre (Cre) and SST-Cre;Gpr120$_{fl/fl}$ mice were used for secretion and proliferation experiments. Since experiments were performed in isolated islets in which only δ cells express SST, SST-Cre;Gpr120$_{fl/fl}$ mice are thereafter referred to as δGpr120KO mice. Genotyping primers are listed in **Supplementary Table 1**. Animals were born at the expected Mendelian ratio. Females of the 4 genotypes were used for qPCR experiments. Gpr120 mRNA levels were significantly reduced in δGpr120KO islets, in accordance with the predominant expression of Gpr120 in δ cells (**Supplementary Fig. 1D**). Gpr120 mRNA levels were similar in islets from WT, Gpr120Flox and SST-Cre mice.

CAMPer and GCaMP6 reporter mice. We used the following Cre drivers to express fluorescent biosensors for calcium (GCaMP6s; Gt(ROSA)26Sor$_{tm96(CAG-GCaMP6s)Hze}$) [29]or cAMP (C57BL/6-Gt(ROSA)26Sor$_{tm1(CAG-ECFP*/Rapgef3/Venus*)Kama}$/J) [30] directly and specifically in α cells (B6;129S4-Gcg$_{em1(cre/ERT2)Khk}$/Mmjax) [31], β cells (B6.FVB(Cg)-Tg(Ucn3-cre)KF43Gsat/Mmucd) [32], or δ cells (B6N.Cg-Sst$_{tm2.1(cre)Zjh}$/J).

2.3 mRNA fluorescence in situ hybridization

mRNAs were visualized by fluorescence in situ hybridization using the RNAscope Fluorescent Multiplex Kit (Advanced Cell Diagnostics, Inc., Hayward, California, USA) on fixed and frozen 12 week-old male C57Bl/6N pancreatic cryosections. Briefly, pancreata were fixed overnight in 4% paraformaldehyde and cryoprotected overnight in 30% sucrose. Tissues were then embedded in OCT, frozen, sectioned at 8 μm, and mounted on Superfrost Plus slides (Life Technologies,

Carlsbad, CA, USA). The following RNAscope probes were used: Mm-Ffar4-O1 (Cat No. 447041), Mm-Ins2-O1-C2 (Cat No. 497811-C2), Mm-Sst-C4 (Cat No. 404631-C4) and Mm-Gcg-C3 (Cat No. 400601-C3). Hybridization and fluorescent detection were performed according to the manufacturer's instructions. 20X images were acquired with a fluorescent microscope (Zeiss, Thornwood, NY, USA). 63X images were acquired with an inverted confocal microscope (Leica Microsystem, Mannheim, Germany).

2.4 Islet isolation

Mouse islets were isolated by collagenase digestion and dextran density gradient centrifugation as described previously [28] and allowed to recover overnight in RPMI 1640 supplemented with 10% (wt/vol) FBS, 100 U/ml penicillin/streptomycin and 11 mM glucose.

2.5 RNA extraction and quantitative RT-PCR

Total RNA was extracted from batches of 120-200 islets using the RNeasy micro kit (Qiagen, Valencia, CA). RNA was quantified by spectrophotometry using a NanoDrop 2000 (Life Technologies) and 0.4-1.0 µg of RNA was reverse transcribed. Real-time PCR was performed by using QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany). Results were normalized to cyclophilin A (Ppia) mRNA levels and normalized to the levels in control islets. Primer sequences are listed in **Supplementary Table 2**.

2.6 Static incubations for insulin secretion

After overnight recovery, islets were incubated in KRBH (pH 7.4) with 0.1% (w/v) FA-free BSA and 2.8 mM glucose for 20 min. Triplicate batches of 20 islets each were then incubated for an additional 20 min in KRBH, 0.1% FA-free BSA, 2.8 mM glucose, followed by a 1-h static

incubation in KRBH in the presence of 2.8 or 16.7 mM glucose, with or without synthetic Gpr120 agonists (10, 20 or 50 µM Cpd A ; 0.1, 1, 5 or 10 µM AZ) or endogenous ligands (ALA, EPA, DHA, 0.1 mM), as indicated in the figure legends. We selected Cpd A and AZ among the different Gpr120 agonists because of their selectivity towards Gpr120 versus Gpr40 [3; 20]. cSST (10 µM) was included at the time of Gpr120 agonist addition as indicated in Figure 6. SST and insulin were measured in the supernatant by RIA. Intracellular insulin content was measured after acid–alcohol extraction.

2.7 Static incubations for glucagon secretion

After overnight recovery, islets were incubated in KRBH (pH 7.4) with 0.1% (w/v) FA-free BSA and 5.5 mM glucose for 20 min. Triplicate batches of 20 islets each were then incubated an additional 20 min in KRBH, 0.1% FA-free BSA, 5.5 mM glucose, followed by a 1-h static incubation in KRBH in the presence of 1 mM glucose, with or without 10 mM L-arginine, Gpr120 agonists (10 or 50 µM Cpd A,; 0.1, 1, 5 or 10 µM AZ) and cSST (10 µM) as indicated in the figure legends. Secreted SST and glucagon were measured in the supernatant by RIA. Intracellular glucagon content was measured after acid–alcohol extraction.

2.8 Calcium and cAMP signaling in α, β, and δ cells

As previously described [32], following isolation islets were cultured overnight after which islets were placed in 35mm glass-bottom dishes (#1.5; MatTek Corporation, Ashland, MA, USA), allowed to attach overnight and imaged in x, y, z and t on a Nikon A1R+ confocal microscope using a 40X lens with a long working distance and 488 excitation and a 525/50 nm BP filter. For calcium imaging, treatments were continuously perfused over the islets using a Masterflex peristaltic pump at 2.5 mL/minute. Each protocol concluded with a 30 mM KCl pulse to

demonstrate viability and responsiveness throughout the treatment. Individual cells in individual z-planes were defined as regions of interest (ROI) and the green fluorescence intensity within the ROIs was plotted over time as a measure of calcium activity. To trace cAMP in δ cells we used islets from a double transgenic offspring of a cross between cAMPER (The Jackson Laboratory, strain #032205) and SST-Cre. Islets were processed and imaged essentially as described above for calcium tracing, but exciting with a 445 laser line while simultaneously detecting CFP (485/40 nm BP) and YFP (525/50 nm BP) emission with two parallel detectors. We used forskolin instead of KCl as our positive indicator of cell viability and ability to mount a cAMP response.

2.9 Metabolic tests

Cpd A (60 mg/kg BW) was administered orally in Cremophor-EtOH-water (1/1/18, v/v/v) to 4-h fasted mice 30 min prior to oral glucose administration (1 g/kg BW) or intraperitoneal L-arginine injection (1.25 g/kg BW). Tail blood glucose was measured using a hand-held Accu-Chek glucometer (Roche, Indianapolis, IN). For glucagon measurements, aprotinin (0.5 KIU/µL) was added immediately after collection and plasma samples were immediately snap frozen in liquid nitrogen. Plasma insulin and glucagon were measured by ELISA.

2.10 Statistical analyses

Data are expressed as mean ± SEM. Significance was tested using standard one-way ANOVA, Brown-Forsythe and Welch ANOVA and corrections, in cases of variance heterogeneity, or two-way ANOVA with post hoc adjustment for multiple comparisons, as appropriate, using GraphPad Instat (GraphPad Software, San Diego, CA). Tukey or Dunnett post hoc tests were performed as indicated in figure legends. $P<0.05$ was considered significant.

3. RESULTS

3.1 Cpd A acutely improves glucose tolerance and potentiates insulin and glucagon secretion in vivo

To assess the effect of Gpr120 activation on insulin secretion *in vivo*, Cpd A was administered orally to C57BL/6N mice 30 min prior to an oral glucose or arginine tolerance test. Cpd A-treated mice displayed significantly lower glycemia 30 min after Cpd A administration and at all time points thereafter (**Fig. 1A & B**). The improved glucose tolerance of Cpd A-treated mice was associated with increased insulin levels at t = 30 min (**Fig. 1C & D**). Likewise, Cpd A potentiated arginine-induced glucagon secretion (**Fig. 1E**). Cpd A-treated mice had significantly lower blood glucose 30 min after arginine administration (**Fig. 1F**), likely resulting from a Cpd A-dependent increase in insulin during the test. These results show that Cpd A improves glucose tolerance and increases insulin and glucagon secretion in mice.

