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The role of CART in islet function

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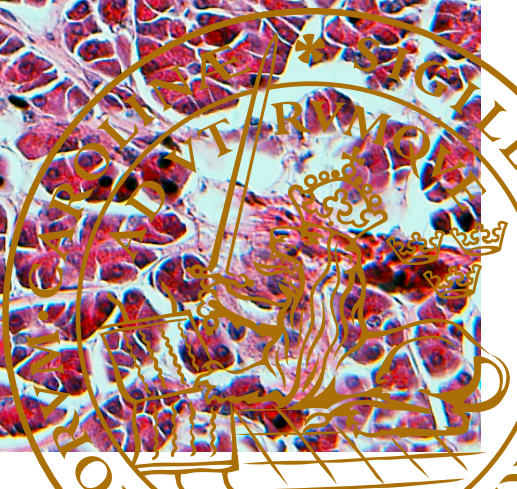
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A high-magnification histological image of a pancreatic islet. The islet is a cluster of endocrine cells, appearing as a lighter, more cellular mass compared to the surrounding exocrine acinar tissue, which is stained a deep red. The islet contains numerous small, dark-staining nuclei, likely representing the islet cells. The surrounding acinar tissue shows a characteristic honeycomb-like structure with many small, clear spaces (acini) and darker-staining nuclei.

The role of CART in islet function

MIA ABELS

FACULTY OF MEDICINE | LUND UNIVERSITY



The role of CART in islet function

Mia Abels



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DOCTORAL DISSERTATION

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To be defended at CRC Aula, Jan Waldenströms gata 35, Malmö.

Friday 18th of November 2016 at 9.00.

Faculty opponent

Professor Patrik Rorsman

University of Oxford, United Kingdom and University of Gothenburg, Sweden

Organisation LUND UNIVERSITY Faculty of Medicine Department of Clinical Sciences Malmö Division of Neuroendocrine Cell Biology Author: Mia Abels	Document name DOCTORAL DISSERTATION	
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Abstract <p>Diminished insulin secretion and dysregulated glucagon secretion are key features of type 2 diabetes (T2D). The overall aim of this thesis was to study the role of cocaine- and amphetamine-regulated transcript (CART) in islet cell function and how CART regulates glucose homeostasis. We found that CART is expressed in human islet cells and that its expression is increased in patients with T2D, as well as in mouse models of T2D. Rat islet CART expression was regulated by glucose and beta cell CART expression was normalised by insulin treatment in diabetic rats. Furthermore, CART increased insulin secretion from both mouse and human islets in a glucose-dependent fashion. This could partly be explained by increased beta cell exocytosis, altered intracellular Ca^{2+} oscillation pattern as well as improved synchronisation of Ca^{2+} oscillations between islet cells. Importantly, CART increased insulin secretion and glucose elimination <i>in vivo</i> in mice. We also showed that CART decreased glucagon secretion in mouse and human islets, as well as <i>in vivo</i> in mice, and that CART reduced exocytosis in alpha cells. To mimic the situation in patients, with increased beta cell CART expression, we generated transgenic mice with beta cell-specific CART overexpression (CARTtg) and studied the impact of increased beta cell CART expression on glucose homeostasis <i>in vivo</i>. Under basal conditions, CARTtg mice were normoglycaemic and normoinsulinemic, but when challenged by streptozotocin treatment or a high fat diet, and in ageing, CARTtg mice displayed increased insulin secretion compared with wild-type littermates. This was accompanied by improved glucose elimination in streptozotocin-treated and aged mice, but not in high fat diet-fed mice, which instead displayed mild insulin resistance. Moreover, viral overexpression of CART in INS-1 (832/13) cells increased insulin secretion. Finally, we found that adipocytes from CARTtg mice had altered metabolism, suggesting that CART may be a mediator of cross-talk between beta cells and adipose tissue. Collectively, our data imply that CART has an important role in beta cell function and in regulation of glucose homeostasis. Hence, the potential for CART-based therapies in T2D should be evaluated.</p>		
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The role of CART in islet function

Mia Abels



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Front cover picture showing haematoxylin and eosin staining of human pancreas.
Picture by Mia Abels.

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Till Rickard & Alfred

Table of contents

Papers included in this thesis	1
Published papers not included in this thesis	2
Summary	3
Populärvetenskaplig sammanfattning	5
Introduction	9
What is the problem?	9
The islets of Langerhans	9
Regulation of blood glucose	10
Insulin	11
Glucose-stimulated insulin secretion	11
Dynamics of insulin secretion	13
Neuronal and hormonal fine-tuning of insulin secretion	15
Glucagon	15
Paracrine regulation of glucagon secretion	15
Intrinsic regulation of glucagon secretion	16
White adipose tissue	17
Function of white adipose tissue	17
Lipolysis	18
Esterification of FFAs and <i>de novo</i> lipogenesis	18
Type 2 Diabetes	19
Genetics of type 2 diabetes	20
Insulin resistance and adipocyte dysfunction	21
Impaired insulin secretion	22
Dysregulated glucagon secretion	23
Treatment of type 2 diabetes	24
CART	25
<i>CARTPT</i> gene structure and regulation	26
CART in CNS and the HPA axis	27
CART in adipose tissue	28
CART in the islets	29
CART receptor and CART KO mice	30
Aims of this thesis	33

Experimental methods	35
Animal models in research	35
Mouse models of diabetes	36
The C57Bl/6J mouse	36
The high fat diet fed mouse	36
The streptozotocin mouse	36
<i>In vivo</i> glucose and insulin tolerance tests	37
Mouse islet isolation and hormone secretion	37
Human islets	38
INS-1 (832/13) cells	38
Immunohistochemistry	39
Main findings	41
Results and discussion	43
Aim I: CART expression in islet cells	43
Aim II: The effect of CART on insulin secretion	46
Aim III: The effect of CART on glucagon secretion	51
Aim IV: The effect of overexpressed beta cell CART	52
Aim V: The effect on insulin sensitivity and adipocyte metabolism in mice overexpressing beta cell CART	58
Anti-diabetic effects of CART	61
Concluding remarks and perspectives	65
Abbreviations	67
Acknowledgments	71
References	73

