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ABSTRACT

CART peptides are anorexigenic and are widely expressed in the central and peripheral nervous systems, as well as in endocrine cells in the pituitary, adrenal medulla and the pancreatic islets. To study the role of CART in islet function, we used CART null mutant mice (CART KO mice) and examined insulin secretion in vivo and in vitro, and expression of islet hormones and markers of β-cell function using immunocytochemistry. We also studied CART expression in normal pancreas. In addition, body weight development and food intake were documented. We found that in the normal mouse pancreas, CART was expressed in numerous pancreatic nerve fibers, both in the exocrine and endocrine portion of the gland. CART was also expressed in nerve cell bodies in the ganglia. Double immunostaining revealed expression in parasympathetic (vasoactive intestinal polypeptide (VIP) –containing) and, in fewer sensory fibers (calcitonin gene-related peptide (CGRP) –containing). Although the expression of islet hormones appeared normal, CART KO islets displayed age dependent reduction of pancreatic duodenal homeobox 1 (PDX-1) and glucose transporter-2 (GLUT-2) immunoreactivity, indicating β-cell dysfunction. Consistent with this, CART KO mice displayed impaired glucose-stimulated insulin secretion both in vivo after an intravenous glucose challenge and in vitro following incubation of isolated islets in the presence of glucose. The impaired insulin secretion in vivo was associated with impaired glucose elimination, and was apparent already in young mice with no difference in body weight. In addition, CART KO mice displayed increased body weight at the age of 40-weeks, without any difference in food intake. We conclude that CART is required for maintaining normal islet function in mice.

Key words:

CART, cocaine- amphetamine-regulated transcript, CART null mutant, type-2 diabetes, islet function, neuropeptides, β-cells, obesity, PDX-1, GLUT2

INTRODUCTION

The neuropeptide cocaine- amphetamine-regulated transcript (CART) is highly expressed in the brain [1-7] and exhibits anorexigenic properties [8, 9] [for review see 10]. Two different splice variants (rat long- and rat short form) of the transcript exist in rodents and, in addition, prohormone processing yields several CART fragments of different lengths. [1, 6, 11]. CART is also expressed in the peripheral nervous system, including sympathetic preganglionic [12, 13], primary sensory [14], and enteric neurons [15, 16]. In addition, CART is expressed in endocrine cells, e.g. pituitary endocrine cells [3, 17], adrenomedullary cells [3, 14, 17], islet somatostatin cells [18, 19], and antral gastrin cells [16] as studied mostly in rats. We recently reported that CART is expressed in several islet cell types, including β-cells, during rat development [19], in the β-cells of type-2 diabetic rats [20, 21], and in nerves in the rat pancreas [19]. Despite the morphological knowledge about CART expression, the functional roles of CART in the periphery are less studied [10]. We recently showed that CART regulate islet hormone secretion [21], although there are reports suggesting no of effect of CART on insulin or glucagon secretion [22, 23]. Furher, a stimulatory effect of CART on pancreatic exocrine secretion [24], an inhibitory effect of centrally administered CART on gastric emptying and gastric acid secretion [25, 26], and a modulatory role for CART on NO induced relaxation of the gut have been described [16]. To increase our knowledge about the role for CART in islets, we studied CART expression in normal mouse pancreas, and used CART knock out (KO) and wild type (WT) mice to study islet function in vivo and in vitro, as well as expression of islet hormones and β-cell function markers, using immunocytochemistry. We also monitored body weight development and food consumption in the mice fed a standard laboratory diet.

MATERIALS AND METHODS

Targeted disruption of the mouse Cart gene.

Cart^{tm1Amgn} mice (Further on called CART KO mice) were generated through targeted disruption of the *Cart* gene. The targeting vector was designed to replace the *Cart* coding sequence with a His/Neo cassette. An EcoRI/BamHI fragment containing all three exons of the mouse *Cart* gene was isolated from a 129sv mouse BAC and subcloned into pRS426 (Fig 1A). The *Cart* targeting vector was then generated via homologous recombination in yeast [27]. The vector was electroporated into RW4 ES cells and neomycin resistant colonies were screened by PCR. PCR positive clones were verified by Southern analysis using 5' and 3' probes outside of the region of homology. ES cells containing a disrupted *Cart* gene were injected into blastocysts and the resulting chimeric animals were bred with black swiss mice to determine germ-line transmission. CART KO mice were maintained on the outbred (Black swiss X 129SvJ) background. Expression analysis of hypothalamic and eye RNA further confirmed that the *Cart* gene had been successfully disrupted (Fig 1C). Mice were genotyped using PCR primers specific for the mutant and wild-type alleles (Fig 1B).

Animals and Tissue processing

Mice were housed under alternate 12-h periods of light and dark with free access to standard rat food and tap water. Initial studies indicated a more pronounced difference in body weight between CART KO mice and WT mice in the males than in the females. Therefore we limited our studies to males aged 8, 12, 20, and 40 w (n= at least 10 of each genotype at each time point). Food consumption was measured once a week for 12 w. Pre-weighed portions of food were given to each cage of animals (6 in each cage). The food remaining after one week was weighed, and the consumed food calculated. For histochemical analysis, the pancreas was

dissected out, fixed overnight in Stefanini's solution (2% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer, pH 7.2), rinsed thoroughly in Tyrode's solution containing 10% sucrose, and frozen on dry ice. Sections (10μm thickness) were cut and thaw-mounted on slides. The experiments were approved by the Regional Animal Ethics Committee in Lund and Malmö.

Immunocytochemistry

Antibodies were diluted in phosphate buffered saline (PBS) (pH 7.2) containing 0.25% bovine serum albumin and 0.25% Triton X-100. Sections were incubated with primary antibodies (Table 1) overnight at 4° C, followed by rinsing in PBS with Triton X-100 for 2 x 10 min. Thereafter secondary antibodies with specificity for rabbit-, guinea pig-, or sheep- IgG, and coupled to either fluorescein isothiocyanate (FITC), Texas-Red, or 7-amino-4-methyl coumarin-3-acetic acid (AMCA) (all from Jackson, West Grove, PA, USA), were applied on the sections. Incubation was for 1h at room temperature. Sections were again rinsed in Triton X-100 enriched PBS for 2 x 10 min and then mounted in PBS:glycerol, 1:1. The specificity of immunostaining was tested using primary antisera preadsorbed with homologus antigen (100μg of peptide per ml antiserum in working dilution) and by omission of primary antibodies. Double immunofluorescence was also used, with combinations of primary antibodies (rabbit antibodies, guinea pig or sheep antibodies). The two primary antibodies were incubated simultaneously overnight at 4° C, followed by rinsing in PBS with Triton X-100 for 2 x 10 min. Thereafter the two different secondary antibodies were incubated simultaneously for 1h at room temperature. In these studies tests for inappropriate binding of the secondary antibodies were performed.