3.2 Gpr120 activation potentiates GSIS and inhibits GSSS in isolated mouse islets

To assess whether intra-islet Gpr120 activation controls islet hormone secretion we measured insulin and SST secretion in isolated WT mouse islets in response to glucose alone or in the presence of synthetic (Cpd A or AZ) or endogenous ω-3 LCFA (ALA, EPA or DHA) Gpr120 agonists. High glucose (16.7mM) significantly increased both insulin (**Fig. 2A & Supplementary Fig. 2A**) and SST (**Fig. 2B**) secretion compared to the low glucose (2.8 mM) condition, as expected. Both Cpd A (**Fig. 2A & B**) and AZ (**Fig. 2C & D**) potentiated GSIS (**Fig. 2A & C & Supplementary Fig. 2A & C**) and simultaneously inhibited GSSS (**Fig. 2B & D**). Interestingly, inhibition of SST secretion by both Cpd A and AZ was already maximal at the lowest concentrations used (**Fig. 2B & D**) whereas stimulation of insulin secretion was dose-dependent

(**Fig. 2. A & C**), suggesting the contribution of SST-independent mechanisms to the stimulation of insulin release. Endogenous Gpr120 agonists (ALA, EPA, DHA) also potentiated GSIS (**Fig. 2E**) and inhibited GSSS (**Fig. 2F**). Although endogenous agonists were less efficient than the synthetic agonists in reducing GSSS, they displayed a greater potentiation of GSIS which is likely due to the combined activation of both Gpr120 and Gpr40 signaling pathways and non receptor-mediated effects due to intracellular metabolism. Cpd A, AZ and ω-3 FAs did not affect insulin content at any of the concentrations tested (**Supplementary Fig. 2B, D & F**).

To test the selectivity of Cpd A towards Gpr120, we treated Gpr120KO islets with the highest concentration of Cpd A (50 µM). The potentiation of GSIS induced by Cpd A in WT islets was absent in Gpr120KO islets (**Fig. 2G & Supplementary Fig. 2G**) without affecting insulin content (**Supplementary Fig. 2H**), confirming the requirement for Gpr120 in the insulinotropic effect of Cpd A. Likewise, the inhibitory effect of Cpd A on GSSS was largely abrogated in Gpr120KO islets (**Fig. 2H**). Residual Cpd A-mediated inhibition of SST secretion in Gpr120KO islets suggests mild off-target effects of Cpd A in these experiments. Interestingly, GSSS was significantly higher in Gpr120KO versus WT islets with no difference in basal (2.8 mM glucose) SST secretion (**Fig. 2H**). This might be explained by the paracrine/autocrine action of islet-derived Gpr120 ligands that reduce SST secretion in WT but not Gpr120KO islets.

In addition to their involvement in GSIS regulation, Gpr120 and Gpr40 share 10% sequence homology and are activated by an overlapping set of ligands [22]. Consequently, most Gpr120 agonists activate Gpr40 at high concentrations. To verify that Gpr40 signaling does not contribute to the effect of Cpd A on GSIS, we measured insulin secretion in response to 50 µM Cpd A in Gpr40KO islets. The effects of Cpd A on GSIS (**Fig. 2I & Supplementary Fig. 2I**), GSSS (**Fig.**

2J) and insulin content (**Supplementary Fig. 2J**) were similar in WT and Gpr40KO islets, ruling out a contribution of Gpr40 signaling.

3.3 Gpr120 activation potentiates arginine-stimulated glucagon secretion and inhibits SST secretion in low glucose conditions in isolated mouse islets

Glucagon secretion was stimulated by 10 mM arginine in the presence of 1 mM glucose, and Cpd A dose-dependently potentiated arginine-induced glucagon secretion (**Fig. 3A & Supplementary Fig. 3A**) while inhibiting SST secretion (**Fig. 3B**). Similarly, AZ dose-dependently potentiated arginine-induced glucagon secretion (**Fig. 3C & Supplementary Fig. 3C**). Neither Cpd A nor AZ affected glucagon content at any of the concentrations tested (**Supplementary Fig. 3B & D**). Arginine-induced glucagon secretion was significantly decreased in Gpr120KO but not Gpr40KO islets (**Fig. 3D & F & Supplementary Fig. 3E & G**). Gpr120KO (**Fig. 3E)**, but not Gpr40KO (**Fig. 3G**) islets also exhibit higher SST secretion in response to arginine. These results are consistent with the presence of islet-derived Gpr120 agonists that promote glucagon and inhibit SST secretion in WT and Gpr40KO but not Gpr120KO islets. As expected, the glucagonotropic effect of Cpd A was lost in Gpr120KO (**Fig. 3D & Supplementary Fig. 3E**) but maintained in Gpr40KO (**Fig. 3F & Supplementary Fig. 3G**) islets without effects on glucagon content (**Supplementary Fig. 3F & H**). Hence, Gpr120 but not Gpr40 is required for the potentiation of glucagon secretion by Cpd A.

3.4 Gpr120 mRNA is enriched in δ cells in the mouse pancreas.

To determine which islet cell types express Gpr120 mRNA we performed RNA in situ hybridization on sections of adult mouse pancreas. Double-fluorescence labelling with probes to insulin, glucagon and SST in conjunction with the Gpr120 probe was used to confirm Gpr120-

expressing cell identity. Gpr120 RNA transcripts were detected predominantly in δ **(Fig. 4A & B)** and α cells **(Fig. 4E & F)**, with comparatively few in β cells (**Fig. 4C & D**). Hence, although this method is not quantitative, these results confirm that in the adult mouse pancreas Gpr120 expression is enriched in islet δ cells [24].

3.5 Gpr120 signaling in δ cells mediates the insulinotropic and glucagonotropic effects of Cpd A in isolated mouse islets

To determine the specific contribution of δ cell Gpr120 signaling in the insulinotropic and glucagonotropic effect of Cpd A, we measured hormone secretion in static incubations in response to Cpd A in isolated islets from δGpr120KO mice compared to 3 control groups (WT, Gpr120Flox and SST-Cre). Cpd A dose-dependently increased GSIS in WT, Gpr120Flox and SST-Cre islets, but not in δGpr120KO islets (**Fig. 5A)**. Accordingly, Cpd A was unable to inhibit GSSS in δGpr120KO islets (**Fig. 5B**). Cpd A increased arginine-induced glucagon secretion in WT, Gpr120Flox and SST-Cre islets, but not in δGpr120KO islets (**Fig. 5C)**. Cpd A significantly inhibited arginine-induced SST secretion in WT and Gpr120Flox islets but not in δGpr120KO islets (**Fig. 5D**). We were unable to determine the effect of Cpd A in SST-Cre islets as arginine-induced SST secretion was largely absent (**Fig. 5D**). Taken together, these results show that δ cell Gpr120 signaling mediates the insulinotropic and glucagonotropic effects of Cpd A.

3.6 The regulation of islet hormone secretion by Cpd A is partially preserved upon SST receptor blockade

The stimulation of insulin and glucagon secretion by Cpd A in static incubations is inversely correlated with SST release (**Figs. 2, 3 & 5**) and is eliminated upon deletion of Gpr120 in δ cells

(**Fig. 5**). Given the known paracrine, inhibitory effect of SST on insulin and glucagon secretion [33], our observations suggest that Gpr120 activation alleviates SST inhibition of insulin and glucagon secretion. To directly assess this possibility, we tested the effects of Cpd A on hormone secretion in the presence of the competitive antagonist of all SST receptor isoforms, cyclosomatostatin (cSST). The potentiation of GSIS (**Fig. 6A**) and arginine-stimulated glucagon secretion (**Fig. 6B**) by Cpd A was only partially eliminated in the presence of cSST. Although we did not directly confirm the effectiveness of cSST treatment, the large increase in GSSS in the presence of cSST (**Fig. 6C**) suggests that the SST negative feedback loop was indeed alleviated. Together these data suggest that the increase in insulin and glucagon secretion in response to Cpd A likely involves a Gpr120-mediated, δ cell-derived signal in addition to SST.

3.7 Gpr120 activation inhibits cAMP generation in δ cells but does not modulate intracellular calcium

Our results so far suggest that the stimulation of insulin and glucagon secretion following Gpr120 activation is indirect and mediated by the δ cell. To exclude the possibility that Gpr120 agonists directly stimulate insulin and/or glucagon secretion via calcium mobilization as shown in various cell types [5; 13; 19-21; 23; 34], we measured calcium signaling in α, β and δ cells using a calcium reporter (GCaMP6) expressed under the control of the glucagon (Gcg), urocortin 3 (Ucn3) or somatostatin (SST) promoter, respectively. Cpd A and AZ did not affect calcium traces induced in α cells by an amino acid mixture in the presence of basal (5.5 mM) glucose (**Fig. 7A**). Nevertheless, the same α cells did mount robust calcium responses to AVP and KCl, as previously reported [32]. Similarly, although elevated glucose levels (10 and 16.8 mM) increased calcium in β (**Fig. 7B**) and δ (**Fig. 7C**) cells, respectively, Cpd A and AZ were without effect. We

subsequently exposed islets to SST to confirm that the GCaMP6 reporter was sensitive to inhibition in calcium levels. We then asked whether Gpr120 agonists modulate cAMP levels in δ cells using a cAMP reporter (CAMPER) expressed under the control of the somatostatin (SST) promoter. Elevated cAMP levels in response to forskolin in the presence of 16.8 mM glucose were reduced upon addition of either Cpd A (**Fig. 8A & Supplementary Video 1**) or AZ (**Fig. 8B & Supplementary Video 2**). Taken together these findings demonstrate that the Gpr120 signal transduction pathway in δ cells involves inhibition of cAMP generation independent of calcium mobilization.

