

ARTICLE

# 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 hyperglucagonaemia are culprits in type 2 diabetes. Cocaine- and amphetamine-regulated transcript (CART, encoded by *Cartpt*) affects islet hormone secretion and beta cell survival in vitro in rats, and *Cart*<sup>-/-</sup> 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 rodent models of diabetes. Insulin and glucagon secretion was examined in isolated islets and in vivo in mice. Ca<sup>2+</sup> oscillation patterns and exocytosis were studied in mouse islets.

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**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<sup>2+</sup> signalling pattern in mouse islets from fast to slow oscillations and improved synchronisation 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/interpretation** 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 the ability of CART 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 <sup>2+</sup> ] <sub>i</sub>	Cytoplasmic Ca <sup>2+</sup> concentration
ΔC <sub>tot</sub>	Capacitance increase
AIR	Acute insulin response
CART	Cocaine- and amphetamine-regulated transcript
DEX	Dexamethasone
GSIS	Glucose-stimulated insulin secretion
ICC	Immunocytochemistry

ISH	In situ hybridisation
K <sub>G</sub>	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*<sup>−/−</sup> 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*<sup>−/−</sup> mice are obese [15, 19], and genetic variations in *CARTPT* associate 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 is 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 non-diabetic and type 2 diabetic individuals, 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.

## Methods

**Human tissue** Human pancreases from nine type 2 diabetic patients (five men, four women; median age 54 years, BMI  $28.9 \pm 7 \text{ kg/m}^2$ ) and ten controls (four men, six women; median age 56 years, BMI  $25.8 \pm 2 \text{ kg/m}^2$ ) were used for morphometric analysis. Human islets were provided by the 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, Sunnyvale, CA, USA or gift from L. Thim [Novo Nordisk, Malmö, Denmark]) was used.

**Immunocytochemistry, in situ hybridisation and image analysis** Immunocytochemistry was performed using characterised antibodies as detailed [13, 22]. The specificity of CART antibodies (Cocalico, Stevens, PA, USA) was verified in *Cart*<sup>−/−</sup> mice and with pre-absorption with CART55-102 [23]. For in situ hybridisation (ISH), 30-mer oligodeoxyribonucleotide probes for *Cart* mRNA were used in human [24] and rat [12] specimens as described previously [25]. Hybridisation in the presence of excess unlabelled probe on adjacent sections was used as a negative control. CART-immunoreactive alpha cells and beta cells, and CART ISH-labelled area/whole-islet area were quantified blinded in the indicated samples in all islets in three separate sections as described [12].

**Western blot** Western blot was performed as described previously [26] using CART primary antibody (Cocalico) diluted 1:1000 with CART55-102 (Novo Nordisk) 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* (8th edn) was followed. Female Sprague Dawley rats (225 g) were used. Six rats received dexamethasone (DEX), 2 mg/kg/day i.p. for 10 days [12, 27]. Six rats received DEX and insulin detemir (Levemir, Novo Nordisk), 10 U/day for 10 days. Controls ( $n=6$ ) received NaCl 154 mmol/l. In addition, the genetically modified mouse strains (1) HNF1 $\alpha$  dominant-negative (*Hnf1* $\alpha$  dn) mice [28] and (2) *ob/ob* mice with respectively matched control mice, as well as C57Bl/6J mice (Taconic, Bomholt, Denmark) fed a high-fat diet (HFD) or control diet for 6 months were used (all female mice, aged 10–14 weeks,  $n=5$ –8 in each group) [29]. The pancreases were processed as described previously [12].

**Real-time quantitative PCR** Total RNA was isolated using All Prep DNA/RNA kit (Qiagen, Hilden, Germany). RNA, 1  $\mu\text{g}$ , was reverse-transcribed to cDNA with QuantiTect Reverse Transcription kit (Qiagen). Quantitative RT-PCR was performed using the ABI Prism 7900 HT system (Applied Biosystems, Foster City, CA, USA) with TaqMan technology and assay Hs00182861\_m1 for *Cart* and 4333768F for *Hprt1*, which served as an endogenous control. Gene expression was analysed with the  $\Delta\Delta\text{C}_t$  method.