Image analysis and morphometry

Immunofluorescence was examined in epi- fluorescence microscope (Olympus, BX60). By changing filters the location of the different secondary antibodies in double staining was determined. Images were captured with a digital camera (Olympus, DP50). Density of PDX-1 immunostaining was examined using Image Pro Plus software. The immunostained area and the total islet area were calculated and density of immunostaining was expressed as the percentage of the total islet area. In each animal (CART KO mice n=5, WT; mice n=5) 5 - 10 islets were analysed. To measure islet size (in 40 w old mice), images were analyzed as previously described [28].

Studies on insulin secretion in vivo

The in vivo studies were performed in late morning 4h after removal of food from the cages. The animals were anesthetized with an intraperitoneal injection of midazolam (Dormicum®, Hoffman-La-Roche, Basel, Switzerland, 0.2 mg/mouse) and a combination of fluanison (0.4 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm®, Janssen, Beerse, Belgium). Thirty minutes later, a blood sample was taken from the retrobulbar, intraorbital, capillary plexus in heparinized tubes, and D-glucose (British Drug Houses, Poole, UK; 1g/kg) was rapidly injected intravenously. The volume load was 10 µl/g body weight. At specific times points after injection, blood samples (75µl each) were collected. A total of five samples were taken during each experiment. The removal of this amount of blood has previously been shown not to alter baseline glucose levels in mice [29]. Blood was kept in heparinized tubes; then, following immediate centrifugation, plasma was separated and stored at –20°C until analysis.

Studies on insulin secretion in vitro

Islets were isolated by standard collagenase digestion, and handpicked under a stereo microscope. They were then kept overnight in RPMI-1640 medium, containing 11.1 mM glucose, 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, at 37°C in 95% air and 5% CO_2. Islets were thereafter kept in HEPES balanced salt solution (HBSS; 114 mM NaCl, 4.7 mmol KCl, 1.16 mM $MgSO_4$, 20 mM HEPES, 2.5 mM $CaCl_2$, 0.1% BSA; pH 7.35) containing 3.3 mM glucose for 60 minutes in an incubator at 37°C. Then, 3 islets at a time were transferred to a multi-well plate kept on ice and containing 200 μl per well of the same buffer but with the addition of glucose at different concentrations, or in the second series of experiments with addition of glucose at different concentrations. When all islets had been transferred, the plate was again placed in an incubator at 37°C; after 60 minutes, a sample from the buffer was removed for measurement of insulin.

Assays

Insulin was determined radioimmunochemically with the use of a guinea pig anti-rat insulin antibody, ^{125}I-labelled human insulin as tracer and rat insulin as standard (Linco Research, St Charles, MO, USA). Free and bound radioactivity was separated by use of an anti-IgG (goat anti-guinea pig) antibody (Linco). The sensitivity of the assay is 12 pmol/l and the coefficient of variation (CV) is less than 3% within assays and less than 5% between assays. Plasma glucose concentrations were determined with the glucose oxidase technique.

Data analysis.

Data and results are reported as means±SEM. From the intravenous glucose tolerance test, the acute insulin response (AIR) was calculated as mean suprabasal insulin levels for min 1 and 5 (i.e., $(ins_{5\ min} + ins_{1\ min})/2 - ins_{0\ min}$) and the area under the curve of insulin concentration (AUC_{ins}) was calculated using the trapezoidal rule. Glucose elimination rate after the glucose

injection (K_G, the glucose tolerance index) was calculated as the slope for the interval 1-20 min after glucose injection of the logarithmic transformation of the individual plasma glucose values.

Statistics

Statistical comparisons between CART KO and WT animals were performed with Student's un-paired t-test and in the in vitro studies with ANOVA. Differences with $p<0.05$ were considered significant.

RESULTS

Animals, body weight, and food consumption

CART KO mice were viable and litter sizes were normal. Male CART KO mice had increased body weight compared to the WT (Fig 2). Thus, at 40w CART KO mice weighed 14% more than the WT mice ($p<0.05$); at 12w and 20w there was a tendency, however not reaching statistical significance, for higher body weight in CART KO mice. Food consumption was monitored for 12w, from 16-28 w of age and did not reveal any difference between CART KO mice (0.15 $\pm$ 0.014 g/day/g body weight; n=6) and WT mice (0.15 $\pm$ 0.014 g/day/g body weight; n=6).

Immunocytochemistry

CART in pancreas of WT mice

Numerous CART immunoreactive (IR) nerve fibres were observed around blood vessels and ducts, around acini of the exocrine parenchyma (Fig 3C and 4A and G), among islet endocrine cells (Fig 3A-B and E), and within ganglia (Fig 3D) that also regularly contained CART IR nerve cell bodies. Sometimes such ganglia were situated close to islets, forming neuro-islet complexes [30] (Fig 4D). Double immunostaining for CART and VIP revealed a high degree of coexpression in nerve fibers as well as nerve cell bodies (Fig 4A-F) Furthermore, CART was expressed in a subpopulation of CGRP IR nerve fibers (Fig 4G-I). In addition, weakly CART IR endocrine cells were occasionally found at the islet periphery. Double immunostaining for CART and somatostatin revealed that a small subpopulation of the δ-cells were CART IR (data not shown).

