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CART is overexpressed in human type 2 diabetic islets and inhibits glucagon secretion and increases insulin secretion

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ABSTRACT

Aims/Hypothesis Insufficient insulin release and hyperglucagonemia are culprits in type 2 diabetes. Cocaine- and amphetamine-regulated transcript (CART) affects islet hormone secretion and beta cell survival *in vitro* in rats and CART-/- mice have diminished insulin secretion. We aimed to test if CART is differentially regulated in human type 2 diabetic islets and if CART affects insulin- and glucagon secretion *in vitro* in humans and *in vivo* in mice.

Methods CART expression was assessed in human type 2 diabetic and non-diabetic control pancreases and diabetic rodent models. Insulin- and glucagon secretion was examined in isolated islets and *in vivo* in mice. Ca^{2+} oscillation patterns and exocytosis was studied in mouse islets.

Results We report an important role of CART in human islet function and glucose homeostasis in mice. CART was found to be expressed in human alpha and beta cells and in a subpopulation of mouse beta cells. Notably, CART expression was several-fold higher in islets of type 2 diabetic humans and rodents. CART increased insulin secretion *in vivo* in mice and in human and mouse islets. Furthermore, CART increased beta cell exocytosis, altered the glucose-induced Ca^{2+} signalling pattern in mouse islets from fast to slow oscillations and improved synchronization of the oscillations between different islet regions. Finally, CART reduced glucagon secretion in human and mouse islets, as well as *in vivo* in mice via diminished alpha cell exocytosis.

Conclusions We conclude that CART is a regulator of glucose homeostasis and could play an important role in the pathophysiology of type 2 diabetes. Based on its properties to increase insulin secretion and reduce glucagon secretion CART-based agents could be a therapeutic modality in type 2 diabetes.

Keywords: CART-peptide, Cocaine- and amphetamine-regulated transcript, glucagon, insulin, islets, type 2 diabetes

Abbreviations:

$[Ca^{2+}]_i$: cytoplasmic Ca^{2+} concentration

ΔC_{tot}: capacitance increase

AIR: acute insulin response

CART/CARTPT: cocaine- and amphetamine-regulated transcript

DEX: dexamethasone

ESM: electronic supplementary material

GSIS: glucose-stimulated insulin secretion

ICC: immunocytochemistry

ISH: in situ hybridization

IVGTT: intravenous glucose-tolerance test

K_G: glucose tolerance index

INTRODUCTION

Insufficient insulin secretion and exaggerated glucagon secretion are key features of type 2 diabetes [1-3]. The pancreatic islets are vital regulators of glucose homeostasis, and islet dysfunction is a primary cause of type 2 diabetes [4]. Islet hormone secretion is controlled by the metabolic state, e.g. plasma levels of glucose, amino acids and fatty acids. Furthermore, the islets are influenced by regulatory peptides expressed in islet cells or released from nerve fibres innervating the islets [5].

The anorexigenic regulatory peptide cocaine- and amphetamine-regulated transcript (CART) [6-10] is expressed in islet cells and islet nerve fibres in animals [11-17]. CART regulates islet hormone secretion and protects beta cells from glucotoxicity-induced cell death *in vitro* in rat islets [12, 18]. Importantly, CART is required for normal islet function: CART-/- mice exhibit islet dysfunction with diminished insulin secretion and reduced glucose elimination [15]. On the other hand, expression of CART is augmented in beta cells in rat models of type 2 diabetes [12]. Moreover, CART-/- mice are obese [15, 19], and genetic variations in the *CARTPT* gene associates with obesity in humans [20, 21].

It is not known if CART affects human islet function or if CART is expressed in human islets and affected by type 2 diabetes. Furthermore, it is not known how CART affects glucose homeostasis *in vivo*. To address this, we examined islet CART expression in nondiabetic and type 2 diabetic subjects, as well as the effect of CART on islet hormone secretion in human islets. Furthermore, we studied the effect of exogenous CART in mice to pinpoint the mechanisms underlying the effect of CART on insulin secretion.

MATERIAL AND METHODS

Human tissue

Human pancreases from 9 type 2 diabetic patients (5 males, 4 females; median age 54, BMI 28.9±7kg/m^2) and 10 controls (4 males, 6 females; median age 56, BMI 25.8±2kg/m^2) were used for morphometric analysis. Human islets were provided by Nordic Network for Clinical Islet Transplantation, Uppsala University. All procedures were approved by Uppsala and Lund University ethics committees.

CART peptide

Rat CART55-102 peptide (American Peptide Co Inc, Sunnyvale, CA, or Novo Nordisk A/S, Målöv, Denmark (gift from Dr Lars Thim)) was used.

Immunocytochemistry, *in situ* hybridization and image analysis

Immunocytochemistry was performed using characterized antibodies as detailed [13, 22]. Specificity of CART antibodies (Cocalico, Reamstown, PA) was verified in CART-/- mice and with pre-absorption with CART55-102 [23]. 30-mer oligodeoxyribonucleotide probes for *CART* mRNA were used for *in situ* hybridization (ISH) in human [24] and rat [12] specimens as detailed [25]. Hybridization in the presence of excess unlabelled probe on adjacent sections was used as negative control. CART immunoreactive alpha cells and beta cells, and CART ISH-labelled area/whole islet area was quantified blinded in the indicated samples in all islets in three separate sections as described [12].

Western-blot

Western-blot was performed as detailed [26] using CART primary antibody (Cocalico, Stevens, PA) diluted 1:1000 with CART55-102 (Novo Nordisk A/S, Målöv, Denmark) as a positive control.

Animal models of type 2 diabetes

Experiments were approved by the regional animal ethics committee and the Guide for the care and use of laboratory animals, (Eighth edition) was followed. Female Sprague-Dawley (SD) rats (225g) were used. Six rats received dexamethasone (DEX), 2mg/kg/day *i.p.* for 10 days [12, 27]. Six rats received DEX and Levermir (10 U/day, Novo Nordisk A/S, Målöv, Denmark) for 10 days. Controls (n=6) received saline. In addition (female mice, n=5-8), HNF1αdn mice [28], *ob/ob* mice, C57Bl/6J mice (Taconic, Bomholt, Denmark) fed a high fat diet for 6 months and matched C57Bl/6J control mice were used [29]. Pancreases were processed as detailed [12].

Real-time quantitative PCR

Total RNA was isolated using All Prep DNA/RNA kit (Qiagen GmbH, Hilden, Germany). 1 µg of RNA was reverse-transcribed to cDNA with QuantiTect Reverse Transcription kit (Qiagen GhbH, Hilden, Germany). Quantitative RT-PCR was performed on ABI Prism 7900 HT system (Applied Biosystems, Foster City, CA) with TaqMan technology using assay Hs00182861_m1 for CART and 4333768F for HPRT1, serving as endogenous control. Gene expression was analysed with the ΔΔCt method.

***In vitro* islet studies**

Secretion studies in human and mouse islets (1h incubations) with insulin secretagogues, w/wo CART55-102 as indicated were performed as detailed [15, 30, 31]. One experiment represents one donor/animal and 6-8 technical repeats were used for each experiment. For *Cart* mRNA expression studies, rat islets were cultured 24h in 5, 11.1 or 25 mmol/l glucose in RPMI medium (1% serum).

Intravenous glucose-tolerance test (IVGTT)

IVGTT was performed as detailed [15] in female C57Bl/6J mice w/wo 150 nmol/kg CART55-102 and 4 nmol/kg GLP-1 co-injected with glucose (0.75g/kg) *i.v.* Controls received saline. Acute insulin response (AIR) was calculated as mean suprabasal insulin levels for min 1 and 5 (i.e., $(ins_{5\ min} + ins_{1\ min})/2 - ins_{0\ min}$). Glucose elimination rate (K_G) was calculated as the slope for 1-20min of the logarithmic transformation of individual plasma glucose values [15].

Glucagon secretion *in vivo*

Female C57Bl/6J mice were fasted overnight and given 150 nmol/kg CART55-102 *i.v.* Blood samples were taken from the retro-orbital plexus after 2 min. Controls received saline.

Electrophysiology

Depolarization-evoked exocytosis was recorded by capacitance measurements using standard whole-cell configuration w/wo CART55-102 as indicated, as detailed [30]. Islets from female C57Bl/6J mice were used. Exocytosis was induced by 500ms depolarization from -70mV to 0mV.

Ca^{2+} signalling and analysis of cell synchronization

Changes of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$) were recorded as previously described [32]. In brief, mouse islets or islet cells were loaded with Fura-2 LR acetoxymethyl ester (TEFLabs, Inc., Austin, TX) and transferred to poly-L-lysine-coated coverslips in a 50-µl chamber on the stage of an Eclipse TE2000U microscope (Nikon, Tokyo, Japan) equipped for ratiometric epifluorescence recordings of $[Ca^{2+}]_i$ [32]. The chamber was superfused at 160

µl/min with 37 °C buffer containing (in mmol/l) 138 NaCl, 4.8 KCl, 1.2 $MgCl_2$, 1.3 $CaCl_2$, 3 glucose, 25 HEPES with pH set to 7.40, and 1 mg/ml BSA. $[Ca^{2+}]_i$ is expressed as the background-corrected Fura-2 LR 340/380 nm fluorescence excitation ratio.