**In vitro islet studies** Secretion studies in human and mouse islets (1 h incubations) with insulin secretagogues, with and without CART55-102 as indicated, were performed as described previously [15, 30, 31]. One experiment represents one donor/animal, with 6–8 technical repeats for each experiment. For *Cart* mRNA expression studies, rat islets

were cultured for 24 h in 5, 11.1 or 25 mmol/l glucose in RPMI 1640 medium (1% serum).

**IVGTT** IVGTT was performed as previously described [15] in female C57Bl/6J mice with or without 150 nmol/kg CART55-102 and 4 nmol/kg glucagon-like peptide 1 (GLP-1) co-injected, i.v., with glucose, 0.75 g/kg. Controls received NaCl. Acute insulin response (AIR) was calculated as mean suprabasal insulin level of insulin at mins 1 and 5 (i.e.  $[ins_5 \text{ min} + ins_1 \text{ min}] / 2 - ins_0 \text{ min}$ ). The glucose elimination rate ( $K_G$ ) was calculated as the slope for 1–20 min of the logarithmic transformation of individual plasma glucose values [15].

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

**Electrophysiology** Depolarisation-evoked exocytosis was recorded by capacitance measurements using standard whole-cell configuration with or without CART55-102 as indicated [30]. Islets from female C57Bl/6J mice were used. Exocytosis was induced by 500 ms depolarisation from  $-70$  mV to 0 mV.

**Ca<sup>2+</sup> signalling and analysis of cell synchronisation** Changes in the cytoplasmic Ca<sup>2+</sup> 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, Austin, TX, USA) and transferred to poly-L-lysine-coated coverslips in a 50  $\mu\text{l}$  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  $\mu\text{l}/\text{min}$  with 37°C buffer containing 138 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l MgCl<sub>2</sub>, 1.3 mmol/l CaCl<sub>2</sub>, 3 mmol/l glucose and 25 mmol/l 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 synchronisation of  $[Ca^{2+}]_i$  signals between different non-overlapping regions-of-interest (ROI) in the islet, we calculated the linear correlation (Pearson's  $r$ ) 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 using Igor Pro software version 6.37 (WaveMetrics, Lake Oswego, OR, USA).

**Glucose insulin and glucagon measurements** Glucose was measured using Infinity Glucose (Ox) (Thermo Scientific, Lexington, MA, USA) [15]. Insulin and glucagon levels were determined with ELISA (Mercodia, Uppsala, Sweden).