4. DISCUSSION

The objectives of this study were to clarify the role of islet Gpr120 in the regulation of islet hormone secretion, and to delineate the specific contribution of δ-cell Gpr120 signaling. We first showed that Gpr120 activation improves glucose tolerance and increases insulin and glucagon secretion in vivo in mice. Accordingly, we found that Gpr120 agonists potentiate GSIS and arginine-stimulated glucagon secretion and concomitantly inhibit SST secretion in isolated mouse islets. Importantly, the activity of the Gpr120 agonist, Cpd A, is dependent on islet Gpr120 but not Gpr40. We demonstrated that Gpr120 mRNA is enriched in δ cells and that the insulinotropic and glucagonotropic effects of Cpd A are lost in δ-cell specific Gpr120 KO islets. Finally, we showed that Gpr120 activation inhibits forskolin-induced cAMP generation in δ cells but does not modulate calcium fluxes in either α, β or δ cells. Overall, this study supports a predominant contribution of inhibitory Gpr120 signaling in δ-cells in the control of both insulin and glucagon secretion.

We found that acute oral administration of Cpd A increases glucose tolerance and insulin and glucagon secretion (**Fig. 1**). These data are in agreement with the study by Sundstrom et al. [20] demonstrating improved glucose tolerance and increased insulin secretion following administration of the Gpr120 specific agonists AZ and Metabolex 36 in mice. Interestingly, Sundstrom et al. [20] demonstrated that the glucose lowering and insulinotropic effect of orally administered AZ and Metabolex 36 is dependent on gut-derived GLP-1. Hence, although our study supports a role of islet Gpr120 in the control of insulin and glucagon secretion in response to Cpd A, enteroendocrine hormone secretion may also contribute to the in vivo effects of Gpr120 agonism. Unfortunately, the specific contribution of Gpr120 signaling in islet δ cells in vivo could not be assessed in our δGpr120KO mouse model as Gpr120 is deleted in all SST-expressing cells of the body. Conversely, Gpr120-dependent GLP-1 release in vivo would not be expected to contribute positively to the glucagonotropic effects of Cpd A as GLP-1 has been show to inhibit glucagon secretion from α cells [35].

In isolated islets from WT mice, the synthetic Gpr120 agonists Cpd A and AZ, and the endogenous agonists ALA, EPA and DHA dose-dependently potentiate GSIS and inhibit GSSS. Furthermore, the effects of Cpd A are lost in Gpr120 but not Gpr40 KO islets (**Fig. 2**). These findings are in line with previous studies showing an insulinotropic effect of different Gpr120 agonists in vitro in isolated rodent islets and insulin-secreting cell lines (INS-1E and BRIN-BD11) [19; 21; 22]. In contrast Oh et al. [3] did not report a significant effect of Cpd A on GSIS in isolated mouse islets. However, the maximum dose tested was 10 µM, whereas in our hands a significant insulinotropic effect was detected beginning at 20 µM (**Fig. 2**). Similarly, Stone et al. [24] did not observe an increase in insulin secretion in response to the Gpr120 agonist Metabolex 36 despite an inhibitory effect on GSSS. Our observation that low concentrations of Cpd A inhibit GSSS but do not

stimulate GSIS might explain why Stone et al. observed a reduction in GSSS without a concomitant increase in GSIS at their chosen concentration of Metabolex 36.

Deletion of Gpr120 increases glucose- and arginine-stimulated SST secretion (**Fig. 2 & 3**). Although a constitutive, ligand-independent, activity of Gpr120 could potentially account for these observations, we favor a role of endogenous, islet-derived, Gpr120 ligands in controlling δ-cell function. This possibility would be analogous to the paracrine/autocrine action of FA [36] and 20-HETE [37] which are released by the β cell and contribute to Gpr40-mediated potentiation of GSIS. Whether islet-derived fatty acids also activate Gpr120 in the δ cell will require further studies. Contrary to whole-body Gpr120KO islets, δGpr120KO islets did not secrete more SST in response to glucose compared to WT islets (**Fig. 5**). This may be explained by the lower levels of SST expression in several tissues of SST-Cre mice, due to the Cre knock-in [38]. Another possibility is that residual Gpr120 activity in δ cells of δGpr120KO islets might contribute to the partial repression of SST secretion through the action of islet-derived Gpr120 ligands.

Cpd A and AZ dose-dependently potentiate arginine-stimulated glucagon secretion (**Fig. 3**). Furthermore, the effect of Cpd A is dependent on Gpr120 but not Gpr40. Although Suckow et al. [23] also reported an increase in glucagon secretion in response to palmitate and DHA, the glucagonotropic effect in their study required both Gpr120 and Gpr40. The difference between our results and those of Suckow et al. is likely due to the activity of the Gpr120 ligands at the concentrations tested. Hence, activation of Gpr120 alone can be sufficient to promote glucagon secretion. In the absence of exogenous Gpr120 agonists, arginine-stimulated glucagon secretion is reduced in Gpr120- but not Gpr40-deficient islets pointing to a possible role of endogenous Gpr120 ligands in the control of glucagon secretion.

We showed that the insulinotropic and glucagonotropic effects of Cpd A are mainly driven by δ-cell Gpr120 signaling, as they are not observed in δGpr120KO islets (**Fig. 5)**. The enriched expression of Gpr120 mRNA in δ cells detected in pancreatic islet sections (**Fig. 4**) and by other groups [18; 25-27] is consistent with these findings. Nevertheless, Gpr120 mRNA is also detected in α cells, and at low levels in β cells. Importantly, as Gpr120 may be subject to biased agonism, whether agonists distinct from Cpd A recruit signalling pathways (e.g. Gq/11) in β- or α-cells to promote hormone secretion remains unanswered. As GSSS is repressed in WT but not δGpr120KO islets, we inferred that Cpd A-induced insulin and glucagon secretion results mainly from the inhibition of SST secretion. However, the partial preservation of the stimulatory effect of Cpd A on hormone secretion in the presence of cSST (**Fig. 6**) suggests that other, Gpr120-dependent δ cell-derived signals may also play a role. Of note, neuropeptide Y family proteins, including peptide YY and pancreatic polypeptide (PPY) are expressed in δ cells [26] and negatively regulate GSIS [39; 40] and hence are potential δ cell-derived signals that could partially mediate the effects of Gpr120 activation on insulin secretion. Surprisingly, the effect of Cpd A on glucagon secretion is considerably stronger than its effect on insulin secretion, despite limited arginine-induced compared to glucose-induced SST secretion. Although, as discussed above, δ cell-derived signals other than SST might contribute to glucagon secretion, we favor the possibility that inhibition of SST secretion through activation of Gpr120 in δ cells contributes to the glucagonotropic effect of Cpd A. Indeed, α cells are highly sensitive to δ cell-derived SST (SST14) due to expression of the SST receptor subtype, SSTR2 [41]. Furthermore, recent studies suggest that α-cell activity is intimately regulated by δ cells, which exert a tonic inhibition through SST [42].

The loss of Cpd A response in δGpr120KO islets (**Fig. 5**) in the absence of changes in calcium fluxes in α and β cells in response to Cpd A and AZ (**Fig. 7**) suggest that under these experimental conditions, the δ cell is the primary site of action of Gpr120 in islets. Notwithstanding the possible contribution of δ-cell-derived products other than SST to insulin and glucagon secretion, the inhibition of SST secretion upon Gpr120 agonism suggests that in this context, the receptor is mainly coupled to Gαi/o subunits. This possibility is supported by the inhibition of forskolin-induced cAMP production and the absence of calcium mobilization in δ cells in response to Cpd A and AZ (**Fig. 8**). It is also consistent with previous studies in isolated mouse islets demonstrating a role of Gαi/o in Gpr120-mediated inhibition of SST secretion [24] and a decrease in islet cAMP levels in response to AZ and Metabolex 36 [20].

In conclusion, our results show that the insulinotopic and glucagonotropic effects of islet Gpr120 activation are mediated by Gpr120-Gαi/o signaling in δ cell, in part but not exclusively through inhibition of SST secretion. These findings contribute to our understanding of the role of FA in the regulation of islet hormone secretion and of the mechanisms of action of putative anti-diabetic drugs targeting Gpr120.