To estimate the synchronization of $[Ca^{2+}]_i$ signals between different non-overlapping regions-of-interest (ROI) in the islet, we calculated the linear correlation (Pearson R) between all possible ROI pairs and displayed the correlation coefficients in a matrix. The correlation matrices were subsequently used to construct undirected graphs representing functional connectivity. These graphs show the geometric location of each ROI with lines added between the ROI pairs with significant correlation coefficients ($P<0.001$) equal to or exceeding a threshold value of 0.8. Correlation analyses and the construction of correlation matrices and connectivity maps were performed with standard tools in Igor Pro software (Wavemetrics Inc, Lake Oswego, OR).

Glucose, insulin and glucagon measurements

Glucose was measured using Infinity™ Glucose (Ox) (Thermo Scientific, Lexington, MA) [15]. Insulin and glucagon was determined with ELISA (Mercodia, Uppsala, Sweden).

Statistics for experimental studies

Human insulin secretion data was analysed using Wilcoxon signed-rank test. All other data were analysed using 1-way ANOVA, followed by Bonferroni´s test, or unpaired Student's t-test.

RESULTS

CART is expressed in human beta cells and alpha cells

Islet CART expression was demonstrated using immunocytochemistry (ICC) (**Fig 1a**), *in situ* hybridization (ISH) (**Fig 1b, ESM Fig 1**) and qPCR (n=10, not shown). Western-blot confirmed processing of active CART55-102 (**Fig 1c**) and ICC localized CART to alpha and beta cells (**Fig 1a**).

Islet CART expression is increased in type 2 diabetic patients and diabetic mouse models

CART mRNA expression was 3-fold ($p<0.009$) higher in islets from type 2 diabetic patients (n=9) (**Fig 1b, d**) and type 2 diabetic patients had 3-fold ($p<0.008$) more CART-positive beta cells (**Fig 1a, e**) and 2-fold ($p<0.007$) more CART-positive alpha cells (**Fig 1a, f**) than controls (n=10). CART-positive beta cells were also several-fold more abundant in 3 models of diabetic mice (**Fig 1h-j**), whereas CART was absent in mouse alpha cells (**ESM Fig 2**).

Beta cell CART is regulated by glucose *in vivo* and *in vitro* in rats

Since CART expression was elevated in islets from type 2 diabetic patients and diabetic mice, we hypothesized that islet CART expression is regulated by glucose. To test this, we used dexamethasone-treated rats (DEX-rats), previously shown to have overexpressed beta cell CART [12], and DEX-rats treated with insulin. DEX-rats displayed 50% higher plasma glucose than controls ($p<0.05$) and more CART-positive beta cells ($p<4x10^{-6}$, **Fig 2a-b**), as well as upregulated islet *Cart* mRNA ($p<0.001$, **Fig 2c** and **ESM Fig 3**); CART staining was evident in delta cells and inert to DEX treatment, as previously reported (**Fig 2a**) [12] and CART was not expressed in alpha or PP cells (**ESM Fig 4**). Insulin treatment of DEX-rats normalized glucose levels ($p<0.05$ vs DEX-rats) and prevented the up regulation of beta cell

CART (**Fig 2a-c**). The frequency of CART-positive beta cells correlated positively with plasma glucose (r^2=0.28, p<0.02) and *CART* mRNA increased in rat islets cultured in 11 mmol/l (2.8-fold, p<0.03) and 25 mmol/l (3.2-fold, p<0.03) compared to 5 mmol/l glucose (**Fig 2d**). Western blot confirmed that biologically active CART55-102 is processed in DEX-rats and controls (**Fig 2e**). Thus, beta cell CART expression is increased by hyperglycaemia *in vivo* and *in vitro*.

CART stimulates insulin secretion and inhibits glucagon secretion in human islets

As it is not known whether CART affects insulin- or glucagon secretion in humans, we treated human islets with CART during 1h static incubations. CART (100 nmol/l) enhanced glucose-stimulated insulin secretion (GSIS) by 24±11% (n=19 donors, p<0.03) at 16.7 mmol/l glucose (**Fig 3a)**. This dose was previously shown to increase insulin secretion in INS-1 (832/13) cells [12]. Importantly, CART stimulated GSIS also in islets from diabetic donors (22±5%, n=7 donors, p<0.02) (**Fig 3b)**. CART had no effect on insulin secretion at low glucose (2.8 mmol/l) and a slight but non-significant effect at intermediate glucose (8.3 mmol/l; **Fig 3a, b**). Finally, CART reduced glucagon secretion by 30±11% (n=4 donors) at 1 mmol/l glucose, but was without effect at a non-stimulatory glucose concentration (8.3 mmol/l; **Fig 3c**). Thus, CART increased insulin-, and inhibited glucagon secretion in human islets in a glucose-dependent fashion.

CART stimulates insulin secretion, reduces glucagon secretion and improves glucose elimination *in vivo* in mice

To examine the effect of exogenous CART on whole body glucose homeostasis, CART (150 nmol/kg) was given together with glucose (0.75g/kg) during IVGTTs in mice (n=16). CART provoked a 2-fold increase in first phase insulin secretion (acute insulin response (AIR)

783±99 vs. 365±26 pmol/l in controls; p<0.001; **Fig 3d, e**) and improved glucose elimination (K_G) (CART-treated: 4.0±0.2; controls: 3.2±0.2 %/min; p<0.05; **Fig 3f, g**). Next, we tested the effect of CART on GLP-1-stimulated GSIS. CART alone provoked a similar response in insulin secretion to that of GLP-1 (AIR 2140±382 vs. 2349±365 pmol/l; p=0.28). Notably, CART caused a 30% further increase in insulin secretion on top of that achieved by GLP-1 alone (AIR 2871±522 vs. 2349±365 pmol/l, p<0.02) (n=16; **Fig 3h**). To test if CART affects glucagon secretion, fasted mice were given 150 nmol/kg CART *i.v*. This provoked a 40% reduction in glucagon secretion after 2 min (n=8; p<0.001) compared to controls (n=7; **Fig 3i)**. In summary, CART increased both GSIS and GLP-1-stimulated GSIS, reduced glucagon secretion and enhanced glucose elimination in mice.

CART increases insulin and inhibits glucagon secretion *in vitro* in mice

Next, we explored the effect of CART on insulin secretion stimulated with secretagogues other than glucose, as well as the effect on glucagon secretion in static incubations of isolated mouse islets. Similar to the findings in human islets, CART (10 nmol/l) increased GSIS at 16.7 (1.7-fold, p<0.004, **Fig 4a**), but not at 2.8 or 8.3 mmol/l glucose. CART had no effect on insulin secretion induced by 10 mmol/l alpha-ketoisocaproic acid (alpha-KIC; an activator of mitochondrial metabolism), 100 μmol/l carbachol, or 35 mmol/l K^+ at 11.1 or 16.7 mmol/l glucose (**Fig 4b**). In keeping with the *in vivo* data, CART increased (30%, p<0.05) GLP-1-stimulated (100 nmol/l) GSIS (**Fig 4c**). To test the effect of long-term exposure to CART, as evident in type 2 diabetic patients and diabetic mice, islets were pre-incubated with 100 nmol/l CART for 24h before the insulin secretion assay. This provoked increased GSIS at 16.7 (40%. p<0.05), but not at 2.8 mmol/l glucose (**Fig 4d**). Finally, CART (10 nmol/l) reduced glucagon secretion at 2.8 (-26%, p<0.05), 5.5 (-36%, p<0.01) and 11.1 mmol/l

glucose (-50%, p<0.001) (**Fig 4e**). Thus, CART increased glucose- and GLP-1-stimulated insulin secretion and reduced glucagon secretion in mouse islets.

CART enhances exocytosis of insulin and reduces exocytosis of glucagon *in vitro* in mice

To assess the effect of CART on exocytosis at the single cell level in beta and alpha cells, we used patch clamp in intact mouse islets. In control beta cells, the capacitance increase (ΔC_{tot}) induced by a train of depolarisations amounted to 139±23 fF. Beta cells treated with CART (10 nmol/l, 1h) showed a slight, but non-significant increase in exocytosis (**Fig 4f-i**). When pre-treated for 24h, insulin exocytosis increased 2-fold compared to controls (287±102 fF, p<0.05) (**Fig 4f-i**). Control alpha cells responded with a capacitance increase of 338±98 fF. Already after 1h treatment, 10 nmol/l CART robustly reduced glucagon exocytotic capacity by 62% to 130±22 fF (p<0.05) (**Fig 4j-l**).