Papers included in this thesis

This thesis is based on the following original paper and manuscripts, referred to in the text by their roman numerals indicated below:

- I. **Abels M**, Riva M, Bennet H, Ahlqvist E, Dyachok O, Nagaraj V, Shcherbina L, Fred RG, Poon W, Winzell MS, Fadista J, Lindqvist A, Kask L, Sathanoori R, Dekker-Nitert M, Kuhar MJ, Ahrén B, Wollheim CB, Hansson O, Tengholm A, Fex M, Renström E, Groop L, Lyssenko V, Wierup N. *CART is overexpressed in human T2D islets and inhibits glucagon secretion and increases insulin secretion*. Diabetologia 2016, 59(9):1928-37.
- II. **Abels M**, Riva M, Shcherbina L, Thorén Fischer A-H, Wierup N. *Overexpressed beta cell CART increases insulin secretion in mouse models of insulin resistance and diabetes*. In manuscript.
- III. **Abels M***, Banke E*, Spégel P, Riva M, Henriksson E, Thorén Fischer A-H, Göransson O, Degerman E, Wierup N. *Altered adipocyte metabolism in mice with beta cell-specific overexpression of CART*. In manuscript. *Both authors contributed equally to this study.

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Published papers not included in this thesis

Sharoyko V, **Abels M**, Sun J, Nicholas LM, Mollet I, Stamenkovic J, Göhring I, Malmgren S, Storm P, Fadista J, Spéjel P, Metodiev MD, Larsson NG, Eliasson L, Wierup N, Mulder H. *Loss of TFB1M results in mitochondrial dysfunction that leads to impaired insulin secretion and diabetes*. Human Mol Genet 2014, 23(21):5733-49.

Nitert MD, Dayeh T, Volkov P, Elgzyri T, Hall E, Nilsson E, Yang BT, Lang S, Parikh H, Wessman Y, Weishaupt H, Attema J, **Abels M**, Wierup N, Almgren P, Jansson PA, Rönn T, Hansson O, Eriksson KF, Groop L, Ling C. *Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes*. Diabetes 2012, 61(12):3322-32.

Salami F, **Abels M**, Hyöty H, Vaziri-Sani F, Aronsson CA, Vehik K, Delli A, Hagopian WA, Rewers M, Ziegler AG, Simell O, Akolkar B, Krischer J, She J, Lernmark Å. *Detection of lactobacilli in monthly mail-in stool samples from 3-18 months old infants at genetic risk for type 1 diabetes*. Int J Probiotics Prebiotics 2012, 7(3-4):135-144.

Summary

Diminished insulin secretion and dysregulated glucagon secretion are key features of type 2 diabetes (T2D). The overall aim of this thesis was to study the role of cocaine- and amphetamine-regulated transcript (CART) in islet cell function and how CART regulates glucose homeostasis. We found that CART is expressed in human islet cells and that its expression is increased in patients with T2D, as well as in mouse models of T2D. Rat islet CART expression was regulated by glucose and beta cell CART expression was normalised by insulin treatment in diabetic rats. Furthermore, CART increased insulin secretion from both mouse and human islets in a glucose-dependent fashion. This could partly be explained by increased beta cell exocytosis, altered intracellular Ca^{2+} oscillation pattern as well as improved synchronisation of Ca^{2+} oscillations between islet cells. Importantly, CART increased insulin secretion and glucose elimination *in vivo* in mice. We also showed that CART decreased glucagon secretion in mouse and human islets, as well as *in vivo* in mice, and that CART reduced exocytosis in alpha cells. To mimic the situation in patients, with increased beta cell CART expression, we generated transgenic mice with beta cell-specific CART overexpression (CARTtg) and studied the impact of increased beta cell CART expression on glucose homeostasis *in vivo*. Under basal conditions, CARTtg mice were normoglycaemic and normoinsulinemic, but when challenged by streptozotocin treatment or a high fat diet, and in ageing, CARTtg mice displayed increased insulin secretion compared with wild-type littermates. This was accompanied by improved glucose elimination in streptozotocin-treated and aged mice, but not in high fat diet-fed mice, which instead displayed mild insulin resistance. Moreover, viral overexpression of CART in INS-1 (832/13) cells increased insulin secretion. Finally, we found that adipocytes from CARTtg mice had altered metabolism, suggesting that CART may be a mediator of cross-talk between beta cells and adipose tissue. Collectively, our data imply that CART has an important role in beta cell function and in regulation of glucose homeostasis. Hence, the potential for CART-based therapies in T2D should be evaluated.

Populärvetenskaplig sammanfattning

I Sverige finns idag över 300 000 personer med diabetes och antalet ökar stadigt. Även om diabetes är en sjukdom som man kan leva med länge innebär sjukdomen ofta ett stort lidande för patienten samtidigt som det är en ekonomisk belastning för samhället att ta hand om dessa patienter. Ökad kunskap om sjukdomen och utveckling av nya mediciner är därför viktigt för att kunna bromsa denna negativa utveckling. I denna avhandling beskrivs hur hormonet CART kan påverka faktorer som är viktiga vid utveckling av diabetes och för diabetesbehandling.

Det finns flera varianter av diabetes och den vanligaste varianten, typ 2-diabetes (tidigare kallad åldersdiabetes), utgör cirka 90 % av alla fall. Majoriteten av de som insjuknar i typ 2-diabetes är överviktiga och sjukdomen är vanligtvis en konsekvens av en stillasittande livsstil och dåliga kostvanor, ofta i kombination med ärftliga faktorer. Det är inte ovanligt att typ 2-diabetespatienter även har andra livsstilsrelaterade hälsoproblem, såsom högt blodtryck och höga blodfetter.