5. ACKNOWLEDGEMENTS

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6. AUTHOR CONTRIBUTIONS

Marine L. Croze: Conceptualization, Methodology, Investigation, Formal Analysis, Writing – Original Draft; **Marcus Flisher**: Methodology, Investigation, Formal Analysis, Visualization; **Arthur Guillaume**: Investigation, Formal Analysis; **Caroline Tremblay**: Methodology, Investigation; **Sabrina Granziera**: Investigation; **Glyn M. Noguchi**: Investigation; **Kevin Vivot**: Methodology, Investigation; **Julien Ghislain**: Conceptualization, Validation, Writing – Review and Editing, Supervision; **Mark O. Huising**: Conceptualization, Validation, Writing – Review and Editing, Supervision, Funding Acquisition; **Vincent Poitout**: Conceptualization, Validation, Writing – Review and Editing, Supervision, Funding Acquisition, Project Administration.

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8. FIGURE LEGENDS

Figure 1. Cpd A potentiates insulin secretion and arginine-induced glucagon secretion in mice. Blood glucose (A), area under the curve (AUC) of blood glucose (B) and plasma insulin (C and D) in mice during an oral glucose tolerance test (1g/kg) performed 30 min after oral administration of Cpd A (60 mg/kg) or vehicle (Cremophor-EtOH-water). Data are mean +/- SEM of 8 animals per group. Plasma glucagon (E) and blood glucose (F) in C57BL/6N mice during an arginine test (1.25 g/kg, ip) performed after oral administration of Cpd A (60 mg/kg) or vehicle. Data are expressed as mean +/- SEM of 7 animals per group. * $p<0.05$, ** $p<0.005$ and *** $p<0.0005$ compared to vehicle group following two-way ANOVA with Tukey post hoc adjustment for multiple comparisons.

Figure 2. Gpr120 activation potentiates GSIS and inhibits GSSS. Insulin secretion presented as a percentage of islet insulin content (A, C, E) and SST secretion measured in parallel on the same isolated islets (B, D, F) were assessed in 1-h static incubations in response to 2.8 or 16.7 mM glucose with or without the Gpr120 agonists Cpd A (10-50 µM) (A, B), AZ (0.1-10 µM) (C, D) or the ω-3 fatty acids, alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (100 µM) (E, F). Insulin and SST secretion in response to 2.8 or 16.7 mM glucose with or without Cpd A (50 µM) was measured in isolated islets from Gpr120KO (G, H) and Gpr40KO (I, J) mice and WT littermate controls. Data represent individual values and are expressed as mean +/- SEM of 4-8 independent experiments. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$ or ****$p<0.0001$ between groups following one-way ANOVA (A-F) or two-way ANOVA (G-J) with Dunnett's (A-F, versus 16.7-EtOH condition) or Tukey's (G-J) post hoc adjustment for multiple comparisons and Welch/Brown-Forsythe correction when necessary to compensate for SD variances.

Figure 3. Gpr120 activation potentiates arginine-stimulated glucagon secretion and inhibits SST secretion. Glucagon secretion presented as a percentage of islet glucagon content (A, C) and SST secretion measured in parallel on the same isolated islets (B) were assessed in 1-h static incubations in response to 10 mM arginine with or without the Gpr120 agonists Cpd A (10-50 µM) (A, B) and AZ (0.1-10 µM) (C). Glucagon secretion in response to 10 mM arginine with or without Cpd A (50 µM) (D, F) and arginine-stimulated SST secretion in the absence of exogenous Gpr120 agonist (E, G) was measured on isolated islets from Gpr120KO (D, E) and Gpr40KO (F, G) mice and WT littermate controls. Data represent individual values and are expressed as mean +/- SEM from 4-8 independent experiments. * $p<0.05$, ** $p<0.005$ or ***$p<0.0005$ between groups following one-way ANOVA (A-C) with Welch/Brown-Forsythe correction when necessary to compensate for SD variances, or two-way ANOVA (D-H) with Dunnett or Tukey post hoc adjustment for multiple comparisons.

Figure 4. Gpr120 mRNA levels are predominantly detected in mouse islet δ and α cells. Gpr120 (green) and SST (A, B), insulin (INS; C, D), or glucagon (GCG; E, F) (magenta) mRNA were detected in adult mouse pancreatic cryosections by fluorescent in situ hybridization. Representative sections are shown at 20 X (A, C, E) and 63 X (B, D, F) magnification. DAPI (blue). Scale bar = 10 µm.

Figure 5. Gpr120 signaling in δ cells mediates Cpd A-dependent potentiation of GSIS, arginine-stimulated glucagon secretion and inhibition of SST secretion. Normalised insulin secretion presented as a percentage of the 16.7-EtOH condition (A) and SST secretion measured in parallel on the same isolated islets (B) were assessed in 1-h static incubations in response to 2.8 or 16.7 mM glucose with or without Cpd A (10 or 50 µM) on isolated islets from WT, $Gpr120^{fl/fl}$

(Flox), SST-Cre (Cre) and SST-Cre, Gpr120$_{fl/fl}$ (δKO) mice. Normalised glucagon secretion presented as a percentage of the ARG-EtOH condition (C) and SST secretion measured in parallel on the same isolated islets (D) were assessed in 1-h static incubations in response to 10 mM arginine with or without Cpd A (50 µM) on isolated islets from mice of the 4 genotypes. Data represent individual values and are expressed as mean +/- SEM from 3-8 independent experiments. * $p<0.05$, ** $p<0.005$, ***$p<0.0005$ or ****$p<0.0001$ between groups following two-way ANOVA with Dunnett post hoc adjustment for multiple comparisons.

Figure 6. Cpd A-dependent potentiation of GSIS and arginine-stimulated glucagon secretion are only partially affected by SST receptor blockade. (A) Insulin secretion presented as a percentage of islet insulin content was assessed in 1-h static incubations in response to 2.8 or 16.7 mM glucose with or without the Gpr120 agonists Cpd A (50 µM). (B) Glucagon secretion presented as a percentage of islet glucagon content was assessed in 1-h static incubations in response to 10 mM arginine with or without the Gpr120 agonists Cpd A (50 µM). (C) SST secretion was assessed as in (A). Data represent individual values and are expressed as mean +/- SEM of 5-6 independent experiments. * $p<0.05$, *** $p<0.0005$ or ****$p<0.0001$ between groups following two-way ANOVA with Tukey post hoc adjustment for multiple comparisons between all groups.

Figure 7. Gpr120 agonists do not evoke calcium signals in mouse α, β or δ cells. The calcium sensor GCaMP6 was used to measure calcium activity in individual α (A), β (B) and δ (C) cells in the presence of 5.5 mM glucose and an amino acid mixture (2mM each of glutamine, arginine and alanine) (A) or 2.8 mM glucose (B, C) followed by Cpd A (50 µM), AZ (50 µM) and SST (100 nM) in the presence of 5.5 mM glucose with amino acid mixture (A) or 10 (B) and 16.8 (C)

mM glucose. KCl (30 mM) induced depolarization (A, B, C) and AVP (10 nM) (A) served as positive controls.

Figure 8. Gpr120 agonists inhibit forskolin-induced elevation in cAMP levels in mouse δ cells. The cAMP sensor cAMPER was used to measure cAMP levels in individual δ cells in islets from an SST-Cre x lsl-cAMPER cross. Cells were exposed to 2.8 mM glucose or 16.8 mM glucose with forskolin (1 μM) and Cpd A (50 μM) (A) or AZ (10 μM) (B) at the times indicated.