CART alters the $[Ca^{2+}]_i$ signalling pattern in intact mouse islets

We next measured changes of $[Ca^{2+}]_i$ in intact mouse islets and dispersed islet cells loaded with Fura-2 LR. CART had no effect on slow (~0.2/min) $[Ca^{2+}]_i$ oscillations induced by 16.7 mmol/l glucose (n=10, not shown). However, islets often respond to glucose with fast (period ~20 s) $[Ca^{2+}]_i$ oscillations that sometimes are superimposed on the slow ones. The probability of evoking the fast pattern is increased under conditions of elevated cAMP [33, 34]. Accordingly, islets stimulated by 16.7 mmol/l glucose and 2.5 µmol/l forskolin almost invariably responded with fast or mixed fast and slow $[Ca^{2+}]_i$ oscillations. The average period of the fast oscillations were 19±5 s (n=388 oscillations from 5 experiments). Under these conditions, 100 nmol/l CART consistently transformed the $[Ca^{2+}]_i$ response into large-amplitude slow oscillations without a fast component (period 176±19 s, n=43 oscillations from 5 experiments; P<0.001 for difference from fast pattern; **Fig 5a**). A weaker effect was

sometimes seen with 10 nmol/l CART (**ESM Fig 5**). Interestingly, the CART-induced change in $[Ca^{2+}]_i$ oscillations was associated with improved synchronization of different regions within the islet (n=5; **Fig 5b-d, ESM Fig 5**). Notably, CART had no effect on $[Ca^{2+}]_i$ oscillations in dispersed islet cells (data not shown). Thus, our data show that CART promoted slow $[Ca^{2+}]_i$ oscillations and improved synchronization of $[Ca^{2+}]_i$ signalling, known to influence the kinetics of insulin secretion [35].

DISCUSSION

Agents that stimulate insulin secretion and reduce glucagon secretion are attractive in the search for new treatments for type 2 diabetes. Here we provide evidence for CART being a novel human islet peptide, which holds promise for use as anti-diabetic therapy. This is based upon our data showing that CART inhibits glucagon secretion and stimulates insulin secretion, even on top of GLP-1.

Importantly, CART increased insulin secretion in islets from type 2 diabetic patients, a finding that gains support from previous observations in GK-rat islets [12]. Furthermore, similar to incretins, the stimulatory effect of CART on insulin secretion was glucose-dependent. Notably, and in agreement with our previous *in vitro* data [12] CART augmented GLP-1-stimulated GSIS even further. This, together with the fact that CART-/- mice have diminished insulin secretion suggests that CART is a physiologically important regulator of insulin secretion. Acute CART stimulation was not associated with alterations of beta cell $[Ca^{2+}]_i$ level. However, CART promoted a change in the pattern of $[Ca^{2+}]_i$ oscillations, with fast oscillations being replaced by slow ones, known to underlie pulsatile release of insulin [33, 36]. Furthermore, the change in $[Ca^{2+}]_i$ oscillation pattern was paralleled by improved synchronization of the $[Ca^{2+}]_i$ signal between different islet sub-regions. Synchronization of beta cells is critical for normal insulin secretion kinetics [35] but it is not known how it is modulated by CART. The exact mechanism underlying CART amplification of insulin secretion remains to be established. We suggest that the short-term stimulatory action of CART on glucose-induced secretion may be related to its effect on the islet $[Ca^{2+}]_i$ signalling pattern. This would explain why CART lacks effect when glucose is combined with high K^+ or carbachol, known to induce pronounced, non-oscillatory islet $[Ca^{2+}]_i$ increases [37, 38].

A factor complicating mechanistic studies is that the CART receptor remains unknown [7, 39] The majority of the presented effects of CART on islets were rapid, and evident within 1h, however 24h stimulation with CART was needed to increase exocytosis from single, voltage-clamped β-cells. Whether this effect was secondary to transcriptional effects of CART, as reported previously [40], is not known.

We also showed that CART is an inhibitor of glucagon secretion; *in vivo* in mice, in human islets and in mouse islets via a direct effect on the alpha cell. Of note, insulin [41], somatostatin [42], and GLP-1 [31] are the only hormones reported so far to have such a direct effect. In human islets the glucagon-lowering effect of CART was glucose-dependent and only evident at stimulatory glucose levels. Together with the insulinotropic actions and protective effects against beta cell death [18], this positions CART as a plausible anti-diabetic target.

Furthermore, CART was found to be a novel constituent of human beta and alpha cells. It is of notice that there are large species differences in islet CART expression. Thus, in rats CART is mainly expressed in delta cells [11, 12], whereas mice have CART expression in a small subpopulation of beta cells. Pigs on the other hand have no CART expression in islet cells [16]. Notably type 2 diabetic subjects, as well as four different rodent models of diabetes had increased islet CART expression. Our data suggest that beta cell CART is upregulated as a response to hyperglycaemia since the up regulation of CART in type 2 diabetic rats was prevented by glucose-normalizing insulin therapy and *Cart* mRNA expression was enhanced in rat islets cultured at high glucose. Our data also show that islet CART expression is dynamic; a notion supported by the fact that CART is transiently upregulated in rodent islets during development [13].

In view of the insulinotropic and glucagon-lowering effects of CART, the up regulation of CART in type 2 diabetic islets is most likely a homeostatic compensatory mechanism trying to overcome hyperglycaemia via paracrine actions. Even though CART has been localized to beta- and delta cell secretory granules [12], it is not known if islet CART contributes to circulating levels or if these are altered by type 2 diabetes.

Taken together the present study expands previous knowledge obtained *in vitro* in rodents and shows that CART affects islet hormone secretion in human islets and *in vivo* in mice.
We conclude that CART is an important regulator of glucose homeostasis and, based upon its properties to stimulate insulin- and inhibit glucagon secretion, CART is a peptide with potential of being explored as a future anti-diabetic therapy.

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DUALITY OF INTEREST

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

CONTRIBUTION STATEMENT

All authors contributed to each of the following: (1) substantial contribution to the conception and design, acquisition of data and/or analysis and interpretation of data; (2) drafting the article and/or revising it critically for important intellectual content; and (3) final approval of the version to be published. NW is guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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FIGURE LEGENDS

Fig 1. CART is elevated in islets of diabetic humans and mice.

a: Human pancreatic sections from non-diabetic (C) and type 2 diabetic (T2D) donors, triple-immunostained for CART, insulin, and glucagon. CART is localized to alpha (arrows) and beta cells (arrow heads) in both type 2 diabetic patient and control (scale bar=100 μm). **b:** *In situ* hybridization showing higher expression of *CART* mRNA in a type 2 diabetic patient (arrows indicate labelling, scale bar=20 μm). **c:** Western-blot showing that human islet CART is processed to biologically active CART55-102 (peptide) in isolates containing 50% islets (1) and 75% islets (2).

d-f: Islets from type 2 diabetic patients (n=9) exhibit higher *CART* mRNA expression (quantification of *in situ* hybridization, relative units (RU)) (**d**) and more CART-positive beta cells (**e**) and CART-positive alpha cells (**f**) than controls (n=10). **g:** Double-immunostaining for CART and insulin showing that CART is upregulated in beta cells of *ob/ob* mouse compared to wild type (WT). Arrows indicate beta cells with CART (scale bar=50 μm). **h-j:** The number of CART-positive beta cells is several-fold elevated in three mouse models of type 2 diabetes: *ob/ob* (**h**), high fat diet (HFD) fed mice (**i**) and HNF1αdn mice (**j**), compared to controls. n=5-8 per group.*,$p<0.05$; **,$p<0.01$.

Fig 2. Beta cell CART is regulated by glucose *in vivo* and *in vitro* in rats.

a: Triple-immunostaining for CART, insulin and somatostatin in control rat (C), dexamethasone (DEX)-treated rat, and insulin-treated DEX rat (DEX+INS). White arrows indicate beta cells and red arrows delta cells with CART immunostaining (scale bar=100 μm). CART-positive beta cells **(b)** and islet *Cart* mRNA (**c**, quantification of *in situ* hybridization**)** is upregulated in DEX-rats (n=6), and insulin treatment (n=6) prevents the up regulation. **d:**

Cart mRNA expression is increased in isolated rat islets after 24h culture in 11 mmol/l and 25 mmol/l vs. 5 mmol/l glucose (qPCR, ΔΔCt, n=5). **e:** Western blot showing that islet CART in DEX-rats and controls is of the biologically active form CART55-102 (HT=rat hypothalamus positive control).*,p<0.05, ***,p<0.001.

Fig 3. CART increases insulin secretion, improves glucose elimination, and inhibits glucagon secretion *in vivo* in mice and *in vitro* in humans.

a: CART (100 nmol/l) enhances insulin secretion in human islets (n=19) at 16.7 mmol/l glucose. **b:** A similar effect is seen in islets from diabetic donors (n=7). **c:** CART (100 nmol/l) reduces glucagon secretion at 1 mmol/l glucose. **d-g:** Intravenous injection of CART (150 nmol/kg) provokes a 2-fold increase in first phase insulin secretion **(d)**, enhances acute insulin response (AIR) **(e),** lowers plasma glucose **(f)** and improves glucose elimination (K_G) **(g)** during an intra venous glucose tolerance test (IVGTT) in mice (n=16). CART: black. Saline: white. **h:** CART raises AIR to a similar extent as GLP-1 (4 nmol/kg) and when CART is given together with GLP-1 AIR is further increased (n=16). **i**: *i.v.* injection of CART (150 nmol/kg) reduces glucagon secretion after 2 min in fasted mice (n=8), compared to controls (C, n=7). *,p<0.05, ***,p<0.001.