Man räknar idag med att ungefär 3-4 % av den svenska befolkningen har typ 2-diabetes och det är en siffra som ökar, förmodligen på grund av ökande förekomst av övervikt och fetma. Men även om den största riskfaktorn för typ 2-diabetes är fetma, så insjuknar inte alla som har fetma i diabetes. Anledningen till detta är, trots omfattande forskningsinsatser, fortfarande inte helt klarlagd.

Diabetes innebär att kroppen inte kan reglera sockerhalten i blodet på egen hand. Sockret, som behövs som energi till kroppens celler, får svårare att ta sig in i cellerna. Detta medför att sockerhalten i blodet efterhand stiger, vilket på sikt kan leda till komplikationer såsom njursvikt, hjärt-och kärlsjukdomar och blindhet. Det som behövs är hormonet insulin som hjälper sockret att ta sig från blodet och in i cellerna.

Insulin produceras i speciella celler som finns i ansamlingar i bukspottkörteln. Dessa ansamlingar kallas de Langerhanska öarna. Typ 2-diabetes orsakas oftast av en kombination av att insulinet inte fungerar tillräckligt effektivt och att de Langerhanska öarna frisätter för lite insulin. En anledning till att det frisätts för lite insulin är att de insulinproducerande cellerna i bukspottkörteln fungerar dåligt och i vissa fall dör.

Utöver att ha för lite insulin har många diabetiker även för höga nivåer av hormonet glukagon. Glukagon, som också produceras i celler i de Langerhanska öarna, fungerar på motsatt sätt som insulin och höjer istället blodsockret mellan måltider.

Detta medför att höga nivåer av glukagon bidrar till de redan för höga nivåerna av blodsocker vid diabetes. Nya läkemedel som hjälper bukspottkörteln att frisätta mer insulin och mindre glukagon skulle därför kunna vara till stor nytta.

Vår forskargrupp har tidigare upptäckt att ett hormon som heter "Cocaine- and Amphetamine-Regulated Transcript" (förkortat CART) kan få en insulinproducerande cellinje att frisätta mer insulin samt att CART även kan skydda de insulinproducerande cellerna från att dö. Dessutom har vi visat att nivåerna av CART är högre i insulinproducerande celler hos diabetiska råttor än hos friska råttor. Vilken betydelse CART har hos oss människor är dock ännu inte klarlagt och inte heller vilken betydelse höga nivåer av CART har. Målet med denna avhandling är att studera just detta.

Den första studien visar att CART finns i insulin- och glukagonproducerande celler i bukspottkörteln hos oss människor samt att det även finns högre nivåer av CART i insulinproducerande celler hos personer med typ 2-diabetes. Vi visar även att CART ökar insulinfrisättningen samt minskar frisättningen av glukagon från Langerhanska öar från människor, vilket gör att CART kan reglera blodsockernivån på två olika sätt.

De första experimenten gjordes på mänskliga Langerhanska öar som donerats till forskning. För att visa att CART även fungerar i levande organismer testade vi effekten av CART i möss. Anledningen till att man ofta testar nya ämnen i möss är att de har en ämnesomsättning som människans. Om man i djurförsök kan visa att ämnet fungerar, samt att det inte är giftigt, kan man gå vidare med försök i människor. I möss kunde vi visa att CART ökade insulinfrisättningen, minskade glukagonfrisättningen samt förbättrade blodsockerregleringen. Tillsammans visar dessa resultat att CART är viktigt för reglering av blodsockernivåer i möss. Detta gör att vi har förhoppningar om att CART har samma effekt i människor.

Vårt DNA innehåller våra gener (arvsanlag) och dessa ärvs från våra föräldrar. Generna kan liknas vid ritningar över allt som tillverkas i våra celler, till exempel proteiner. När man vill förstå hur ett protein som tillverkas i kroppen fungerar kan man ändra genen för detta protein. Man kan bland annat ta bort eller göra flera kopior av en specifik gen. Detta gör man rutinmässigt i möss och man brukar kalla den förändrade musen för en musmodell.

I den andra studien ville vi förstå hur ökade nivåer av CART påverkar insulinproducerande celler, något som vi hade upptäckt hos personer med typ 2-diabetes i vår första studie. För att studera detta tillverkade vi en musmodell som hade fler kopior av CART-genen i sina insulinproducerande celler. Vi visar att denna mus har förbättrad frisättning av insulin när den blir gammal samt att den är delvis skyddad mot en viss typ av diabetes. När man ger musen en väldigt fettrik kost får den också ökad insulinfrisättning. Resultaten visar att förhöjda nivåer av CART i insulinproducerande celler gör att dessa fungerar bättre. Därför tror vi att

de ökade CART-nivåerna vid typ 2-diabetes är en skyddsmekanism mot förhöjt blodsocker.

Hos personer med typ 2-diabetes är ämnesomsättningen i fettcellerna ofta förändrad, vilket kan bidra till att förvärra sjukdomen. Vi har i tidigare studier visat att CART påverkar ämnesomsättningen i isolerade fettceller från råttor. I den tredje studien ville vi därför undersöka om vår musmodell hade förändrad ämnesomsättning i fettcellerna.

Vi visar att fettceller från musmodellen har ändrad ämnesomsättning, samt att musen går upp snabbare i vikt när den får en fettrik kost om man jämför med vanliga möss. Detta skulle kunna bero på att de insulinproducerande cellerna frisätter mer CART, vilket gör att de får högre nivåer av CART i blodet. CART kan då transporteras via blodet till fettcellerna och påverka dessa. I så fall skulle CART kunna vara en ny signalbärare mellan de Langerhanska öarna och fettväven. Om detta sker även hos människor är dock för tidigt att säga. Om det visar sig vara så kommer vi att få en ökad förståelse för hur olika organ i kroppen kommunicerar med varandra.

Vi har i denna avhandling alltså visat att hormonet CART påverkar insulin- och glukagonfrisättning samt att CART har en viktig roll i insulinproducerande celler vid utveckling av diabetes. Tillsammans gör dessa egenskaper att jag har stora förhoppningar om att CART-baserade ämnen skulle kunna användas som ett framtida läkemedel mot diabetes.