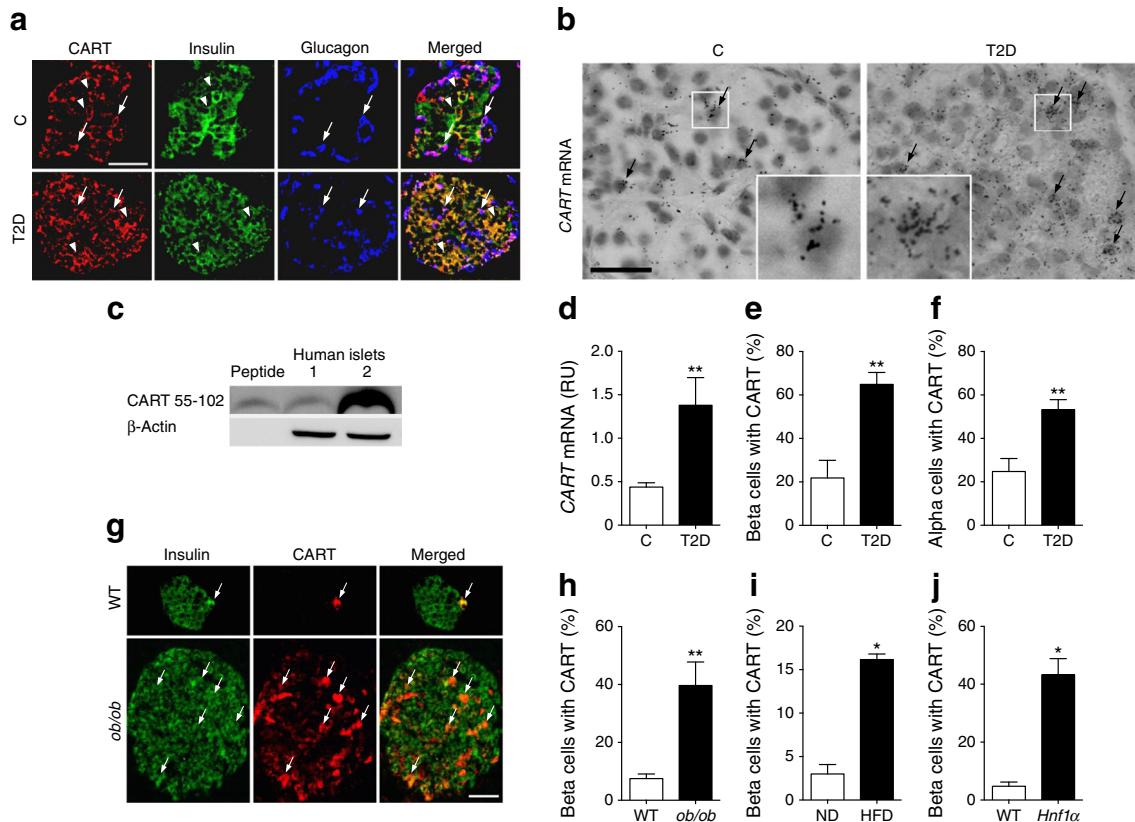
**Statistics and overall design for experimental studies** Human insulin secretion data were analysed using a Wilcoxon signed-rank test. All other data were analysed using one-way ANOVA, followed by Bonferroni's test, or unpaired Student's  $t$  test. Data are expressed as means (SEM). All experiments were performed and analysed in a randomised and blinded fashion when possible. Outliers were identified using Grubbs' test for outliers.

## Results

**CART is expressed in human beta cells and alpha cells** Islet CART expression was demonstrated using immunocytochemistry (ICC) (Fig. 1a), ISH (Fig. 1b, electronic supplementary material [ESM] Fig. 1) and quantitative PCR ( $n = 10$ , not shown). Western blot confirmed processing of active CART55-102 (Fig. 1c) and ICC localised CART to alpha and beta cells (Fig. 1a).

**Islet CART expression is increased in type 2 diabetic patients and mouse models of diabetes** *CART* mRNA expression was threefold higher in islets from type 2 diabetic patients ( $n = 9$ ;  $p < 0.009$ ) (Fig. 1b, d) and type 2 diabetic patients had threefold more CART-positive beta cells ( $p < 0.008$ ; Fig. 1a, e) and twofold more CART-positive alpha cells ( $p < 0.007$ ) (Fig. 1a, f) than controls ( $n = 10$ ). CART-positive beta cells were also several fold more abundant in three mouse models of diabetes (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** As CART expression was elevated in islets from type 2 diabetic patients and diabetic mice, we hypothesised that islet CART expression is regulated by glucose. To test this, we used DEX-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 ( $p < 4 \times 10^{-6}$ , 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 (Fig. 2a), as previously reported [12] and CART was not expressed in alpha or PP cells (ESM Fig. 4). Insulin treatment of DEX-rats normalised glucose levels ( $p < 0.05$  vs DEX-rats) and prevented the upregulation 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*



**Fig 1** CART is elevated in islets of diabetic humans and mice. **(a)** Human pancreatic sections from non-diabetic and type 2 diabetic donors, triple immunostained for CART, insulin and glucagon. CART is localised to alpha (arrows) and beta cells (arrowheads) in both a type 2 diabetic patient and a control (scale bar, 100  $\mu$ m). **(b)** In situ hybridisation showing higher expression of *CART* mRNA in a type 2 diabetic patient (arrows indicate labelling, scale bar, 20  $\mu$ m). **(c)** Western blot showing that human islet CART is processed to biologically active CART55-102 in isolates containing 50% islets (lane 1) and 75% islets (lane 2). **(d–f)** Islets from type 2 diabetic patients ( $n=9$ ) exhibit higher *CART* mRNA expression