Figure 1

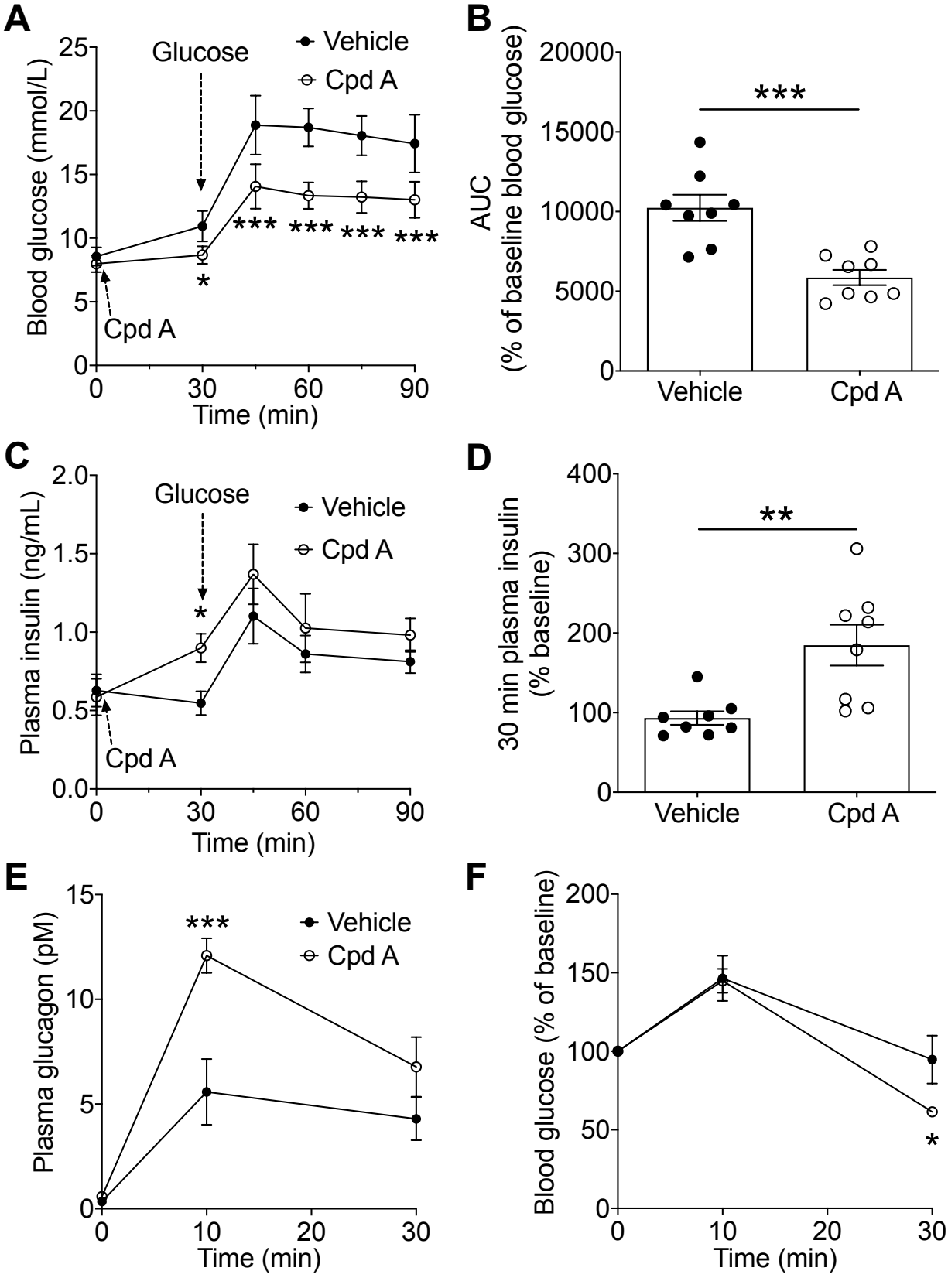

A
Glucose
Vehicle
Cpd A
Blood glucose (mmol/L)
Cpd A
Time (min)
B
AUC
(% of baseline blood glucose)
Vehicle
Cpd A
C
Glucose
Vehicle
Cpd A
Plasma insulin (ng/mL)
Cpd A
Time (min)
D
30 min plasma insulin
(% baseline)
Vehicle
Cpd A
E
Vehicle
Cpd A
Plasma glucagon (pM)
Time (min)
F
Blood glucose (% of baseline)
Time (min)

Figure 2

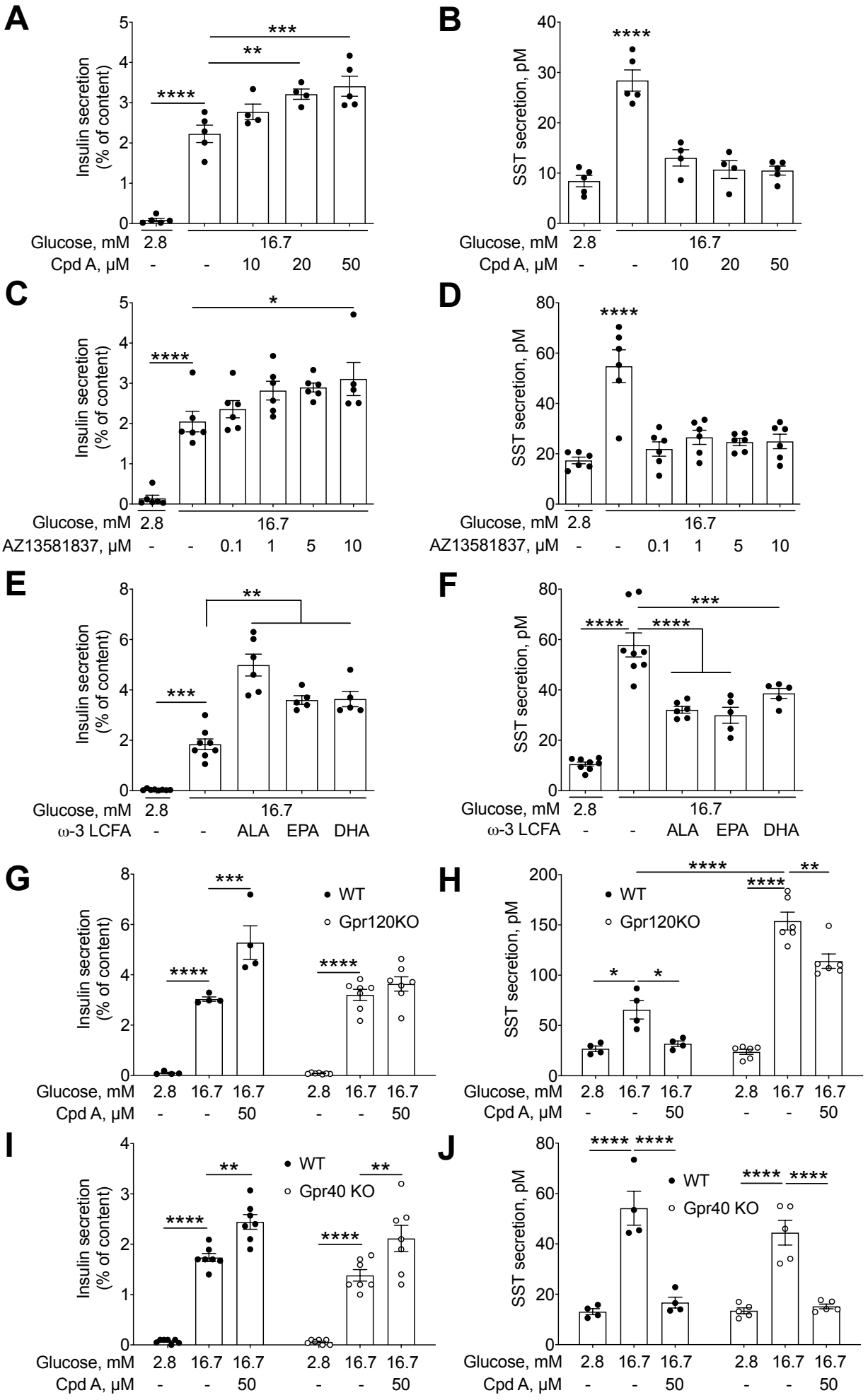

A
Insulin secretion (% of content)
Glucose, mM 2.8 16.7
Cpd A, μM - - 10 20 50
B
SST secretion, pM
Glucose, mM 2.8 16.7
Cpd A, μM - - 10 20 50
C
Insulin secretion (% of content)
Glucose, mM 2.8 16.7
AZ13581837, μM - - 0.1 1 5 10
D
SST secretion, pM
Glucose, mM 2.8 16.7
AZ13581837, μM - - 0.1 1 5 10
E
Insulin secretion (% of content)
Glucose, mM 2.8 16.7
ω-3 LCFA - - ALA EPA DHA
F
SST secretion, pM
Glucose, mM 2.8 16.7
ω-3 LCFA - - ALA EPA DHA
G
Insulin secretion (% of content)
WT
Gpr120KO
Glucose, mM 2.8 16.7 16.7 2.8 16.7 16.7
Cpd A, μM - - 50 - - 50
H
SST secretion, pM
WT
Gpr120KO
Glucose, mM 2.8 16.7 16.7 2.8 16.7 16.7
Cpd A, μM - - 50 - - 50
I
Insulin secretion (% of content)
WT
Gpr40 KO
Glucose, mM 2.8 16.7 16.7 2.8 16.7 16.7
Cpd A, μM - - 50 - - 50
J
SST secretion, pM
WT
Gpr40 KO
Glucose, mM 2.8 16.7 16.7 2.8 16.7 16.7
Cpd A, μM - - 50 - - 50