Introduction

What is the problem?

Diabetes mellitus is a group of diseases with the common manifestation of increased blood glucose levels. Type 2 diabetes (T2D), which accounts for the majority of diabetes cases, is rapidly increasing throughout the world, causing great suffering for patients and a huge economic burden for the society. The major risk factor for T2D is obesity, although family history of diabetes has also been shown to be an important risk factor. In 2014, the prevalence of diabetes was estimated to be 9 % in the adult population [World Health Organization 2014]. The World Health Organization predicts that diabetes will be the 7th leading cause of death in 2030 [Mathers and Loncar 2006]. Therefore, improved diagnostics, new treatment targets and a better understanding of the disease progression is warranted. Blood glucose levels are in the normal state tightly controlled by the two counteracting hormones insulin and glucagon. The increased blood glucose levels in patients with T2D are caused by a combination of reduced insulin sensitivity in insulin target tissues, insufficient insulin secretion and dysregulated glucagon secretion from the endocrine pancreas. Novel agents that target these metabolic disturbances are therefore important in the search of future anti-diabetic medications.

The islets of Langerhans

The pancreas is located in the abdominal cavity (**Fig 1a**) and consists of an exocrine part, producing enzymes for food digestion, and an endocrine part, the islets, producing hormones that regulate blood glucose levels. The pancreatic islets, or islets of Langerhans [Laguesse 1893; Langerhans 1869], consist of five major cell populations; beta cells, alpha cells, delta cells, pancreatic polypeptide (PP)-cells and ghrelin cells, producing the hormones insulin, glucagon, somatostatin, PP, and ghrelin, respectively. The composition of the islets differs between rodents and humans [Steiner, et al. 2010]. Rodent islets consist of approximately 70% beta cells, 20% alpha cells, less than 10% delta cells, less than 5% PP-cells and less than 1% ghrelin cells [Elayat, et al. 1995; Wierup, et al. 2004b]. The human islet contains around 50% beta cells, 35% alpha cells, 10 % delta cells and few PP and ghrelin

cells [Brissova, et al. 2005; Wierup, et al. 2002]. The cytoarchitecture of the islets is also different between species with murine islets having non-beta cells surrounding a beta cell core, whereas in humans beta and non-beta cells are dispersed throughout the islet [Brissova et al. 2005; Cabrera, et al. 2006] (**Fig 1b, c**). The endocrine cells in the islets are well innervated by sympathetic, parasympathetic, and sensory nerve fibres, but also here, differences between human and rodent islets are evident [Ahren, et al. 2006; Rodriguez-Diaz, et al. 2011]. The species differences in islets are most likely a consequence of differences in metabolism and emphasise the importance of studies using human islets.

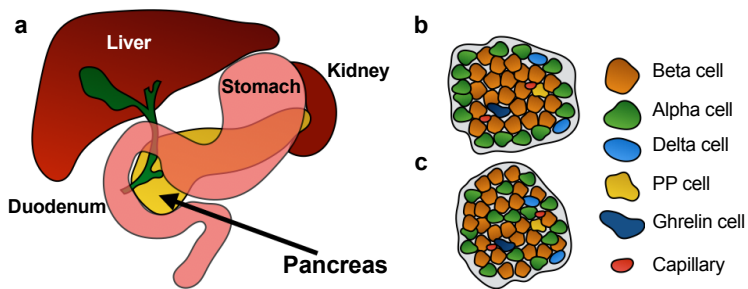


Figure 1. Location of the pancreas in the abdominal cavity (a) and schematic illustration of a mouse islet (b) and a human islet (c). PP: pancreatic polypeptide.

Regulation of blood glucose

Glucose is one of the major energy sources for the cells in the body. Blood glucose levels are therefore tightly controlled, mainly by the two counteracting hormones insulin and glucagon. Blood glucose levels are low just before a meal and increase as glucose, derived from carbohydrates in the food, is absorbed from the intestine. Increased glucose levels trigger release of insulin from the pancreatic islets, which allows glucose to enter the cells, resulting in decreased blood glucose levels. Glucagon levels are, on the other hand, decreased after a meal, which further enhances the postprandial lowering of glucose. Excess glucose is converted into glycogen in the liver and muscles, and to fatty acids in liver; these are then transported to adipocytes and stored as triacylglycerol. After fasting, when blood glucose is low, insulin secretion decreases while glucagon secretion increases. Increased glucagon levels signal to the liver to degrade glycogen into glucose that is secreted into the blood stream, thereby avoiding hypoglycaemia between meals (**Fig 2**). Disturbances in this fine-tuned system may result in deteriorated glucose homeostasis and development of T2D.

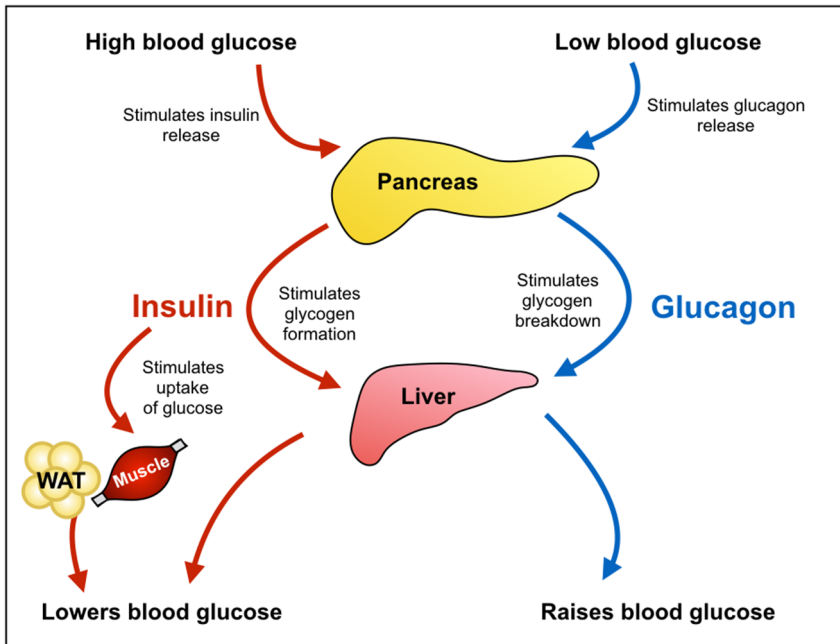


Figure 2. Regulation of blood glucose levels by insulin and glucagon. WAT: white adipose tissue.