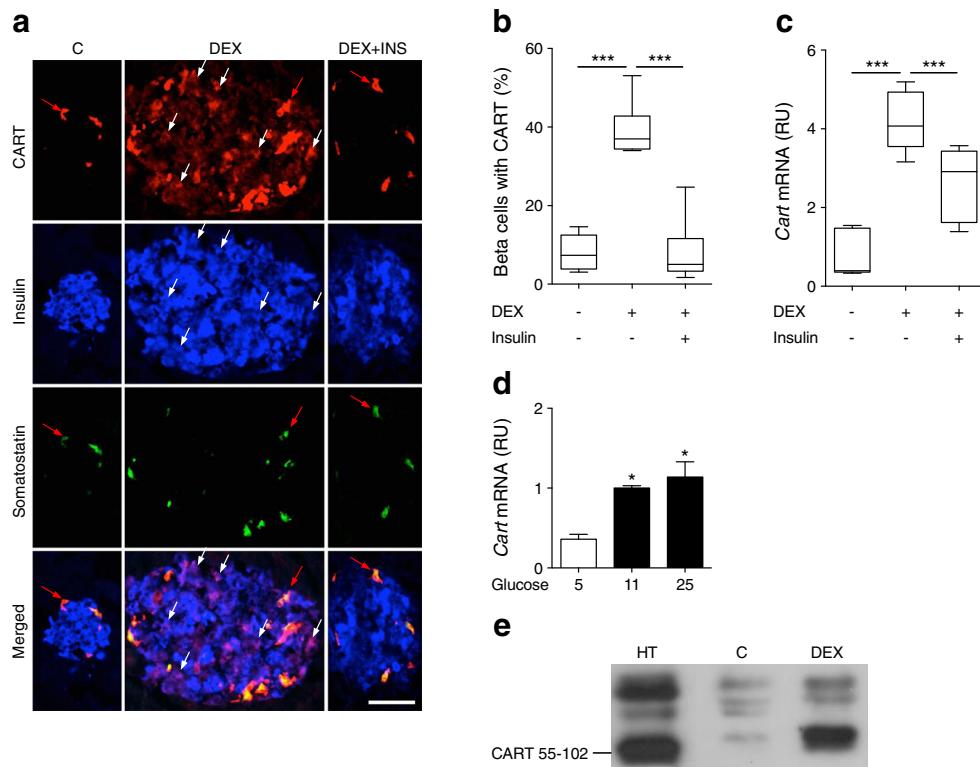
(quantification of *in situ* hybridisation, relative units labelled area/islet area) (**d**) and more CART-positive beta cells (**e**) and CART-positive alpha cells (**f**) than controls ( $n=10$ ). **(g)** Double-immunostaining for CART and insulin showing that CART is upregulated in beta cells of *ob/ob* mice compared with wild type. Arrows indicate beta cells with CART (scale bar, 50  $\mu$ m). **(h–j)** The number of CART-positive beta cells is elevated several fold in three mouse models of type 2 diabetes: *ob/ob* (**h**), HFD-fed mice (**i**) and *Hnf1α* dn mice (**j**), compared with controls.  $n=5–8$  per group. \* $p<0.05$  and \*\* $p<0.01$ . C, control; peptide, biologically active CART55-102; ND, normal diet; RU, relative units; T2D, type 2 diabetes; WT, wild type

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 with 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 1 h static incubations. CART (100 nmol/l) enhanced glucose-stimulated insulin secretion (GSIS) by  $24\pm 11\%$  ( $n=19$  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 also stimulated GSIS in islets from diabetic donors ( $22\pm 5\%$ ,  $n=7$  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  $30\pm 11\%$  ( $n=4$  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 secretion 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 (0.75 g/kg) during IVGTT in mice ( $n=16$ ). CART provoked a twofold increase in first-phase insulin secretion (AIR  $783\pm 99$  vs  $365\pm 26$  pmol/l in controls;  $p<0.001$ ; Fig. 3d, e) and improved  $K_G$  (CART treated:  $4.0\pm 0.2\%/\text{min}$ ; controls:  $3.2\pm 0.2\%/\text{min}$ ;  $p<0.05$ ; Fig. 3f, g). Next, we tested the effect of CART on GLP-1-stimulated GSIS. CART alone