Fig 4. CART enhances insulin secretion and exocytosis and reduces glucagon secretion and exocytosis *in vitro* in mice.

a-e: Insulin and glucagon secretion in mouse islets. CART (10 nmol/l) increases insulin secretion at 16.7 mmol/l glucose **(a)**. CART has no effect on insulin secretion stimulated with K^+ (35 mmol/l), carbachol (100 μmol/l), or alpha-ketoisocaproic acid (alpha-KIC, 10 mmol/l) **(b)**, but CART increases GLP-1-enhanced (100 nmol/l) insulin secretion **(c)**. **d:** 24h pre-treatment with CART (100 nmol/l) enhances insulin secretion at 16.7 mmol/l glucose. **e:**

CART (10 nmol/l) reduces glucagon secretion at 2.8, 5.5, and 11.1 mmol/l glucose. Data from 5-8 experiments, 8 technical replicates in each. **f-l:** Effects of CART on single cell exocytosis in mouse islet cells. Exocytosis was evoked by trains of 10 depolarisations (V), each lasting 500 ms, and monitored as increases in cell capacitance (ΔC) in control beta cells (n=15) **(f)**, beta cells treated with 10 nmol/l CART for 1h (n=12) **(g)**, and beta cells treated with CART for 24h (n=5) **(h)**. Control alpha cells (n=6) **(j)** and alpha cells treated with CART (n=8) for 1h **(k)**. Representative cell recordings are shown. Average total increase in capacitance evoked by the trains (ΔC_{TOT}) in **(i)** beta cells and **(l)** alpha cells. n= number of patched cells, data from 2-6 independent experiments and animals. *p<0.05, **p<0.01, ***p<0.001.

Fig 5. CART modulates the cytoplasmic Ca^{2+} signalling pattern in mouse islets.

Effects of CART on $[Ca^{2+}]_i$ recorded from an individual mouse islet during elevation of the glucose concentration from 3 to 16.7 mmol/l, followed by stimulation with 2.5 μmol/l forskolin. (**a**) $[Ca^{2+}]_i$ response pattern recorded from the entire islet (top panel, black trace) and from indicated smaller regions-of-interest (lower panel, coloured traces). CART transforms fast $[Ca^{2+}]_i$ oscillations into slow ones. (**b**) Time-expanded segments from recording shown in (**a**). Arrows indicate time points used for construction of correlation matrices. (**c**) Correlation of $[Ca^{2+}]_i$ responses in different islet sub-regions at time points indicated in (**b**). These time points are selected to show correlation before (I) and after (II) addition of CART and after washout (III) and reintroduction (IV) of CART. Correlation matrices show color-coded Pearson R coefficients for each possible region-of-interest (ROI) pair. CART improves the synchronization between different islet sub-regions. (**d**) Connectivity maps highlighting the ROI pairs with correlation coefficients exceeding 0.8.

Figure 1

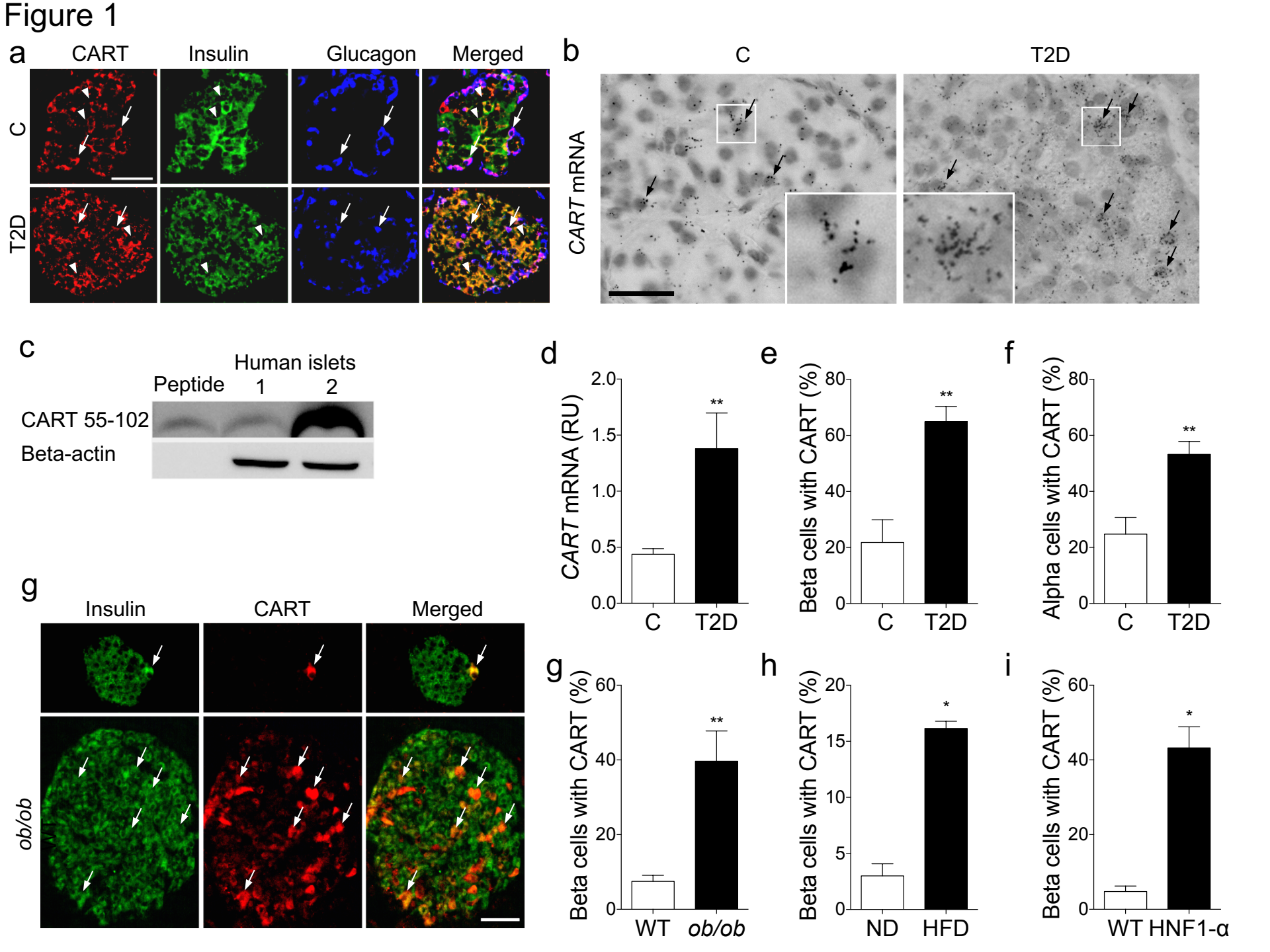
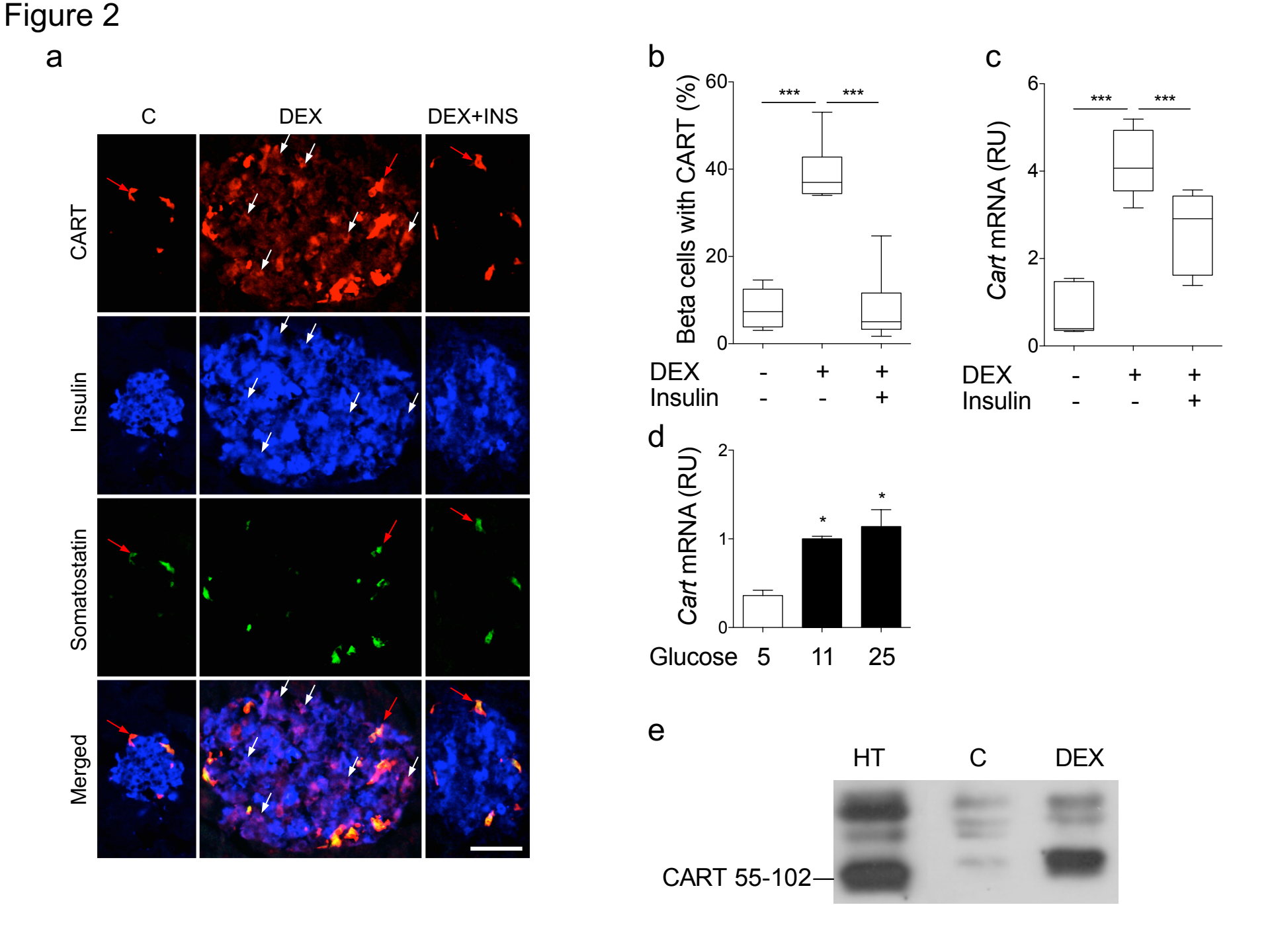