Insulin

Glucose-stimulated insulin secretion

Stimulus secretion coupling in pancreatic beta cells is mainly triggered by glucose in a process known as glucose-stimulated insulin secretion (GSIS). After glucose is taken up by the beta cell via glucose transporters (GLUT2 in mouse beta cells and mainly GLUT1 and GLUT3, but also GLUT2 to some extent, in human beta cells [De Vos, et al. 1995; Schuit 1997]) it is rapidly converted to glucose-6-phosphate by glucokinase. This is the first and the rate-limiting step in the glycolysis, which is the process that converts glucose to pyruvate and generates ATP. Pyruvate is then converted into acetyl-CoA, which enters the citric acid cycle (also known as the Krebs cycle or TCA cycle) resulting in the production of the reducing equivalents NADH and FADH₂. The reducing equivalents are re-oxidised in the electron transport chain, generating ATP. The production of ATP from glycolysis and the electron transport chain increases the ATP:ADP ratio, resulting in closure of ATP-sensitive potassium channels (K_{ATP} channels) [Ashcroft, et al. 1984; Rorsman and Trube 1985]. This results in a depolarisation of the plasma membrane and opening

of L-type voltage-dependent calcium (Ca^{2+}) channels (VDCC), leading to Ca^{2+} influx and triggering of exocytosis of insulin granules (**Fig 3**) [Wollheim and Sharp 1981]. There are, however, several electrophysiological differences between mouse and human beta cells, but the main features of glucose sensing are similar [Rorsman and Braun 2013]. This sequence of events, from glucose stimulation via K_{ATP} channels and opening of VDCC to insulin release, is known as the triggering pathway of insulin secretion [Henquin 2000]. In addition, a second pathway, largely provoked by glucose metabolism, the metabolic amplifying pathway, is estimated to account for around 50% of the GSIS [Henquin 2009, 2011]. It was first described as a K_{ATP} channel independent pathway since it was originally identified during pharmacological bypass of K_{ATP} channels in combination with steady high levels of intracellular Ca^{2+} . It was shown that glucose was further able to increase insulin secretion without an additional increase in intracellular Ca^{2+} concentration [Gembal, et al. 1992; Panten, et al. 1988]. The existence of a metabolic amplifying pathway has also been demonstrated in mice lacking functional K_{ATP} channels [Nenquin, et al. 2004; Ravier, et al. 2009]. Thus, glucose controls insulin secretion via dual mechanisms, however, the cellular mechanisms underlying the K_{ATP} -independent pathway are still incompletely understood.

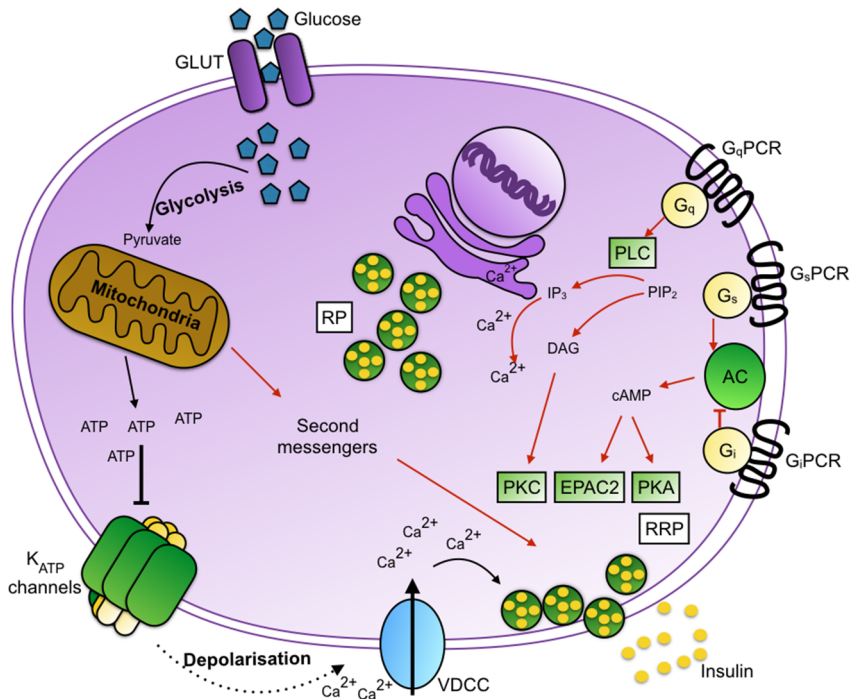


Figure 3. Schematic illustration of insulin secretion from the beta cell. Black arrows indicate the triggering pathway of GSIS. AC: acetylcholine; ATP: adenosine triphosphate; EPAC2: exchange protein directly activated by cAMP 2; GLUT: glucose transporter; GPCR: G-protein coupled receptor; PKA: protein kinase A; RP: releasable pool; RRP: ready releasable pool; VDCC: voltage-gated calcium channels.

Dynamics of insulin secretion

Insulin secretion in response to glucose is biphasic with a rapid first phase lasting 5-10 minutes, suggested to correspond mainly to the triggering pathway, and a second phase, where insulin secretion is sustained; the latter involves both the triggering and the amplifying pathways. The second phase of GSIS in perfused isolated islets differs between species; whereas isolated mouse and human islets display a sustained low suprabasal second phase isolated rat islets, on the other hand, have an increasing second phase that reaches a plateau [Henquin, et al. 2002] (**Fig 4**). There is also an inconsistency between *in vivo* and *in vitro* responses in mice and humans. As mentioned, whereas the second phase of insulin secretion is flat from perfused islets *in vitro*, plasma insulin levels increase during the second phase of insulin secretion during hyperglycaemic clamp in both mice and humans [Henquin et al. 2002]. The discrepancies between species and between *in vitro* and *in vivo* tests are thus important to consider when designing and evaluating secretion experiments.