**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, DEX-rat and insulin (INS)-treated DEX-rat. White arrows indicate beta cells and red arrows delta cells with CART immunostaining (scale bar, 100  $\mu$ m). (b) CART-positive beta cells (quantification of immunohistochemistry) and (c) islet *Cart* mRNA (quantification of in situ hybridisation relative units labelled area/islet area) are upregulated in

DEX-rats ( $n=6$ ), and insulin treatment ( $n=6$ ) prevents the upregulation. (d) *Cart* mRNA expression is increased in isolated rat islets after 24 h culture in 11 mmol/l and 25 mmol/l vs 5 mmol/l glucose (quantitative PCR,  $\Delta\Delta C_t$ , relative to 11 mmol/l,  $n=5$ ). (e) Western blot showing that islet CART in DEX-rats and controls is the biologically active form, CART55-102. \* $p<0.05$  and \*\*\* $p<0.001$ . C, control; HT, rat hypothalamus positive control; RU, relative units; T2D, type 2 diabetes

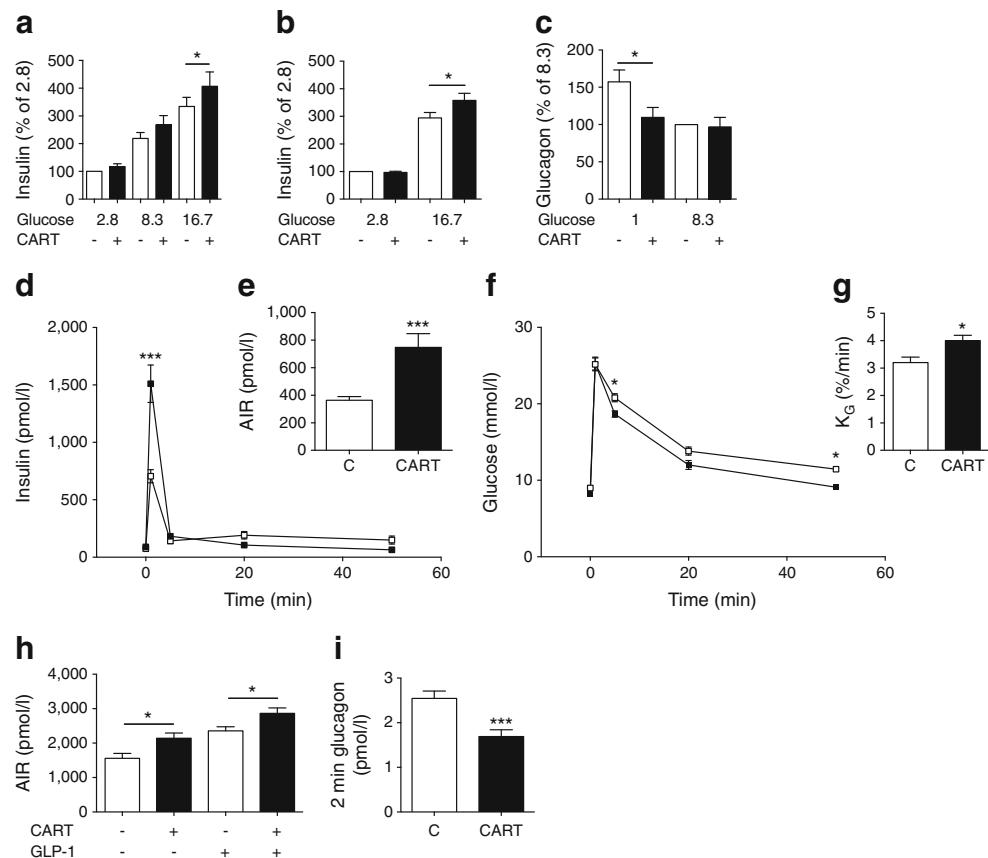
provoked a similar response in insulin secretion to that of GLP-1 (AIR  $2140 \pm 382$  vs  $2349 \pm 365$  pmol/l;  $p=0.28$ ). Notably, CART caused a further 30% increase in insulin secretion above that achieved by GLP-1 alone (AIR  $2871 \pm 522$  vs  $2349 \pm 365$  pmol/l,  $p<0.02$ ) ( $n=16$ ; Fig. 3h). To test if CART affects glucagon secretion, fasted mice were given i.v. CART, 150 nmol/kg. This provoked a 40% reduction in glucagon secretion after 2 min ( $n=8$ ;  $p<0.001$ ) compared with 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  $\mu$ mol/l carbachol, or 35 mmol/l