a
CART
Insulin
Glucagon
Merged
C
T2D
b
C
T2D
CART mRNA
c
Human islets
Peptide
1
2
CART 55-102
Beta-actin
d
CART mRNA (RU)
2.0
1.5
1.0
0.5
0.0
**
C
T2D
e
Beta cells with CART (%)
80
60
40
20
0
**
C
T2D
f
Alpha cells with CART (%)
80
60
40
20
0
**
C
T2D
g
Insulin
CART
Merged
ob/ob
g
Beta cells with CART (%)
60
40
20
0
**
WT
ob/ob
h
Beta cells with CART (%)
20
15
10
5
0
*
ND
HFD
i
Beta cells with CART (%)
60
40
20
0
*
WT
HNF1-α

Figure 2


a
C
DEX
DEX+INS
CART
Insulin
Somatostatin
Merged
b
Beta cells with CART (%)
60
40
20
0
***
***
DEX - + +
Insulin - - +
c
Cart mRNA (RU)
6
4
2
0
***
***
DEX - + +
Insulin - - +
d
Cart mRNA (RU)
2
1
0
*
*
Glucose 5 11 25
e
HT
C
DEX
CART 55-102—

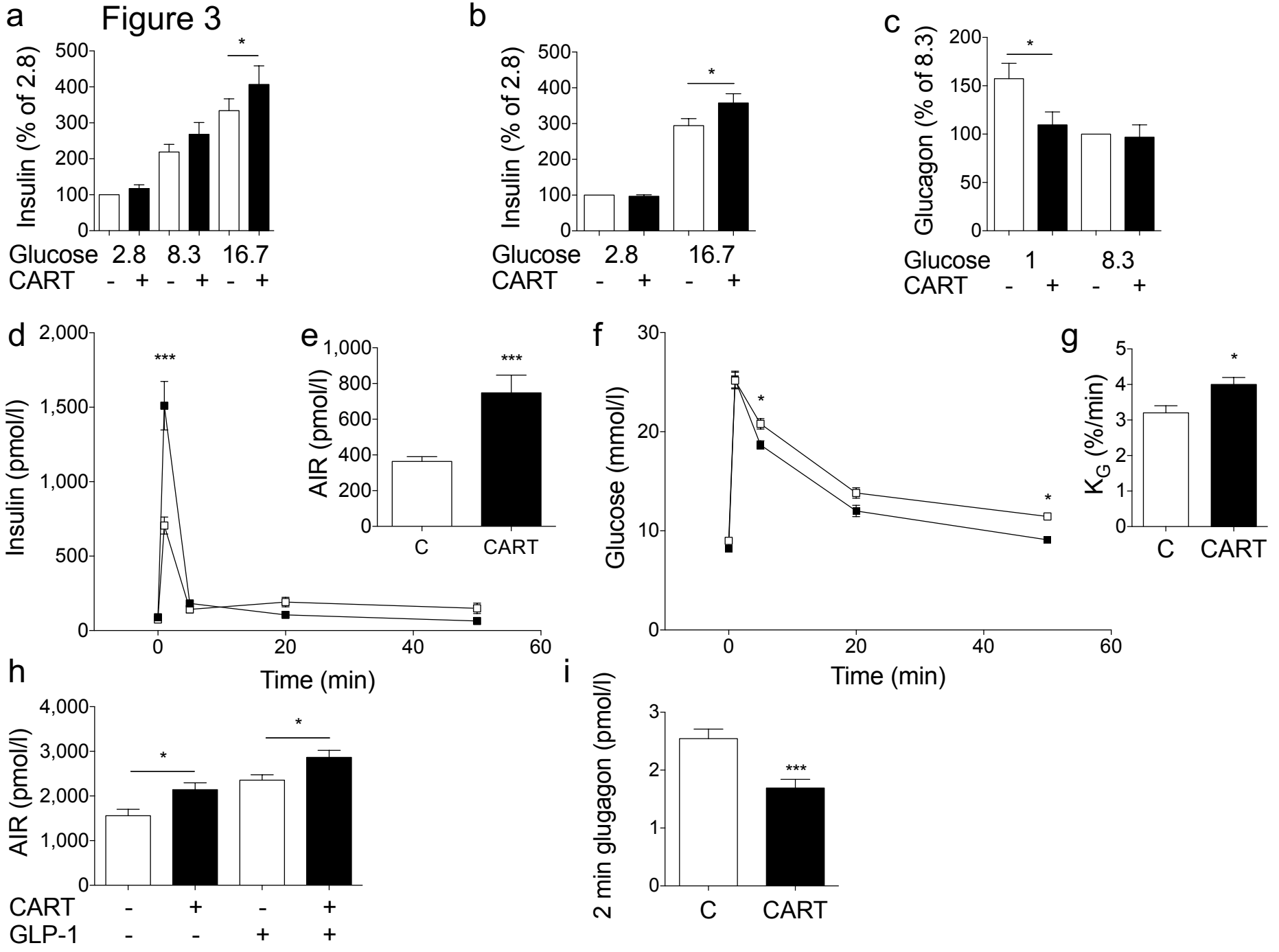


Figure 3
a
Insulin (% of 2.8)
500
400
300
200
100
0
*
Glucose 2.8 8.3 16.7
CART - + - + - +
b
Insulin (% of 2.8)
500
400
300
200
100
0
*
Glucose 2.8 16.7
CART - + - +
c
Glucagon (% of 8.3)
200
150
100
50
0
*
Glucose 1 8.3
CART - + - +
d
Insulin (pmol/l)
2,000
1,500
1,000
500
0
***
0 20 40 60
Time (min)
e
AIR (pmol/l)
1,000
800
600
400
200
0
***
C CART
f
Glucose (mmol/l)
30
20
10
0
*
*
0 20 40 60
Time (min)
g
K_G (%/min)
5
4
3
2
1
0
*
C CART
h
AIR (pmol/l)
4,000
3,000
2,000
1,000
0
*
*
CART - + - +
GLP-1 - - + +
i
2 min glugagon (pmol/l)
3
2
1
0
***
C CART