CART KO pancreas

As expected CART KO mice completely lacked CART IR (data not shown). Immunostainings for the main islet hormones (insulin, glucagon, somatostatin, and PP), did not reveal any obvious differences in cell number, intra-islet topography, or intensity of immunofluorescence between CART KO mice and the WT mice (data not shown). WT islets displayed intense GLUT2 IR confined to the cell membrane of the β-cells (Fig 5A). However, in CART KO islets (examined at 40w) GLUT2 IR was markedly reduced, with many cells displaying only weak cell membrane IR and irregular intra-islet distribution with some areas lacking detectable GLUT2 IR. Further, at the cellular level an abnormal distribution of GLUT2 with sometimes very little membrane staining and instead a weak to moderate cytoplasmic staining was seen (Fig 5B). In WT islets (40w) PDX-1 IR was intense in all β-cell nuclei (Fig 5C). In CART KO islets (examined at 40w) PDX-1 IR in the β-cell nuclei had generally reduced intensity and was in some cells barely detectable (Fig 5D). Morphometrical analysis for PDX-1 IR revealed that the density of IR was 4 times lower in CART KO compared to WT islets (CART KO: (n=5) 3$\pm$2%; WT: (n=5) 12$\pm$2%; $p<0.05$) In 8w mice we could not detect any clear-cut difference in immunostaining pattern of GLUT2 or PDX-1 between WT and mutants. In addition, the islet number appeared normal and the islet size (examined at 40w) was normal in CART KO mice compared to WT mice, being 10640$\pm$1700 μm^2 in CART KO mice (n=10) versus 14400$\pm$1100 μm^2 in wt mice (n=8); NS. Analysis of pancreatic VIP- or CGRP-IR nerves did not reveal any obvious difference in numbers, distribution or IR intensity between WT and mutants at any stage (data not shown).

Insulin and glucose responses to iv glucose challenge

Since CART KO mice had higher body weight at 40w, and since CART was found in pancreatic neurons and islet cells of the WT mice we first examined insulin secretion and glucose elimination after an intravenous glucose challenge in mice at 40 weeks of age. We found that the CART KO mice had higher basal blood insulin (p=0.047) but normal baseline glucose compared to the WT mice. Furthermore, CART KO mice displayed a blunted AIR (CART KO mice 235±305 pmol/l versus 1168±155 pmol/l in WT mice, p=0.02). In contrast, at 50 min after the glucose challenge, the insulin level was almost 3-fold increased compared to the WT (p=0.016). CART KO mice also displayed an impaired glucose elimination; K_G was 2.0±0.3%/min in CART KO mice versus 5.6±0.4 %/min in WT mice (p<0.001) (Fig 6A-B). These mice displayed difference in body weight (CART KO mice 41.9±3.6g vs 34.8±0.2g of WT mice; p=0.041). We then studied young (8w) KO and WT mice, with no significant difference in body weight. At this age there was no difference in basal glucose or insulin between the CART KO mice and the WT mice. However, IVGTT revealed a blunted AIR to glucose in CART KO mice (CART KO mice 73±118 pmol/l versus 1184±420 pmol/l in WT mice; p=0.020). This was associated with an impaired glucose elimination in CART KO mice (K_G 5-20 min was 4.2±0.9%/min in WT mice versus 2.2±0.3%/min in CART KO mice (p=0.039) (Fig 6C-D).

Insulin secretion from isolated islets

Next we examined whether young CART KO mice, with normal weight, had defects at the islet level. Islets from 8w CART KO mice and WT mice were isolated, and glucose stimulated insulin secretion was examined in static incubations. Fig. 7 shows that insulin secretion was lower in islets from CART KO mice than from WT mice and this was observed across the entire range of glucose (p=0.005).

DISCUSSION

In the present investigation we demonstrate that CART deficiency in mice results in islet dysfunction and altered islet expression of markers of β-cell function.

The pattern of CART IR in mouse pancreas, with most of the IR in nerves and less IR in islet cells, differs quantitatively from that in the adult rat pancreas where CART is prominently expressed in islet δ-cells, and less in pancreatic nerves [18, 19]. The present finding in mice of CART expression in CGRP-containing fibers and in VIP-containing pancreatic nerve cell bodies and fibers mirrors our previous observations in the rat pancreas and gut [16, 19] identifying CART as a constituent of intrinsic VIP-containing neurons and of extrinsic CGRP-containing neurons. In the pancreas the VIP-containing nerves are regarded as parasympathetic, and such nerves are known to activate insulin secretion [31]. Interestingly, a recent reported suggested that CART may modulate neuronally mediated excitatory or inhibitory processes in rat exocrine pancreas [24]. It is therefore not inconceivable that this localization of CART may be related to our finding that genetic deletion of CART suppresses glucose-stimulated insulin secretion. It is worth mentioning in this context that CART is released in pigs upon vagal stimulation [22]. Hence, CART may be involved in the regulation of parasympathetic control of islet function.

A main finding in the present study was that genetic deletion of CART is associated with impaired insulin secretion in association with glucose intolerance. The blunted insulin response to glucose was observed both in vivo after an intravenous glucose challenge and in vitro during incubation of isolated islets. In vivo, it was primarily the early insulin response that was impaired in CART KO mice, and this is consistent with the defective glucose

disposal, since it is mainly the early response that is important for glucose disposal [32]. On the contrary, plasma insulin responses at later time points after the intravenous glucose challenge was higher in CART KO mice, which is interpreted as being a consequence of the higher glucose levels seen at these time points. The mechanism of the impaired glucose-stimulated insulin secretion in CART KO mice needs to be established, but is interesting in view of the likelihood that CART is a constituent of pancreatic parasympathetic neurons. The immunocytochemical analyses of the islets in CART KO mice in the present study reveals, however, that it is possible that beta cell dysfunction may have an intrinsic component. Thus, the expression of two important factors involved in beta cell physiology, GLUT-2 and PDX-1, became defective with age in CART KO mice. GLUT-2 is a major determinant of glucose uptake in beta cells [33] whereas PDX-1 is of importance for beta cell development and insulin gene expression [34, 35]. Notably, genetic deletion of either GLUT-2 [36] or PDX-1 [35] is associated with defective insulin secretion and glucose intolerance. It may therefore be hypothesized that neural effects executed by CART is required for normal expression of GLUT-2 and PDX-1 and disturbances in these processes lead to impaired insulin secretion with increasing age. This view is consistent with our finding that also insulin secretion in response to glucose from isolated islets was impaired in CART KO mice. This impairment was observed over a wide range of glucose concentrations, indicating both that the glucose sensitivity and the maximal beta cell response were defective. The fact that impaired islet function is apparent already in young CART KO mice, before the increase in body weight suggests a role of CART directly on the islets, and our data on impaired insulin secretion from isolated CART KO islets also favours such an action. Our recent finding that CART regulates insulin secretion from clonal beta cells and from isolated rat islets, supports a direct effect of CART on control of insulin secretion. Thus, CART augments cAMP-mediated insulin secretion from both clonal cells and islets in a dose-dependent fashion [21].