$K^+$  at 11.1 (Fig. 4b) or 16.7 mmol/l glucose (data not shown). 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 24 h 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%,  $p<0.05$ ) 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 ( $\Delta C_{tot}$ ) induced by a train of depolarisations amounted to  $139 \pm 23$  fF. Beta cells treated with CART (10 nmol/l, 1 h) showed a slight, but non-significant, increase in exocytosis (Fig. 4f-i). When

**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 twofold increase in first-phase insulin secretion (d), enhances AIR (e), lowers plasma glucose (f) and improves  $K_G$  (g) during an IVGTT in mice ( $n=16$ ). (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) Injection of CART (150 nmol/kg) reduces glucagon secretion after 2 min in fasted mice ( $n=8$ ), compared with controls ( $n=7$ ). Black bars/squares, CART; white bars/squares, NaCl. \* $p<0.05$  and \*\*\* $p<0.001$ . C, control



pre-treated for 24 h, insulin exocytosis increased twofold compared with controls ( $287\pm 102$  fF,  $p<0.05$ ) (Fig. 4f–i). Control alpha cells responded with a capacitance increase of  $338\pm 98$  fF. Already after 1 h treatment, 10 nmol/l CART robustly reduced glucagon exocytotic capacity by 62% to  $130\pm 22$  fF ( $p<0.05$ ) (Fig. 4j–l).

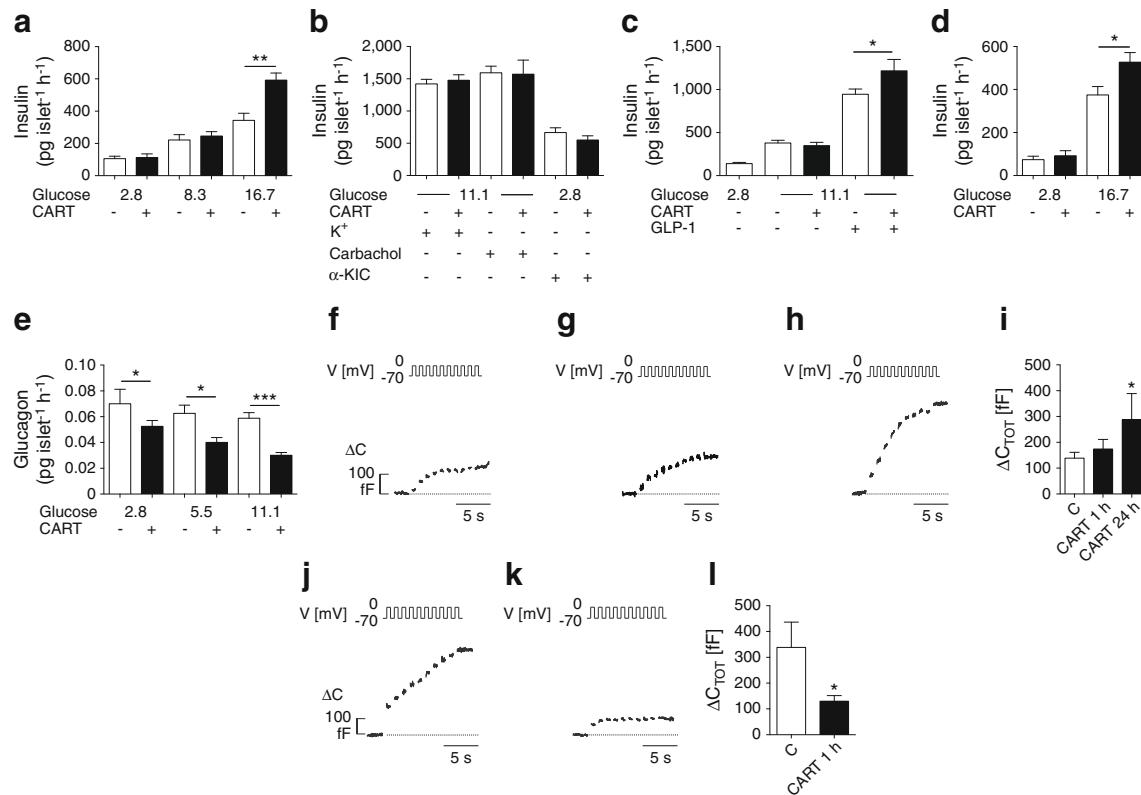
**CART alters the  $[Ca^{2+}]_i$  signalling pattern in intact mouse islets** We next measured changes in  $[Ca^{2+}]_i$  in intact mouse islets and dispersed islet cells loaded with Fura-2 LR. CART had no effect on slow ( $\sim 0.2/\text{min}$ )  $[Ca^{2+}]_i$  oscillations induced by 16.7 mmol/l glucose ( $n=10$ , not shown). However, islets often respond to glucose with fast (period  $\sim 20$  s)  $[Ca^{2+}]_i$  oscillations that are sometimes 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  $\mu\text{mol/l}$  forskolin almost invariably responded with fast or mixed fast and slow  $[Ca^{2+}]_i$  oscillations. The average period of the fast oscillations was  $19\pm 5$  s ( $n=388$  oscillations from five 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  $176\pm 19$  s,  $n=43$  oscillations from five experiments;  $p<0.001$  vs the 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 synchronisation 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 synchronisation 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 diabetes therapy. This is based on 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-