Figure 3

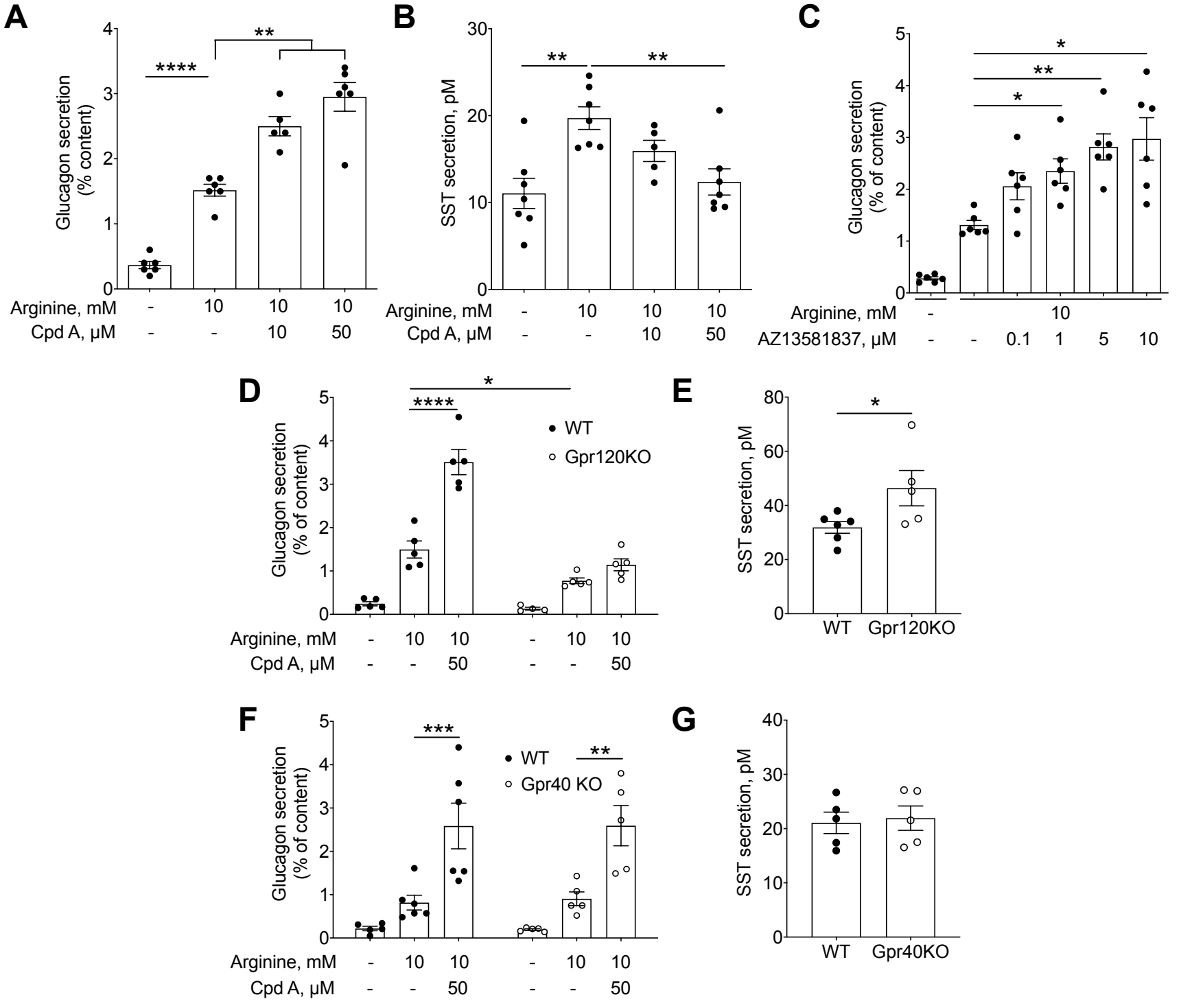

A
Glucagon secretion (% content)
Arginine, mM - 10 10 10
Cpd A, μM - - 10 50
B
SST secretion, pM
Arginine, mM - 10 10 10
Cpd A, μM - - 10 50
C
Glucagon secretion (% of content)
Arginine, mM - 10
AZ13581837, μM - - 0.1 1 5 10
D
Glucagon secretion (% of content)
WT
Gpr120KO
Arginine, mM - 10 10 - 10 10
Cpd A, μM - - 50 - - 50
E
SST secretion, pM
WT
Gpr120KO
F
Glucagon secretion (% of content)
WT
Gpr40 KO
Arginine, mM - 10 10 - 10 10
Cpd A, μM - - 50 - - 50
G
SST secretion, pM
WT
Gpr40KO

Figure 4

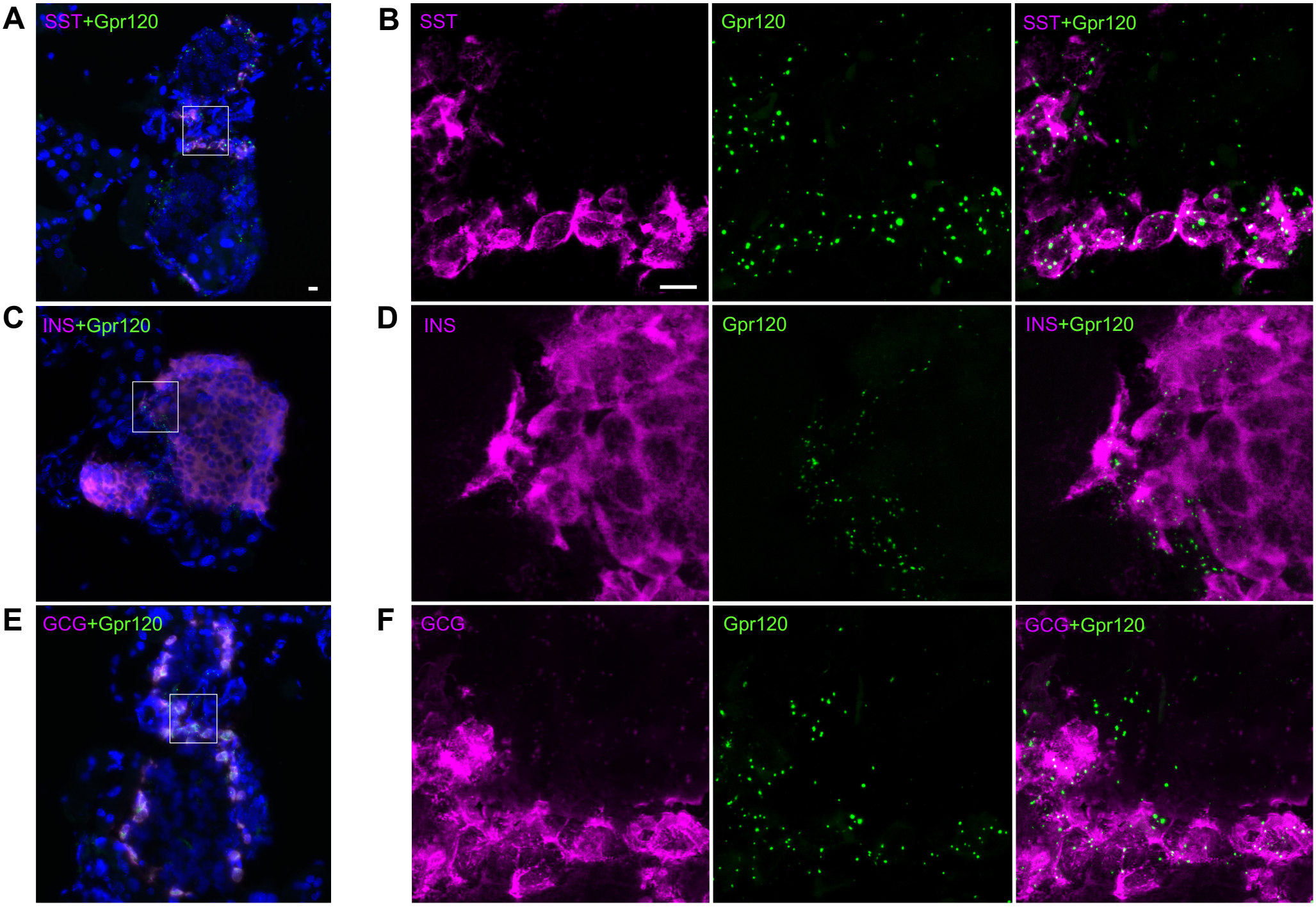

A
SST+Gpr120
B
SST
Gpr120
SST+Gpr120
C
INS+Gpr120
D
INS
Gpr120
INS+Gpr120
E
GCG+Gpr120
F
GCG
Gpr120
GCG+Gpr120