Figure 4

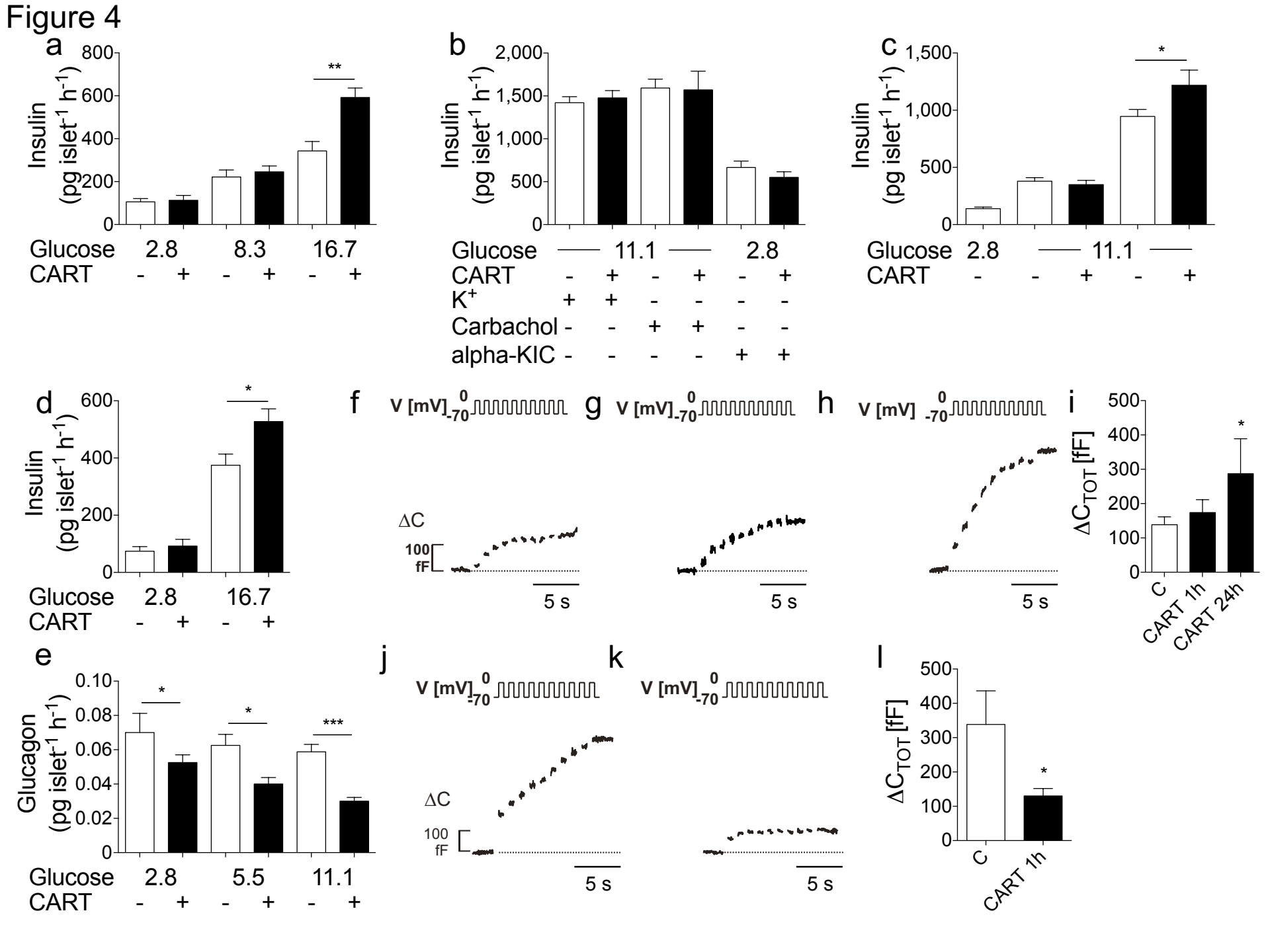
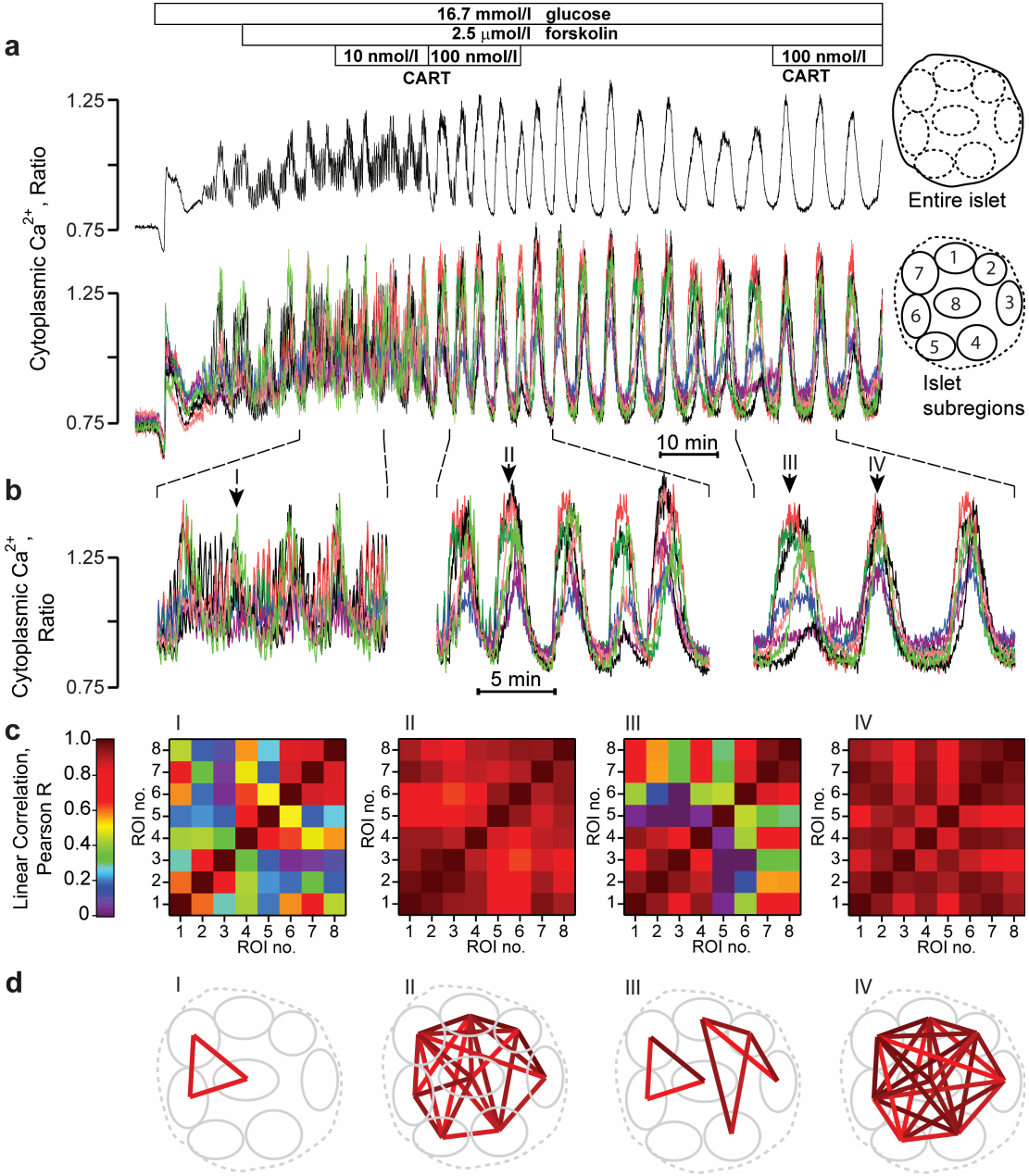
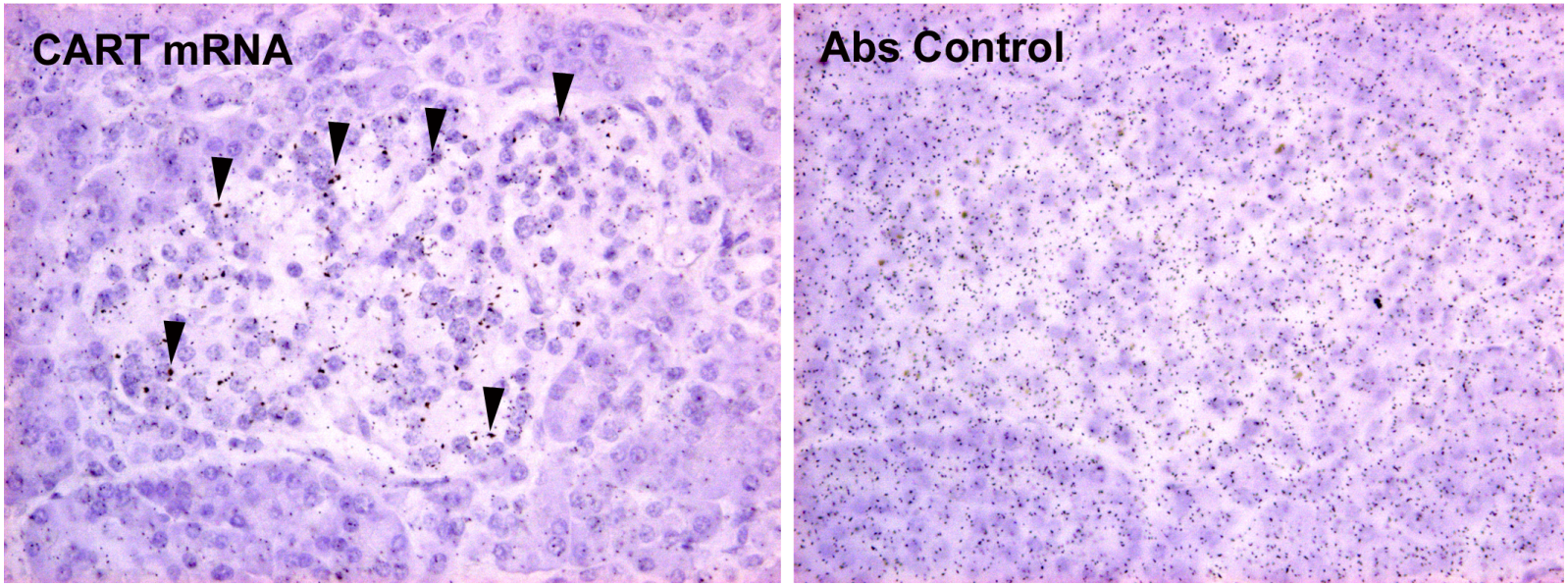
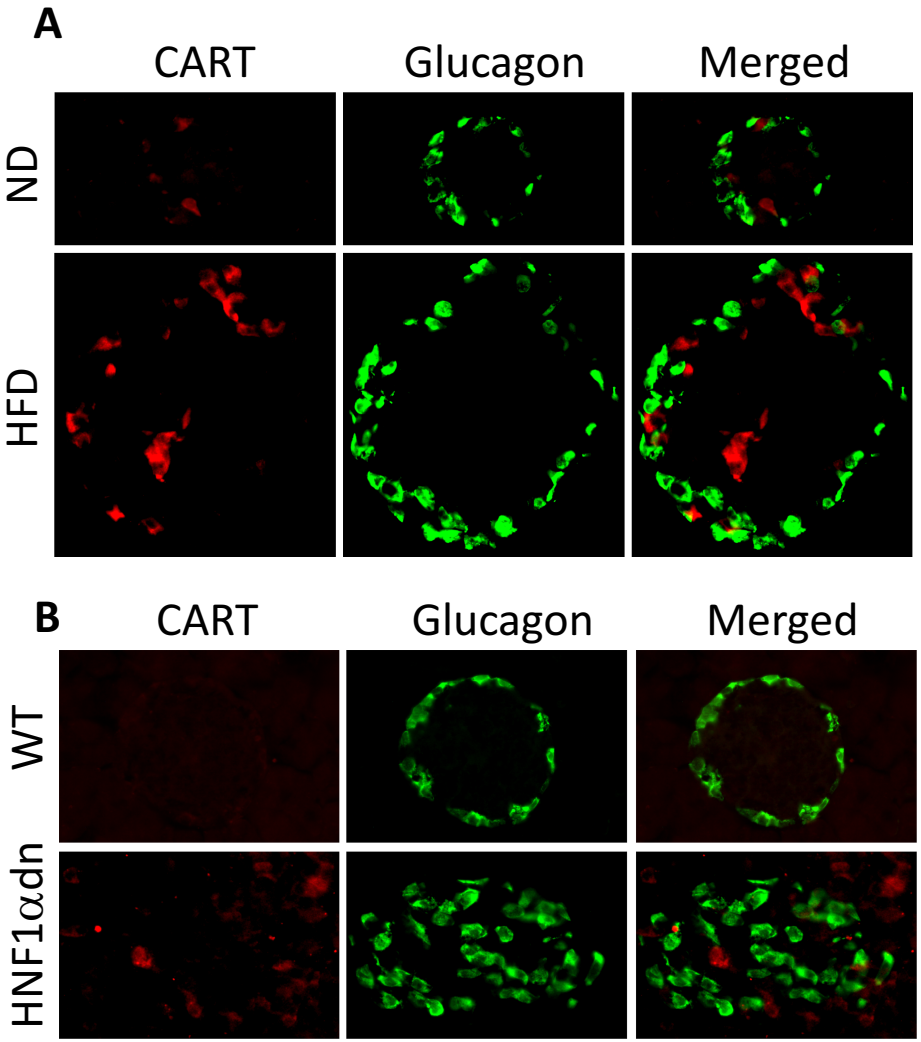