**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<sup>+</sup> (35 mmol/l), carbachol (100 μmol/l) or α-KIC (10 mmol/l) (b), but CART increases GLP-1-enhanced (100 nmol/l) insulin secretion (c). (d) 24 h 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 five to eight experiments, eight technical replicates in each. (f–l)

Effects of CART on single cell exocytosis in mouse islet cells. Exocytosis was evoked by trains of ten depolarisations (V), each lasting 500 ms, and monitored as increases in cell capacitance ( $\Delta C$ ) in control beta cells ( $n=15$ ) (f), beta cells treated with 10 nmol/l CART for 1 h ( $n=12$ ) (g), and beta cells treated with CART for 24 h ( $n=5$ ) (h). Control alpha cells ( $n=6$ ) (j) and alpha cells treated with CART ( $n=8$ ) for 1 h (k). Representative cell recordings are shown. Average total increase in capacitance evoked by the trains ( $\Delta C_{TOT}$ ) in (i) beta cells and (l) alpha cells.  $n$ =number of patched cells, data from two to six independent experiments and animals. \* $p<0.05$ , \*\* $p<0.01$  and \*\*\* $p<0.001$ . C, control

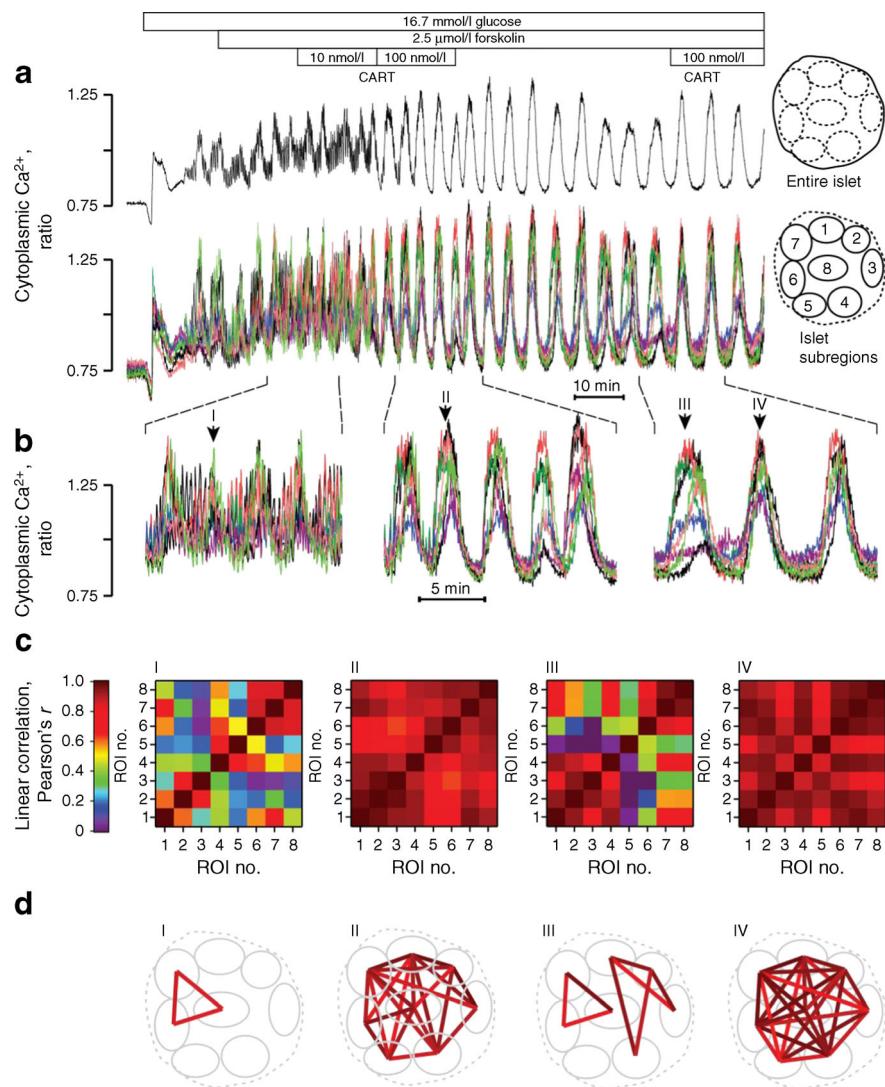
stimulated GSIS even further. This, together with the fact that *Cart*<sup>−/−</sup> mice have diminished insulin secretion, suggests that CART is a physiologically important regulator of insulin secretion. Acute CART stimulation was not associated with alterations of beta cell  $[Ca^{2+}]_i$  level. However, CART promoted a change in the pattern of  $[Ca^{2+}]_i$  oscillations, with fast oscillations being replaced by slow ones, known to underlie the pulsatile release of insulin [33, 36]. Furthermore, the change in  $[Ca^{2+}]_i$  oscillation pattern was paralleled by improved synchronisation of the  $[Ca^{2+}]_i$  signal between different islet subregions. Synchronisation 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<sup>+</sup> or carbachol, known

to induce pronounced, non-oscillatory islet  $[Ca^{2+}]_i$  increases [37, 38].