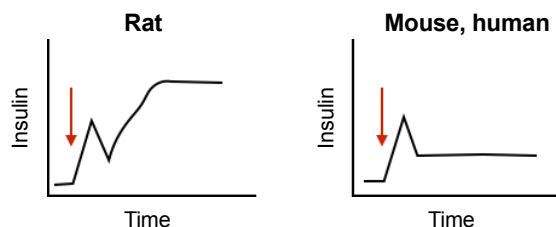


Figure 4. Differences in biphasic insulin secretion between rat vs. mouse and human perfused islets.

The biphasic secretion of insulin can also partly be explained by different pools of insulin granules. To simplify, there are two pools of insulin granules with different cellular location; the ready releasable pool (RRP) with granules that are docked at the plasma membrane and the reserve pool (RP) located in the cytoplasm (**Fig 3**). The RRP only comprises a small fraction, 1-5%, of the granules, whereas the RP makes up the vast majority of the granules. During the first phase of insulin secretion, however, only a small portion of the RRP granules is released. These granules are sometimes referred to as the immediate releasable pool (IRP), which consists of granules that are docked, primed and located near VDCCs. During the second phase, granules in the RRP are primed, and ready to be released in a process that consumes ATP [Rorsman and Renstrom 2003; Straub and Sharp 2002].

In addition to the biphasic response of insulin secretion to glucose, insulin is also released in a pulsatile manner from the pancreas, resulting in oscillatory concentrations of circulating insulin, with pulse intervals of 4-6 min in humans [Porksen, et al. 1997]. This has been shown to maintain insulin sensitivity in healthy subjects [Matthews, et al. 1983]. The pulsatile secretion likely requires a strict coordination among beta cells and islets. It has been suggested that ATP and/or nitric oxide (NO) and carbon monoxide (CO) contributes to the pulsatile insulin secretion from the whole pancreas. Another plausible explanation is that cholinergic innervation from intrapancreatic ganglia, that forms a connected network, synchronises insulin secretion from islets in different parts of the pancreas [Ahren 2000]. Insulin secretion from beta cells within individual islets are synchronised by oscillations in cytoplasmic Ca^{2+} concentrations, which results from spreading of the cell membrane depolarisations via gap junctions [Tengholm and Gylfe 2009].

Insulin secretion from beta cells, islets and the whole pancreas is thus regulated at many different levels with some species differences. Isolated rodent islets are a good tool for studying insulin secretion, however, owing to these differences confirmatory studies in human islets as well as *in vivo* studies are required.

Neuronal and hormonal fine-tuning of insulin secretion

Although glucose is the principal trigger for insulin secretion, other metabolites such as fatty acids and amino acids also regulate insulin release, while yet other mechanisms contribute to the fine-tuning of insulin release. The cytoarchitecture of the pancreatic islets, in combination with the innervation and vascularisation, enables neuronal, hormonal as well as paracrine regulation of insulin secretion. The sympathetic neurotransmitter noradrenaline and its co-transmitters galanin and neuropeptide Y (NPY) released from adrenergic fibres suppress insulin secretion, while the parasympathetic neurotransmitter acetylcholine and its co-transmitters, vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating polypeptide (PACAP) and gastrin-releasing peptide (GRP) released from cholinergic fibres stimulate insulin secretion [Ahren 2000]. In addition, transmitters from sensory nerve fibres, e.g. calcitonin gene-related peptide (CGRP) and substance P (SP) exert a tonic inhibition of insulin secretion [Gram, et al. 2007]. Many transmitters and hormones, including the incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP), acts through G-protein coupled receptors (GPCR). The main G-proteins are inhibitory G-proteins (G_i), G-proteins that stimulate the phospholipase C (PLC) pathway ($G_{o/q}$), and stimulatory G-proteins (G_s). Stimulation through G_s GPCR leads to the activation of adenylate cyclase (AC) and generation of the key second messengers cAMP, which via both protein kinase A (PKA) and EPAC2 acts to enhance insulin secretion (**Fig 3**). Stimulation through G_q GPCR activates PLC that leads to the formation of inositol 1, 4, 5-triphosphate (IP_3) and diacylglycerol. IP_3 promotes Ca^{2+} release from the endoplasmic reticulum and diacylglycerol activates protein kinase C (PKC) thus stimulating insulin secretion. Stimulation through G_i GPCR inhibits AC, thereby reducing the levels of cAMP and inhibiting insulin secretion [Ahren 2009]. Taken together, insulin secretion is a highly regulated process that is fine-tuned by an array of different systems.

Glucagon

Paracrine regulation of glucagon secretion

Glucagon secretion is increased from the islets during fasting conditions when blood glucose levels are low. Glucagon increases glucose output from the liver, thereby balancing blood glucose levels during meals. It has, however, been debated whether glucose concentrations directly regulate glucagon secretion from the alpha cells, or if reduced glucagon secretion at high glucose is a result of paracrine signalling from other islet cells. Both insulin, from the beta cells, and somatostatin, from the delta