a
Insulin (pg islet-1 h-1)
800
600
400
200
0
**
Glucose 2.8 8.3 16.7
CART - + - + - +
b
Insulin (pg islet-1 h-1)
2,000
1,500
1,000
500
0
Glucose 11.1 2.8
CART - + - + - +
K+ + + - - - -
Carbachol - - + + - -
alpha-KIC - - - - + +
c
Insulin (pg islet-1 h-1)
1,500
1,000
500
0
*
Glucose 2.8 11.1
CART - - + - +
d
Insulin (pg islet-1 h-1)
600
400
200
0
*
Glucose 2.8 16.7
CART - + - +
e
Glucagon (pg islet-1 h-1)
0.10
0.08
0.06
0.04
0.02
0
*
*
***
Glucose 2.8 5.5 11.1
CART - + - + - +
f
V [mV] 0 -70
ΔC
100 fF
5 s
g
V [mV] 0 -70
5 s
h
V [mV] 0 -70
5 s
i
ΔCTOT [fF]
500
400
300
200
100
0
*
C
CART 1h
CART 24h
j
V [mV] 0 -70
ΔC
100 fF
5 s
k
V [mV] 0 -70
5 s
l
ΔCTOT [fF]
500
400
300
200
100
0
*
C
CART 1h

Figure 5

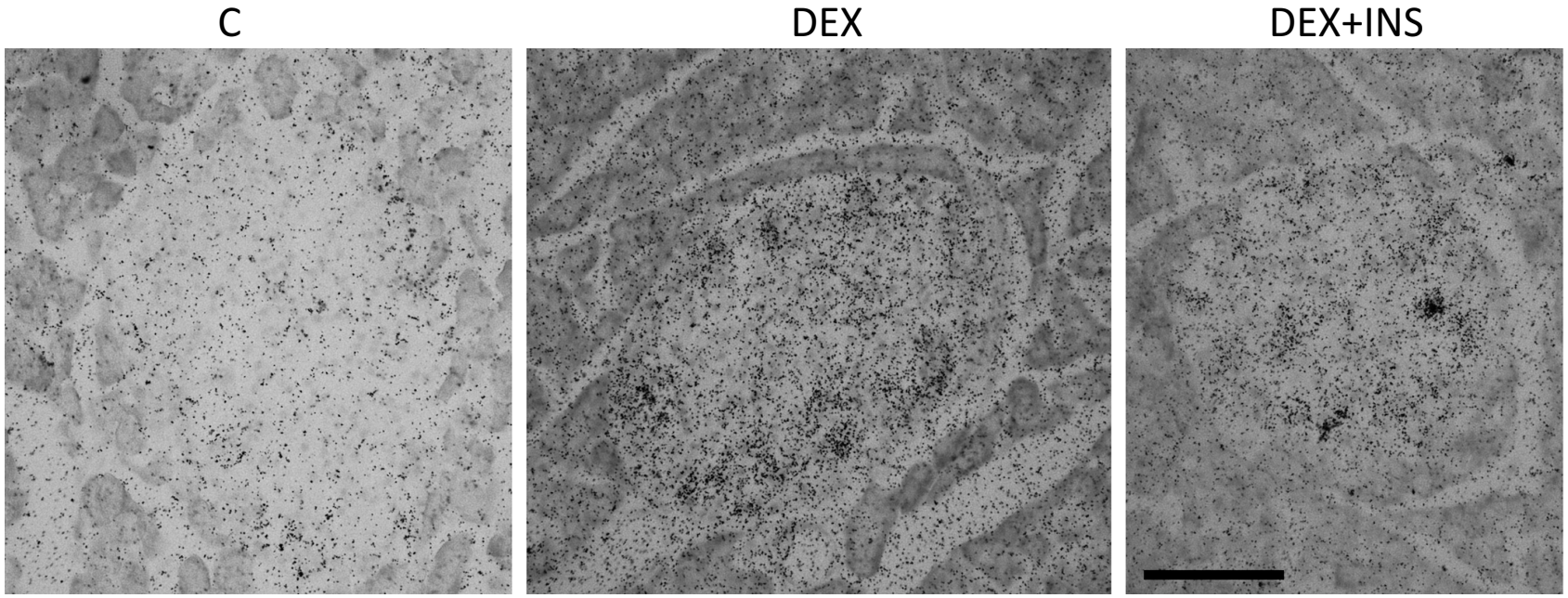

a
16.7 mmol/l glucose
2.5 μmol/l forskolin
10 nmol/l
100 nmol/l
CART
100 nmol/l
CART
Cytoplasmic Ca2+, Ratio
1.25
0.75
1.25
0.75
Entire islet
1
2
3
4
5
6
7
8
Islet subregions
10 min
b
I
II
III
IV
Cytoplasmic Ca2+, Ratio
1.25
0.75
5 min
c
Linear Correlation, Pearson R
1.0
0.8
0.6
0.4
0.2
0
ROI no.
ROI no.
d
I
II
III
IV