Our finding that CART KO mice have increased body weight compared to the WT is in line with the anorectic properties of CART [for review see 10], and indicates that CART is an important player for maintaining energy homeostasis, conceivably by acting at hypothalamic sites. In a recent study [37], using another strain of CART deficient mice, a difference in body weight was observed only in mice fed a high fat diet. The authors reported that at the age of 17w and after 14 weeks (longest time studied) on regular chow there was no significant weight gain in male or female mutants compared to WT mice. In the present study we monitored body weight for a longer time period (up to 40w of age). The present data indicate that CART KO mice may develop higher body weight also when fed standard laboratory food, and that this is late in onset.

Although CART KO mice had a higher body weight than WT mice, we were unable to detect any difference in food intake. This suggests that possible effects on food intake of lifelong loss of CART, even though CART is anorexigenic during acute experimentations, are compensated for by redundant mechanisms. Although Asnicar et al [37] did not report any difference in energy expenditure between CART KO mice and WT mice, humans carrying a mutation in the *Cart* gene have been reported to have reduced metabolic rate and early onset obesity [38]. It is tempting to speculate that the CART KO mice of the strain used here has decreased energy expenditure and hence develop higher body weight than the WT, despite an unchanged food intake.

In conclusion, this study shows for the first time that CART is expressed in the mouse pancreas and is necessary for maintaining normal islet function.

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FIGURE LEGENDS

Figure 1. Targeted disruption of the *Cart* gene and *Cart* gene expression in Cart^{tm1Amgn} mice. A: Schematic representation of the *Cart* targeting construct and *Cart* locus. The three exons of *Cart* encompassing 1.8 kb and containing the entire coding region are indicated by black boxes. In the targeting vector a HIS/neo^{r} fusion gene has replaced the exons. Homologous recombination between the targeting construct and the *Cart* genomic locus resulted in the replacement of the 3 exons of *Cart* containing the coding region of *Cart* with a neo^{r}/his fusion construct. PCR primers (arrows) and southern probes (thick lines) used for genotyping are indicated. B: Representative genotype analysis of Cart^{tm1Amgn} animals. The PCR primers indicated in A. were used to genotype the animals indicated. Animals 75 and 80 exhibited only the wild-type band (240 bp), 77 and 78 both the wild-type and Cart^{tm1Amgn} band (450 bp), and 74 and 76 only the Cart^{tm1Amgn} band indicating that these animals were Cart^{tm1Amgn} homozygotes. C: Expression of *Cart* mRNA in the hypothalamus of Cart^{tm1Amgn} animals. A northern blot of RNA isolated from the hypothalamus of the animals genotyped in B is shown. No *Cart* mRNA is was detected in homozygous Cart^{tm1Amgn} animals. RNA from eye

and pituitary were also examined and a similar result obtained (DNS). The same blot was stripped and re-probed with an internal control (cyclophilin) for loading and RNA integrity.

Fig 2. Body weight development of male WT mice and CART KO mice. Means ±SEM are shown. At 40w of age CART KO mice fed a normal diet exhibit increased body weight compared to WT mice. * $p<0.05$ vs WT mice.

Fig 3. Fluorescence photomicrographs of WT pancreas illustrating neuronal CART (red in all images). A-B: Double immunostaining for CART (A) and insulin (B) illustrating CART IR nerve fibers within the islet. C: CART IR fibers in a small nerve trunk in exocrine tissue. D: CART IR nerve fibers around cell bodies within a pancreatic ganglion. E: Double immunostaining for CART and insulin (blue), illustrating CART IR fibers in a nerve trunk in close contact with the islet. Scale bars = 20 μm, In A for A-C and E, in D for D.

Fig 4. Fluorescence photomicrographs of WT pancreas double stained for CART (A and D) and VIP (B and E). C and F merged. A-C illustrate colocalisation of CART and VIP in a nerve fibre. D-F illustrate colocalisation of CART and VIP in a nerve cell body of a neuro islet complex. The immunostained cell in F is inserted at higher magnification to visualize colocalisation. G-I: WT pancreas double stained for CART (G) and CGRP (H). I merged. CART is expressed in a subpopulation of the CGRP positive varicosites. Scale bars = 20 μm, in A for A-F and in G for G-I.

Fig 5. Fluorescence photomicrographs of CART KO and WT islets (40 w old male mice). In WT islets intense and uniform GLUT2 immunostaining was seen in the membrane of β-cells

(A). In the KO, GLUT2 IR was low and sometimes barely detectable in a subpopulation of the β-cells (B). In WT islets intense PDX-1 immunostaining was seen in β-cell nuclei (C) PDX-1 IR was generally weaker in the CART KO islet (D). Scale bar = 20 μm.

Fig 6. Glucose and insulin levels before and after intravenous administration of glucose (1 g/kg) in CART KO and WT male mice. Left panels display results in mice at 40 weeks of age whereas right panels show results in mice at 8 weeks of age. CART KO mice display a blunted AIR compared to WT mice. * $p<0.05$ AIR KO vs WT. CART KO mice display impaired K_G compared to WT mice. # $p<0.05$ K_G KO vs WT. Means±SEM are shown.

Fig 7. Medium insulin concentrations after 60 min incubation of islets from CART KO or wildtype male mice in the presence of glucose at different concentrations. There were 8 separate incubations, each using three islets (a total of 4 mice were used in each group). Means±SEM are shown.