cells, inhibit glucagon secretion and their secretion are both stimulated by high glucose. It has also been suggested that other factors released from the beta cell, e.g. gamma aminobutyric acid (GABA) and Zn^{2+} , contribute to suppress glucagon secretion when glucose levels are elevated [Rorsman, et al. 1989; Zhou, et al. 2007]. The paracrine signalling concept is supported by reports showing that isolated alpha cells respond by increasing, rather than decreasing, glucagon secretion at high glucose [Franklin, et al. 2005; Olsen, et al. 2005]. In mouse islets, glucagon secretion is maximally inhibited at 7 mM glucose, a concentration that corresponds to the threshold of insulin secretion. Hence, insulin cannot account solely for the inhibition of glucagon at glucose levels below 7 mM. Somatostatin secretion, on the other hand, increases with glucose concentration and parallels the inhibition of glucagon secretion from 0-7 mM glucose [Vieira, et al. 2007]. Somatostatin signals through somatostatin receptors (SSTRs), of which five isoforms exist (SSTR1-5). SSTR2 mediates the effect of somatostatin in mouse alpha cells and SSTR5 mediates the effect in mouse beta cells. The importance of somatostatin as an inhibitor of glucagon secretion has been reinforced by the fact that SSTR2 knockout (KO) mice display increased potassium/arginine-stimulated glucagon secretion [Strowski, et al. 2000]. However, when using inhibitors against SSTR2, which increased glucagon secretion, glucose stimulation could still reduce glucagon secretion from mouse islets [Vieira et al. 2007]. In human islets, somatostatin reduces both insulin and glucagon secretion via direct effects on exocytosis mediated by SSTR2 in both alpha and beta cells [Kailey, et al. 2012]. Collectively, the available data suggest that both paracrine signalling and intrinsic regulation by glucose is important for the regulation of glucagon secretion from alpha cells.

Intrinsic regulation of glucagon secretion

In addition to the regulatory mechanism reviewed in the previous section, there are reports showing that single alpha cells can respond to glucose by a decreasing intracellular Ca^{2+} concentration and glucagon release [Johansson, et al. 1989; Pipeleers, et al. 1985].

The cellular mechanisms underlying glucagon secretion from alpha cells in response to glucose are similar to the mechanisms regulating insulin secretion from beta cells. Interestingly, whereas an increase in glucose concentration increases secretion from beta cells, the opposite is observed in alpha cells. Similar to beta cells, alpha cells are electrically excitable. Glucagon granules undergo exocytosis as a result of increased intracellular Ca^{2+} . One important difference lies in the contribution of Ca^{2+} influx. In beta cells, L-type VDCCs are most important for controlling Ca^{2+} at high glucose, whereas P/Q-type VDCCs are suggested to be important for evoking glucagon secretion in alpha cells at low glucose [Zhang, et al. 2013]. At high glucose, when ATP production increases, a closure of K_{ATP} channels occurs, as in beta cells. This leads to membrane depolarisation and inactivation of Na^+ channels,

which reduce the activity of P/Q VDCCs and hence decreases glucagon secretion. The importance of P/Q VDCC lies most likely in membrane distribution; they are closely associated with the glucagon granules whereas the L-type channels are not. In addition to K_{ATP} -channel-dependent membrane depolarisation, influx of positively charged ions can contribute to the closure of Na^+ channels. This can be achieved by sodium-glucose co-transporter 2 (SGLT2), expressed in alpha cells, but not in beta cells. SGLT2 transports glucose together with Na^+ into the cell at high glucose concentrations [Bonner, et al. 2015].

There are other studies suggesting that a rise in glucose concentration, which increases cellular metabolism and ATP production, leads to reductions of intracellular Ca^{2+} oscillations and a subsequent reduction of glucagon exocytosis. These studies were, however, performed in isolated alpha cells; other studies using intact islets suggest that Ca^{2+} oscillations continue at high glucose concentrations [Briant, et al. 2016]. The existence of these different models for intrinsic regulation of glucagon secretion emphasises the complexity of this system, as well as the need for further research to fully understand how glucagon secretion is regulated.

White adipose tissue

Function of white adipose tissue

White adipose tissue (WAT) is the main organ for energy storage and plays an important role in control of whole body energy homeostasis. The adipose tissue consists of adipocytes, which store energy as triacylglycerol (TAG), as well as endothelial cells, fibroblasts and macrophages. In the fed, postprandial state, the adipocytes take up fatty acids and glucose from the circulation and store them in large lipid droplets, driven by processes termed esterification and *de novo* lipogenesis. During fasting conditions, the stored TAGs are broken down into free fatty acids (FFAs) and glycerol, which are released into the circulation; a process called lipolysis [Frayn 2010]. Besides its important role in storage of energy, WAT has emerged as an important endocrine tissue, releasing hormones and other molecules, collectively known as adipokines. They can in turn affect the metabolism of other tissues, such as the liver and muscle. Two important and well-studied adipokines are leptin and adiponectin [Scherer, et al. 1995; Zhang, et al. 1994]. Thus, the adipose tissue can regulate whole body energy homeostasis, both via storage and release of energy in form of FFAs, and via its endocrine functions.

Lipolysis

Lipolysis, the process when TAGs are hydrolysed into FFAs and glycerol, is mainly activated by catecholamines, e.g. adrenaline, and negatively regulated by insulin [Lafontan and Langin 2009]. The lipolytic machinery consists of adipocyte triglyceride lipase (ATGL) [Zimmermann, et al. 2004], hormone-sensitive lipase (HSL) [Bjorntorp and Furman 1962] and monoacylglycerol lipase (MGL) [Fredrikson, et al. 1986]. When catecholamines bind to and activate beta-adrenergic receptors, AC is activated and cAMP levels are increased. This, in turn, activates PKA, which has numerous downstream targets. PKA phosphorylates perilipin A [Marcinkiewicz, et al. 2006], a protein that covers and stabilises the lipid droplet [Garcia, et al. 2004], to make the TAGs accessible for degradation by ATGL into diacylglycerol (DAG). PKA also activates HSL [Holm 2003], which catalyses the breakdown of DAG to monoacylglycerol (MAG). The last step in the lipolytic process is the breakdown of MAG, by MGL, into glycerol and FFA. Glycerol and most of the FFAs are released into circulation, but some FFAs are re-esterified into TAGs. During the postprandial state, insulin acts anti-lipolytically via activation of the cAMP hydrolysing enzyme phosphodiesterase 3B (PDE3B) [Degerman, et al. 1998], which is activated by protein kinase B (PKB, also known as Akt). Activation of PDE3B leads to reduced levels of cAMP, which in turn leads to reduced activity of PKA, thus inhibiting lipolysis (**Fig 5**).