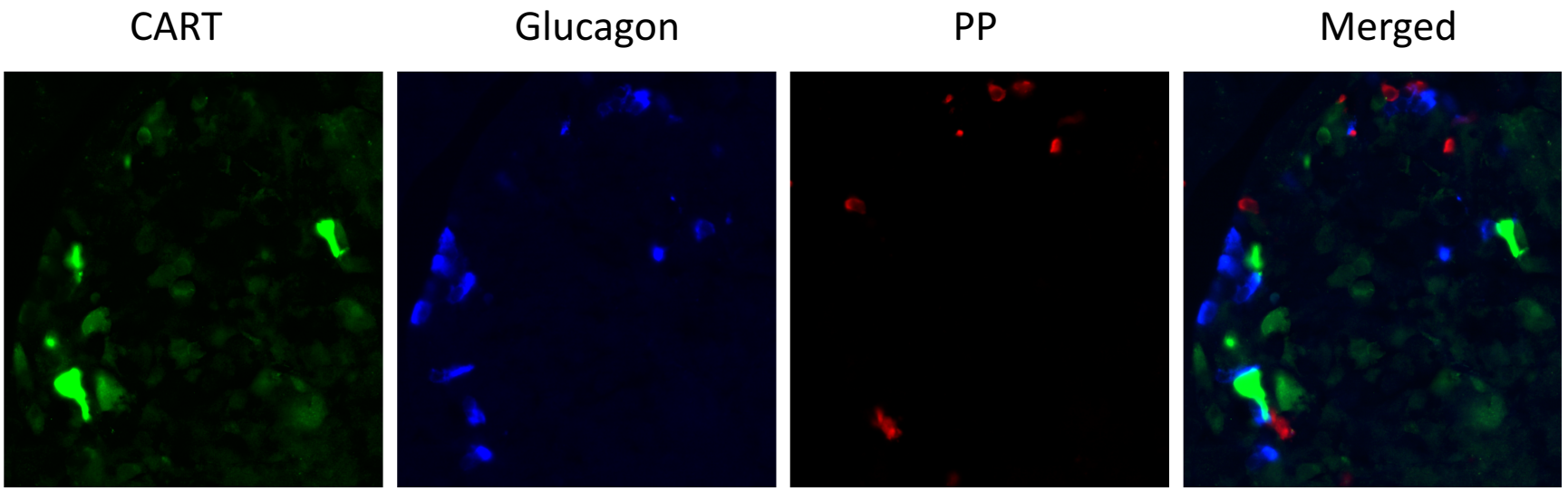
ESM Fig 1. *In situ* hybridisation autoradiographs of human pancreatic sections. Left: Labelling for CART mRNA is evident within the islet. Right: Exactly the same area as in left in a consecutive section. Randomly scattered labelling only is seen after hybridising with excess of unlabelled probe. Arrowheads indicate labelling.



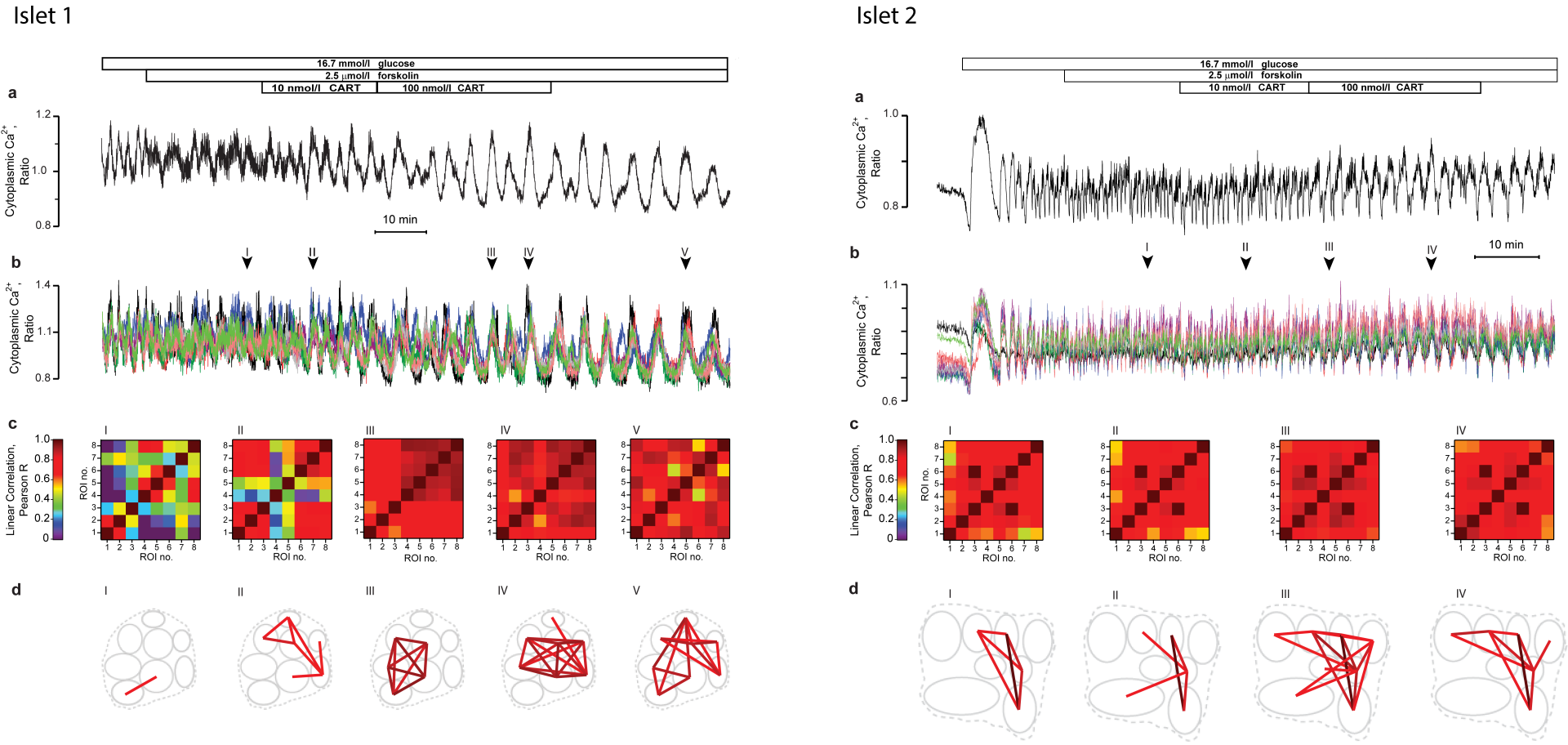
ESM Fig 2. Immunostainings of CART and glucagon in mouse pancreatic sections showing that alpha cells are devoid of CART. **A**: Mice fed a high fat diet (HFD) or a normal diet (ND). **B:** HNF1αdn and WT mice. **C**: *ob/ob* and WT mice. Immunocytochemistry was performed using characterized antibodies as previously detailed (Wierup et al, 2004). Primary antibodies guinea pig-anti-glucagon (1:5000, code: 8708, EuroDiagnostica, Malmö, Sweden) and rabbit-anti-CART (1:1280, 12/D, Cocalico Corp, Reamstown, USA) and secondary antibodies Texas red-anti-rabbit and Cy2-anti-guinea pig (1:400, Jackson, West Grove, PA USA) were used.



ESM Fig 3. *In situ* hybridisation autoradiographs showing higher expression of *Cart* mRNA in islets of dexamethasone treated rats (DEX) compared to control rats (C), and dexamethasone and insulin treated rats (DEX+INS). Scale bar=200µm.



ESM Fig 4. Immunostaining of CART, glucagon and PP in pancreatic section from DEX-treated rat showing that alpha cells and PP-cells are devoid of CART. Immunocytochemistry was performed using characterized antibodies as previously detailed (Wierup et al, 2004). Primary antibodies guinea pig-anti-glucagon (1:5000, code: 8708, EuroDiagnostica, Malmö, Sweden), sheep-anti-PP (1:400, code: AHP 515, Serotec, Oxford, U.K) and rabbit-anti-CART (1:1280, 12/D, Cocalico Corp, Reamstown, USA) and secondary antibodies Cy2-anti-rabbit and Texas Red-anti-sheep (1:400, Jackson, West Grove, PA USA or AMCA-anti-guinea pig (dilution 1:100, Jackson) were used.



ESM Fig 5. CART modulates the cytoplasmic Ca^{2+} signalling pattern in mouse islets.
Additional examples of the effects of CART on $[Ca^{2+}]_i$ recorded from an individual glucose-stimulated mouse islets exposed to 2.5 µmol/l forskolin. (**a**) $[Ca^{2+}]_i$ response pattern recorded from the entire islet and (**b**) from smaller sub-regions. (**c**) Correlation of $[Ca^{2+}]_i$ responses in the different islet sub-regions at the time points indicated in (b). The correlation matrices show color-coded Pearson R coefficients for each possible region-of-interest (ROI) pair. CART improves the synchronization between different islet sub-regions. (**d**) Connectivity maps highlighting the ROI pairs with correlation coefficients exceeding 0.8.