Fig 1

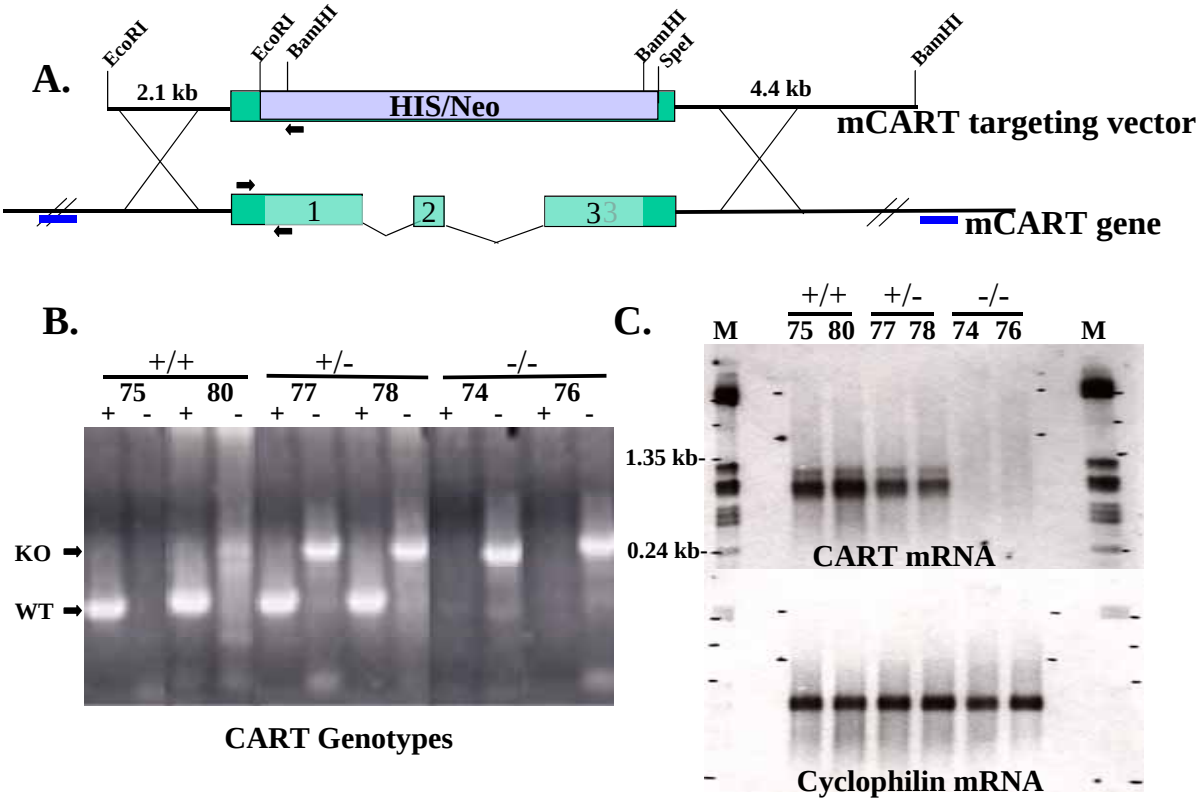
A.
EcoRI
EcoRI
BamHI
BamHI
SpeI
BamHI
2.1 kb
HIS/Neo
4.4 kb
mCART targeting vector
1
2
3
mCART gene
B.
+/+
+/-
-/-
75
80
77
78
74
76
KO
WT
CART Genotypes
C.
M
+/+
+/-
-/-
75 80
77 78
74 76
M
1.35 kb-
0.24 kb-
CART mRNA
Cyclophilin mRNA