Figure 5

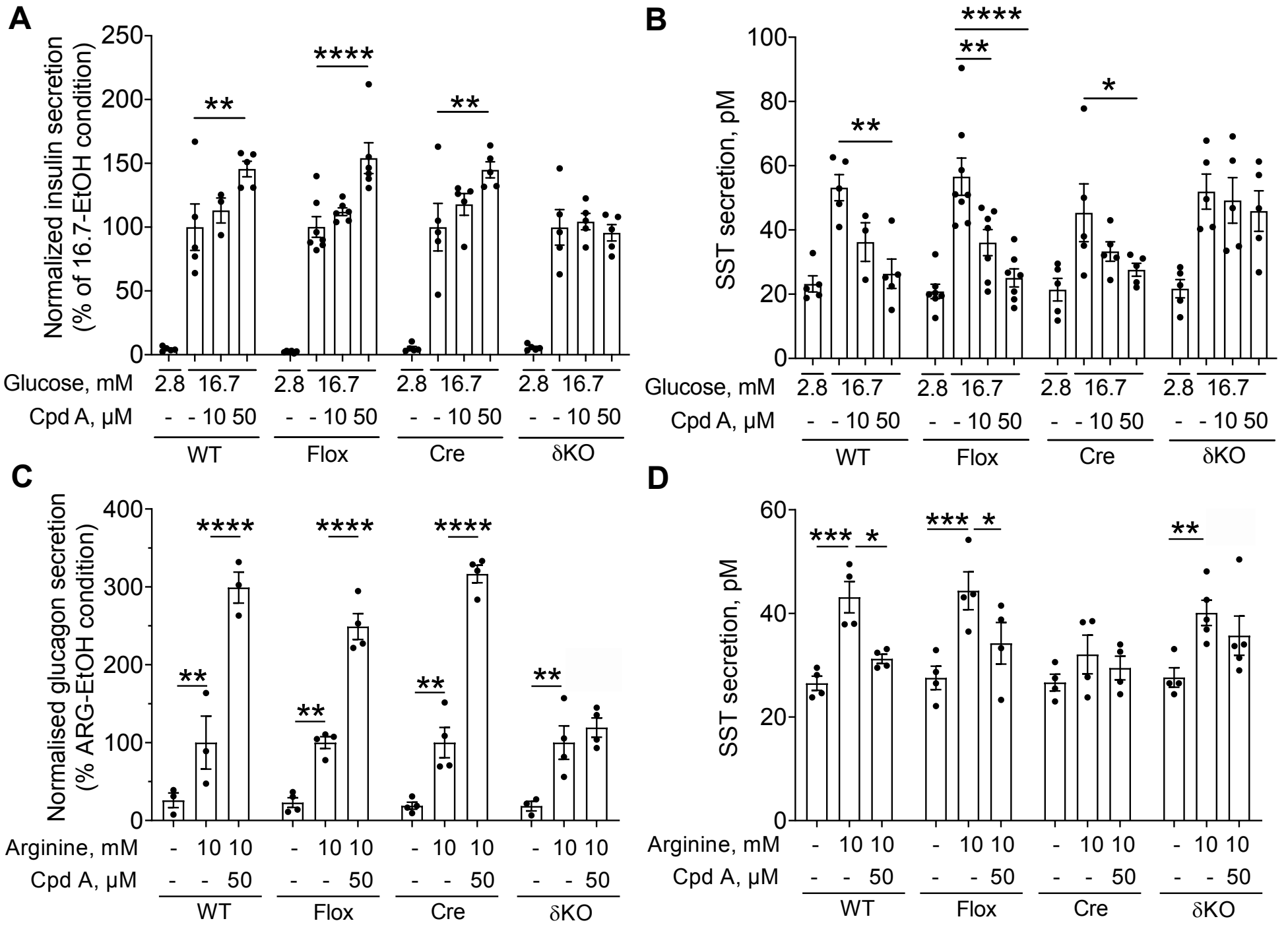

A
Normalized insulin secretion (% of 16.7-EtOH condition)
250
200
150
100
50
0
**
****
**
Glucose, mM 2.8 16.7 2.8 16.7 2.8 16.7 2.8 16.7
Cpd A, μM - - 10 50 - - 10 50 - - 10 50 - - 10 50
WT Flox Cre δKO
B
SST secretion, pM
100
80
60
40
20
0
**
****
**
*
Glucose, mM 2.8 16.7 2.8 16.7 2.8 16.7 2.8 16.7
Cpd A, μM - - 10 50 - - 10 50 - - 10 50 - - 10 50
WT Flox Cre δKO
C
Normalised glucagon secretion (% ARG-EtOH condition)
400
300
200
100
0
**
****
**
****
**
****
**
Arginine, mM - 10 10 - 10 10 - 10 10 - 10 10
Cpd A, μM - - 50 - - 50 - - 50 - - 50
WT Flox Cre δKO
D
SST secretion, pM
60
40
20
0
***
*
***
*
**
Arginine, mM - 10 10 - 10 10 - 10 10 - 10 10
Cpd A, μM - - 50 - - 50 - - 50 - - 50
WT Flox Cre δKO

Figure 6

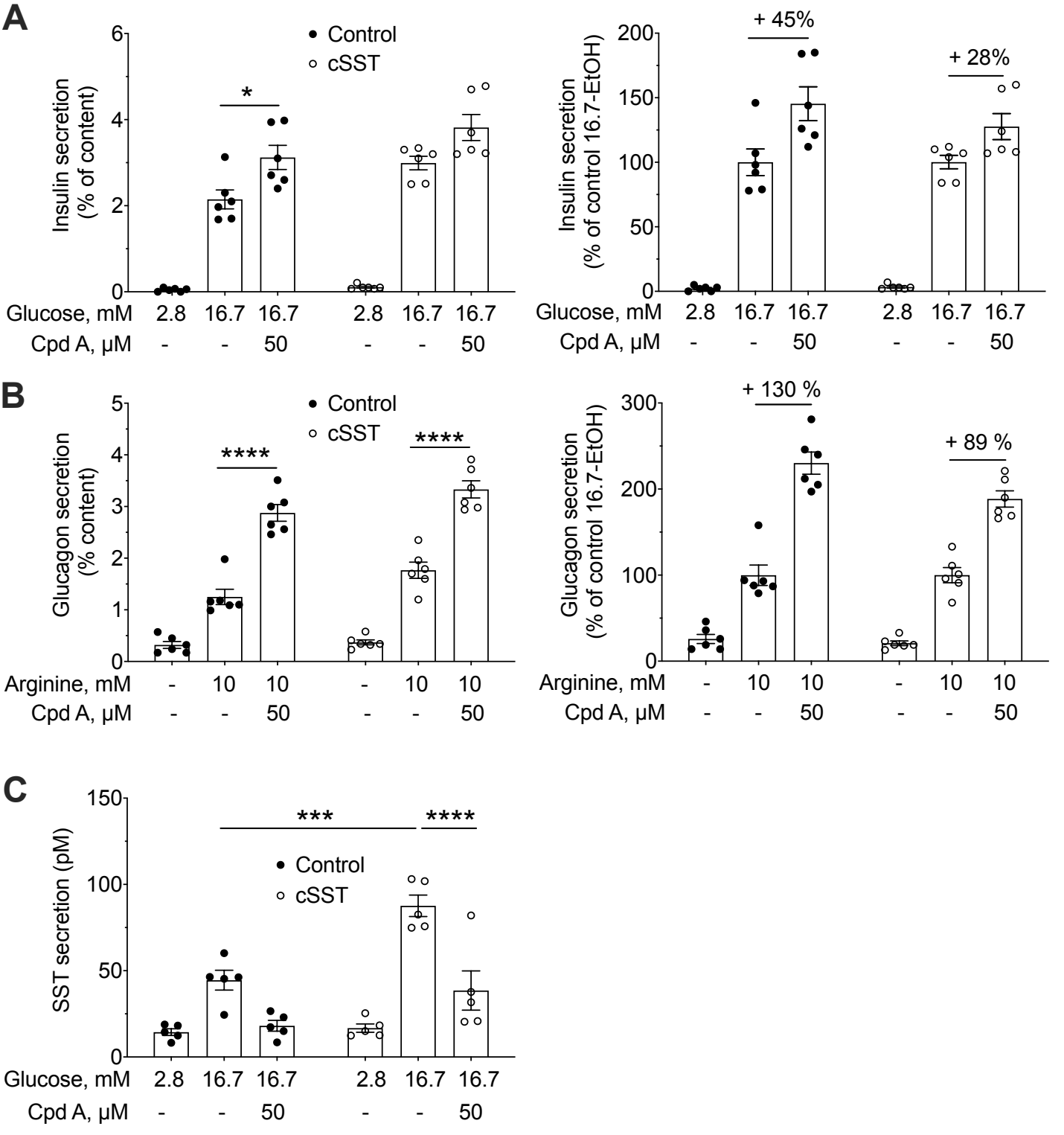

A
Control
cSST
Insulin secretion (% of content)
*
Insulin secretion (% of control 16.7-EtOH)
+ 45%
+ 28%
Glucose, mM 2.8 16.7 16.7 2.8 16.7 16.7
Cpd A, μM - - 50 - - 50
B
Glucagon secretion (% content)
****
****
Glucagon secretion (% of control 16.7-EtOH)
+ 130 %
+ 89 %
Arginine, mM - 10 10 - 10 10
Cpd A, μM - - 50 - - 50
C
SST secretion (pM)
***
****
Glucose, mM 2.8 16.7 16.7 2.8 16.7 16.7
Cpd A, μM - - 50 - - 50