A factor complicating the mechanistic studies is that the CART receptor remains unknown [7, 39]. The majority of the effects of CART on islets were rapid, and evident within 1 h, however 24 h stimulation with CART was needed to increase exocytosis from single voltage-clamped beta 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 target for diabetes therapy.

**Fig. 5** CART modulates the cytoplasmic  $\text{Ca}^{2+}$  signalling pattern in mouse islets. Effects of CART on  $[\text{Ca}^{2+}]_i$  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  $\mu\text{mol/l}$  forskolin. (a)  $[\text{Ca}^{2+}]_i$  response pattern recorded from the entire islet (top panel, black trace) and from indicated smaller ROIs (lower panel, coloured traces). CART transforms fast  $[\text{Ca}^{2+}]_i$  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  $[\text{Ca}^{2+}]_i$  responses in different islet subregions at the 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 colour-coded Pearson's  $r$  coefficients for each possible ROI pair. CART improves the synchronisation between different islet subregions. (d) Connectivity maps highlighting the ROI pairs with correlation coefficients exceeding 0.8



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 in mice CART is expressed in a small subpopulation of beta cells. Pigs have no CART expression in islet cells [16]. Notably, individuals with type 2 diabetes, 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 as the upregulation of CART in rat models of type 2 diabetes was prevented by glucose-normalising 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, which is 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 upregulation of CART in type 2 diabetic islets is most likely a homeostatic compensatory response trying to overcome hyperglycaemia via paracrine actions. Even though CART has been localised 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.

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 on its properties of stimulating insulin secretion and inhibiting glucagon secretion, CART is a peptide that should be explored as a potential future therapy for diabetes.

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