Fig 2

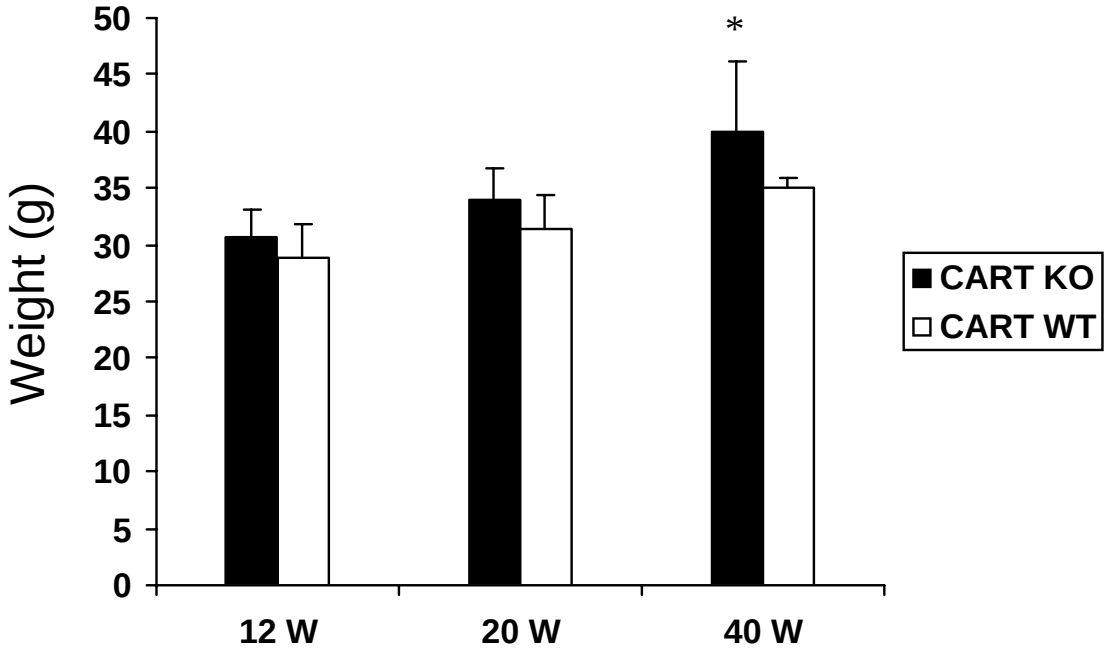

Fig3

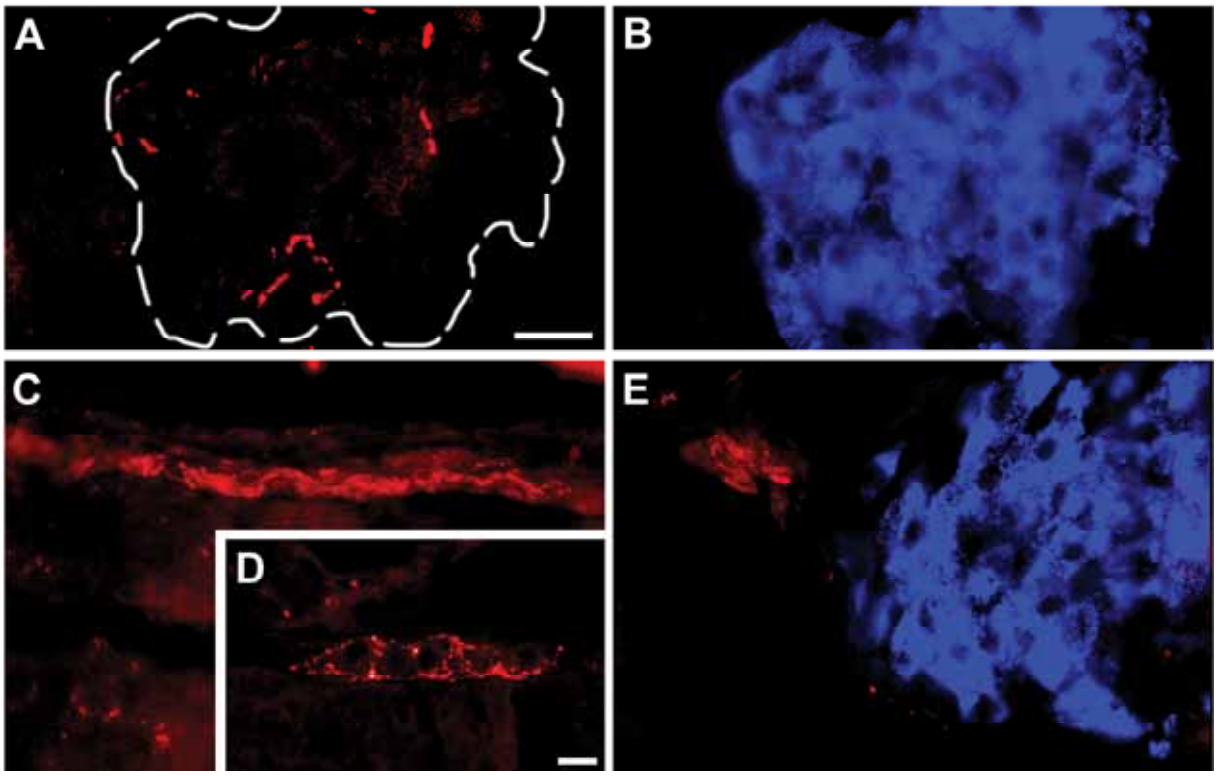

Fig 4

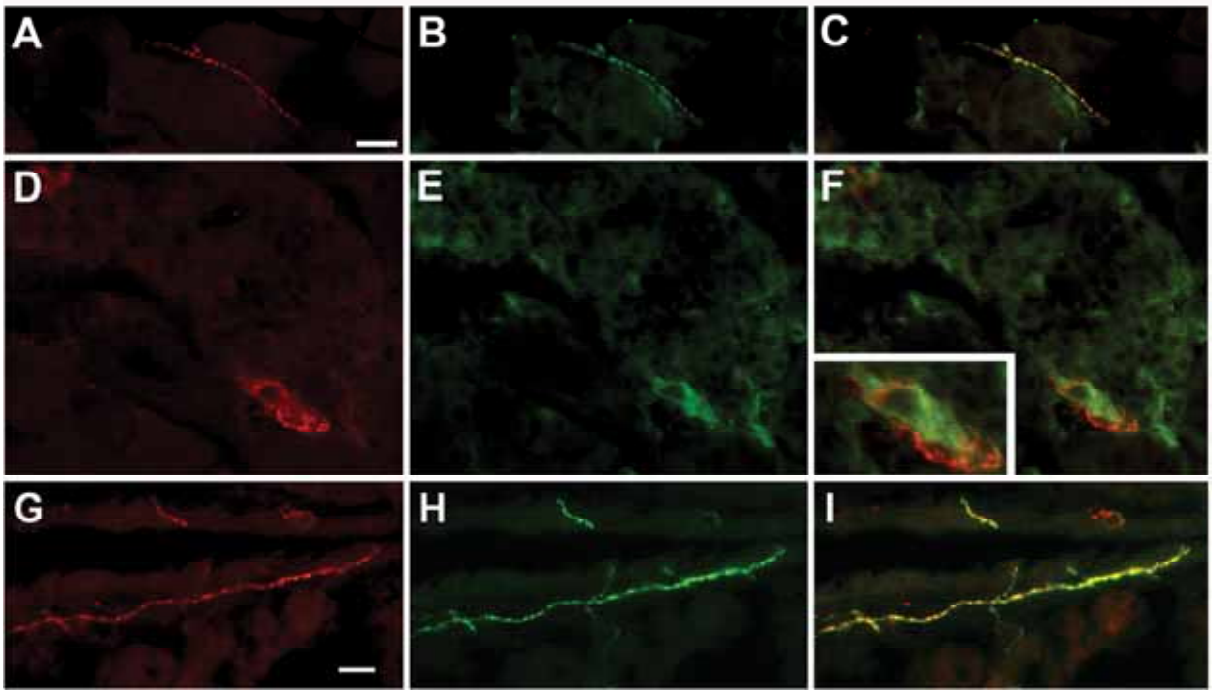

Fig 5

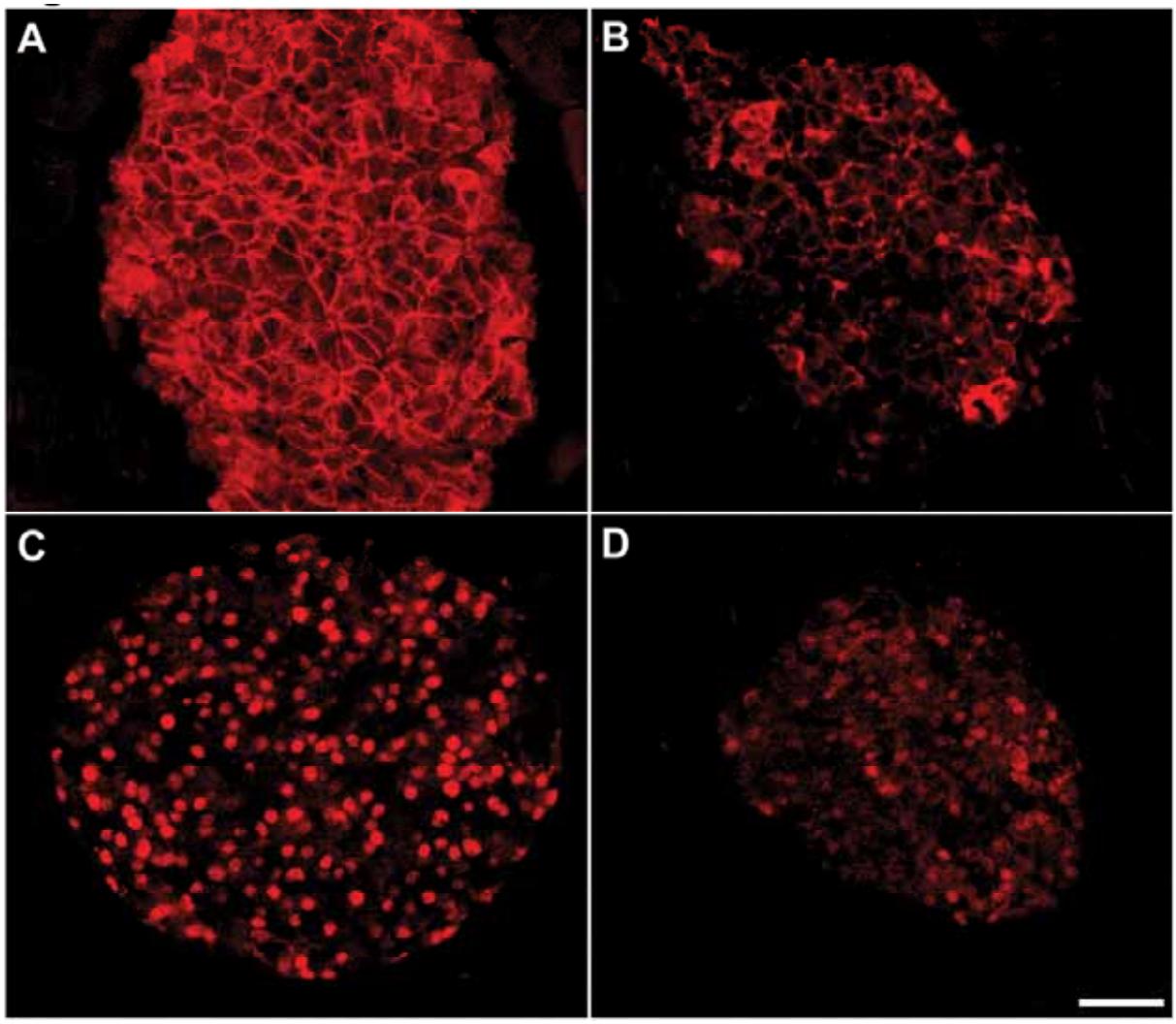

Fig 6

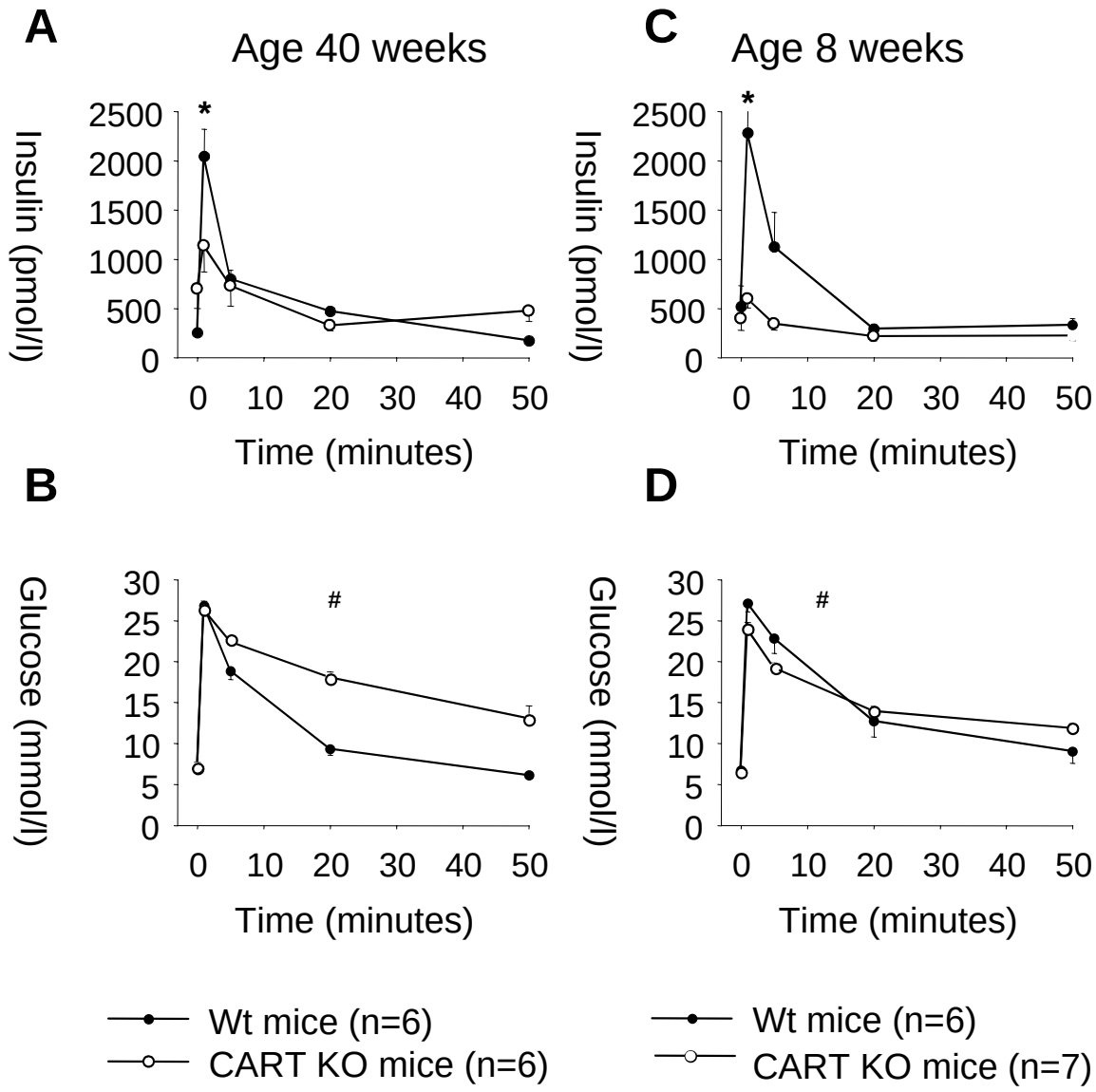
A
Age 40 weeks
Insulin (pmol/l)
2500
2000
1500
1000
500
0
*
0 10 20 30 40 50
Time (minutes)
C
Age 8 weeks
Insulin (pmol/l)
2500
2000
1500
1000
500
0
*
0 10 20 30 40 50
Time (minutes)
B
Glucose (mmol/l)
30
25
20
15
10
5
0
#
0 10 20 30 40 50
Time (minutes)
D
Glucose (mmol/l)
30
25
20
15
10
5
0
#
0 10 20 30 40 50
Time (minutes)
Wt mice (n=6)
CART KO mice (n=6)
Wt mice (n=6)
CART KO mice (n=7)