Figure 7

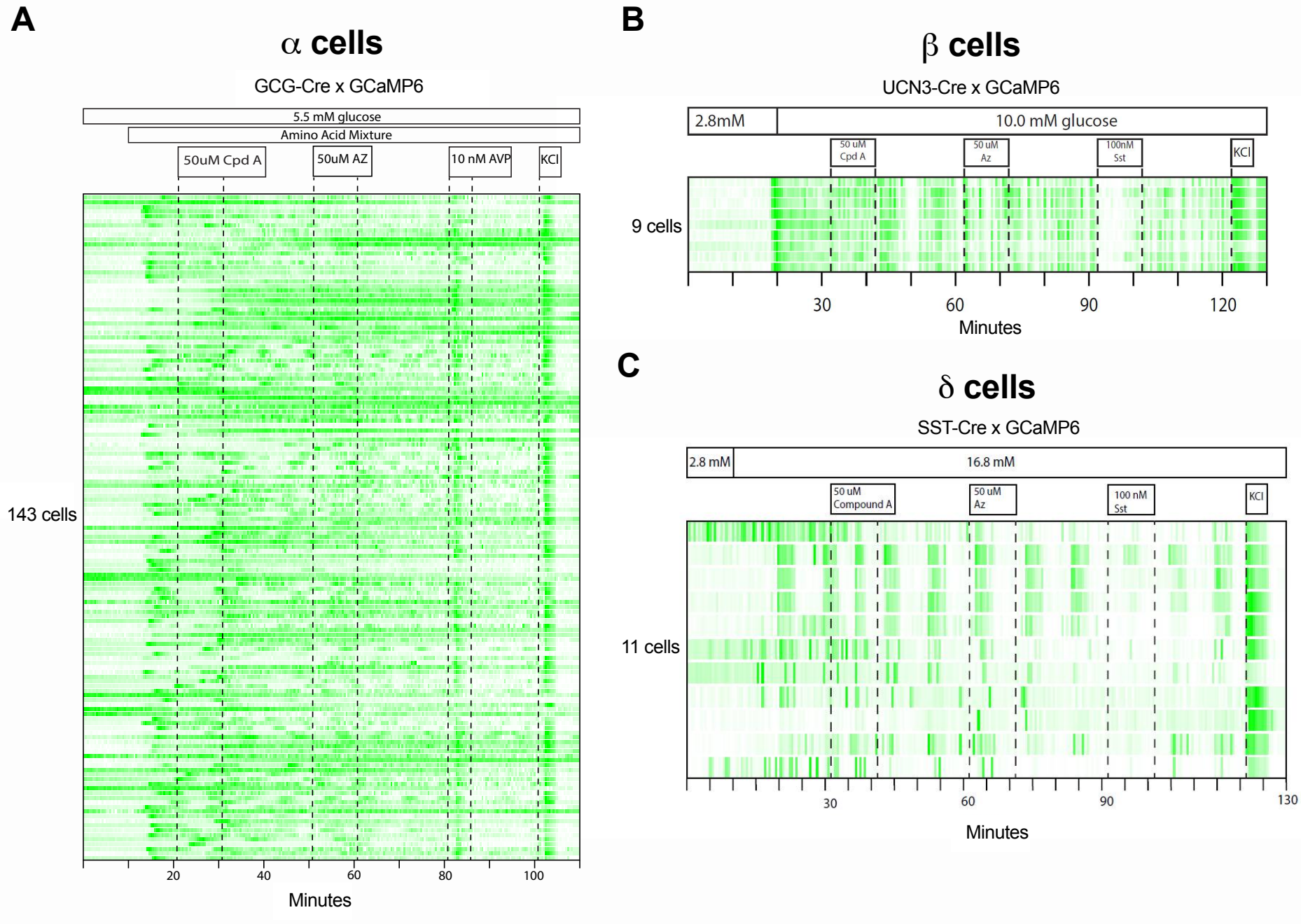
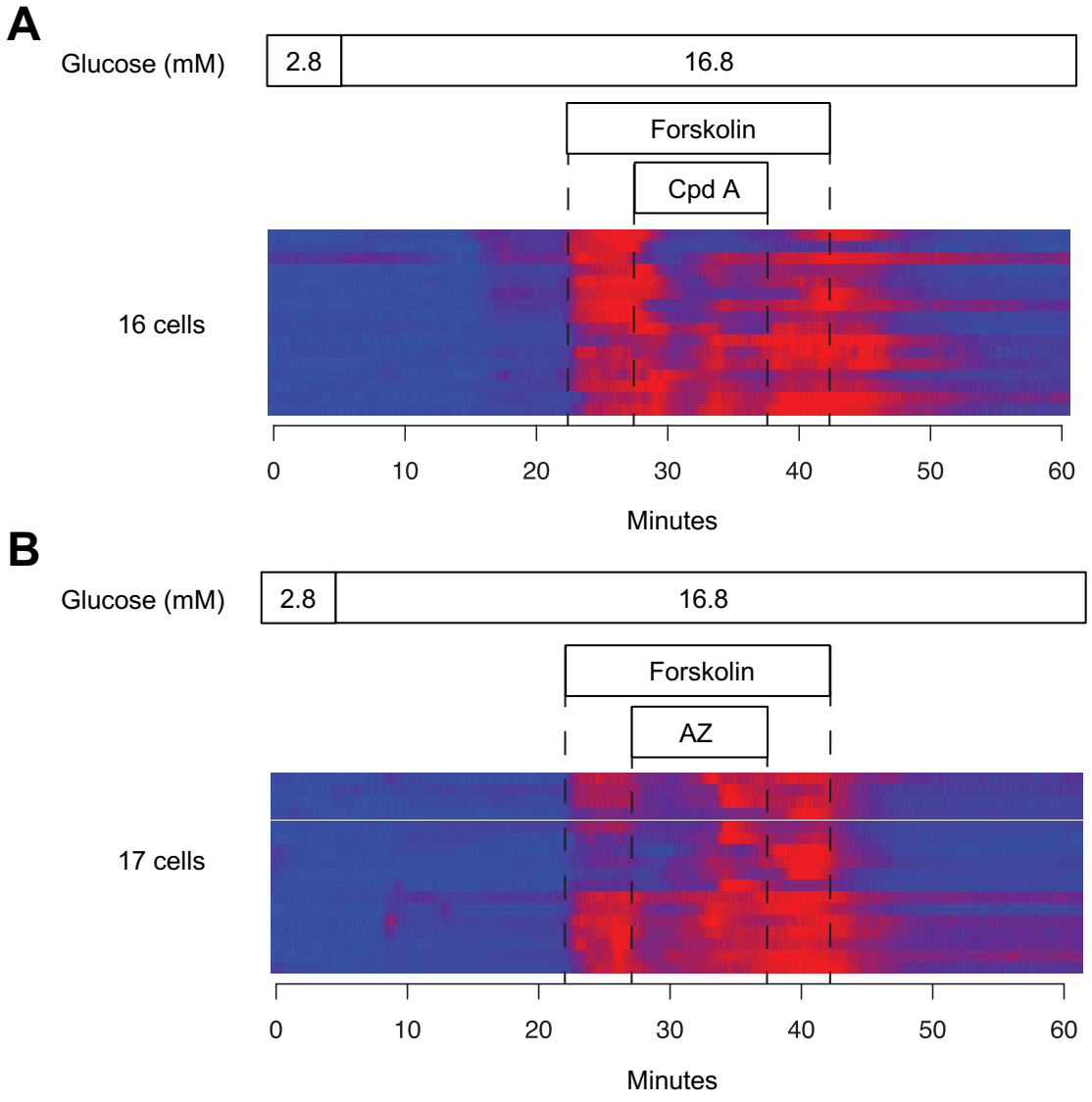

A
α cells
GCG-Cre x GCaMP6
5.5 mM glucose
Amino Acid Mixture
50uM Cpd A
50uM AZ
10 nM AVP
KCl
143 cells
20
40
60
80
100
Minutes
B
β cells
UCN3-Cre x GCaMP6
2.8mM
10.0 mM glucose
50 uM Cpd A
50 uM Az
100nM Sst
KCl
9 cells
30
60
90
120
Minutes
C
δ cells
SST-Cre x GCaMP6
2.8 mM
16.8 mM
50 uM Compound A
50 uM Az
100 nM Sst
KCl
11 cells
30
60
90
130
Minutes

Figure 8


A
Glucose (mM)
2.8
16.8
Forskolin
Cpd A
16 cells
0
10
20
30
40
50
60
Minutes
B
Glucose (mM)
2.8
16.8
Forskolin
AZ
17 cells
0
10
20
30
40
50
60
Minutes