Fig 7

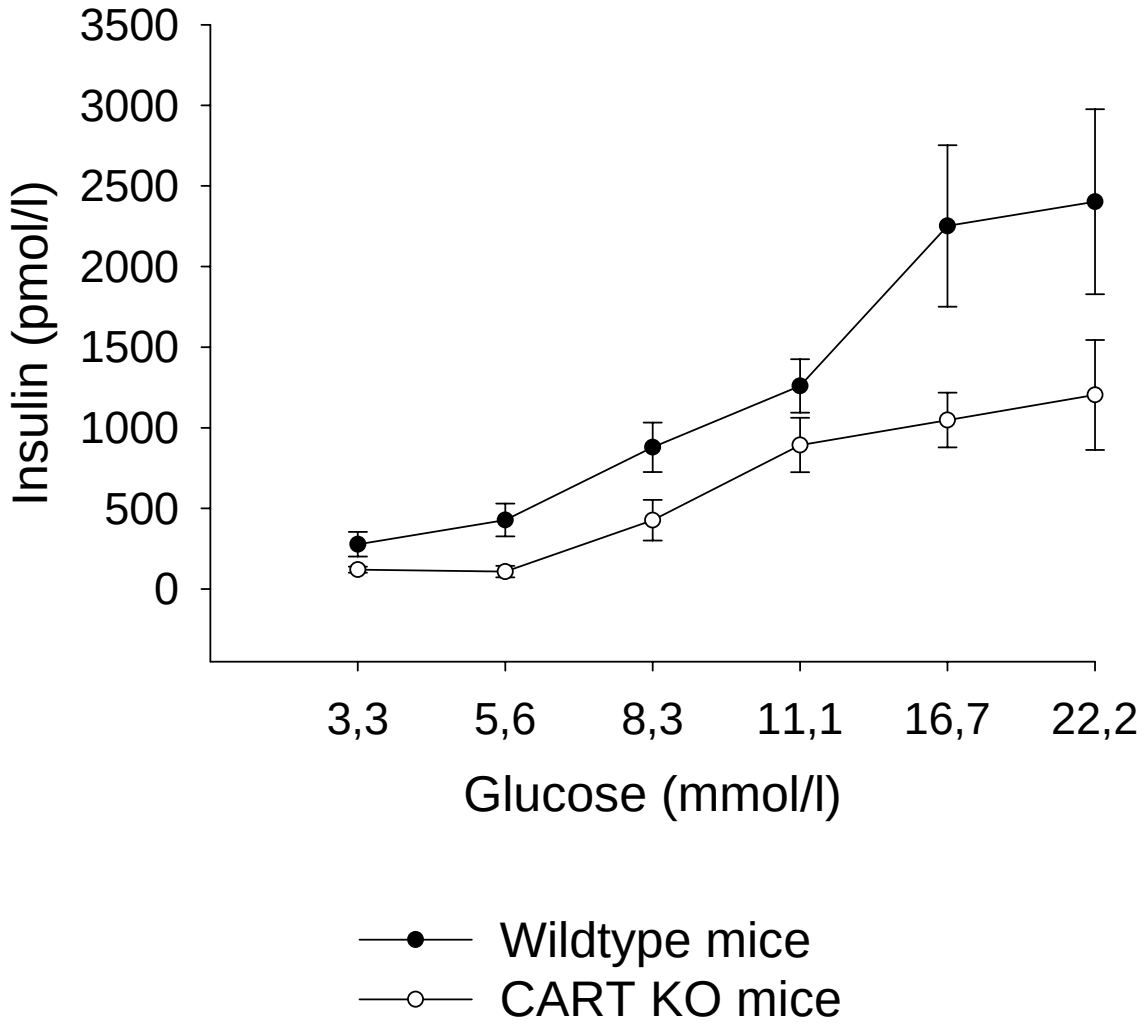
3500
3000
2500
2000
1500
1000
500
0
Insulin (pmol/l)
3,3
5,6
8,3
11,1
16,7
22,2
Glucose (mmol/l)
Wildtype mice
CART KO mice

Table 1 Details of the antibodies.

Antigen	Code	Raised in	Dilution	Source
CART	12/D	Rabbit	1:1280	Cocalico Corp., Reamstown, PA, USA
Proinsulin	9003	Guinea-pig	1:2560	EuroDiagnostika, Malmö, Sweden
Glucagon	8708	Guinea-pig	1:5120	EuroDiagnostika
Somatostatin	N-SOM	Rabbit	1:800	DiaSorin Inc., Stillwater, MN, USA
Pancreatic polypeptide	AHP 515	Sheep	1:640	Serotec, Oxford, UK
VIP	8701	Guinea-pig	1:1280	EuroDiagnostika
GLUT2	AB 1342	Rabbit	1:640	Chemicon, Temecula, CA, USA
PDX-1 CGRP	PDX-1 M8513	Rabbit Guinea-pig	1:2000 1:640	From Dr M. German, UCSF, CA, USA EuroDiagnostika