Esterification of FFAs and *de novo* lipogenesis

The most important way of storing TAGs is through esterification of FFAs. *De novo* lipogenesis, the process of converting non-lipid metabolites such as glucose into TAGs, is less prominent in humans [Leiter 1982]. Circulating TAGs are hydrolysed by lipoprotein lipase (LPL) into FFAs, which are then taken up by the adipocyte. Inside the cell, FFAs are esterified with glycerol-3-phosphate (G3P), derived from glucose, in a process controlled by acyltransferases, and stored as TAGs. Insulin promotes storage of TAGs by stimulating the production of LPL, as well as the production and activation of acyltransferases. Insulin further promotes the uptake of glucose, via relocation of GLUT4 to the plasma membrane, a process controlled by Akt/PKB. The glucose is mainly utilised for cellular metabolism in the adipocyte, but excess levels of glucose can be stored as TAGs via *de novo* lipogenesis. *De novo* lipogenesis starts by glucose entering into the glycolysis and is converted to pyruvate, which is subsequently converted into acetyl-CoA. Acetyl-CoA is the substrate that generates malonyl-CoA in a process controlled by acetyl-coenzyme A carboxylase (ACC) [Brownsey, et al. 2006]. Palmitate is generated from acetyl-CoA and malonyl-CoA, by fatty acid synthase (FAS), and esterified with G3P, derived from glucose, to generate TAGs. Insulin activates *de novo* lipogenesis through activation of ACC via Akt/PKB. One proposed mechanism of ACC

activation is via inhibition of AMP-activated protein kinase (AMPK), an inhibitor of ACC and *de novo* lipogenesis [Berggreen, et al. 2009] (**Fig 5**).

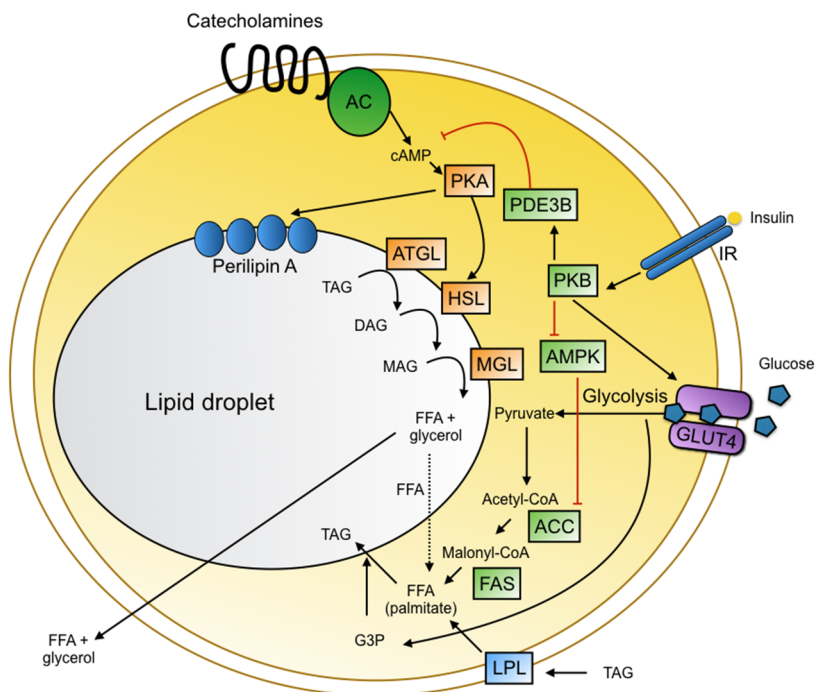


Figure 5. Schematic illustration of an adipocyte describing the main steps of glycolysis, re-estriification and *de novo* lipogenesis and how they are regulated by external stimuli. AC: acetylcholine; ACC: acetyl CoA carboxylase; AMPK: AMP-activated protein kinase; ATGL: adipocyte triglyceride lipase; DAG: diacylglycerol; FAS: fatty acid synthase; FFA: free fatty acid; G3P: glycerol-3-phosphate; GLUT4: glucose transporter 4; HSL: hormone sensitive lipase; IR: insulin receptor; LPL: lipoprotein lipase; MAG: monoacylglycerol; MGL: monoacylglycerol lipase; PDE3B: phosphodiesterase 3B; PKA: protein kinase A; PKB: protein kinase B; TAG: triacylglycerol.

Type 2 Diabetes

Diabetes Mellitus is a set of metabolic diseases with hyperglycaemia as the common denominator. Diabetes has traditionally been divided into type 1 (T1D, also known as insulin-dependent or juvenile diabetes), and type 2 diabetes (T2D, insulin-independent or adult-onset diabetes) [Alberti and Zimmet 1998]. T2D, which accounts for the majority of diabetes cases (~90 %), is increasing rapidly throughout the world. Another variant of diabetes, often misdiagnosed as type 1 or type 2, is latent autoimmune diabetes in the adult (LADA) [Pozzilli and Di Mario 2001;

Tuomi, et al. 1999], Other, less common, variants of diabetes are those caused by single gene mutations named maturity onset diabetes of the young (MODY) [Fajans, et al. 2001], gestational diabetes, and secondary diabetes caused by medication, pancreatitis or pancreatic cancer [Alberti and Zimmet 1998]. This thesis will focus on studies of T2D.

T2D is a complex disease with two key metabolic disturbances: insulin resistance and impaired islet cell function. The disease development of T2D is yet not completely understood, e.g. which of the two metabolic disturbances comes first, a debate that continues to this day [Cerasi 2011]. The time point of T2D diagnosis is often preceded by a long undiagnosed period of asymptomatic glucose intolerance, complicating studies of T2D development. Furthermore, T2D is a multifactorial disease, where genetic, epigenetic and environmental factors contribute to disease progression. The strongest risk factors are overweight and obesity, but smoking, ethnicity, and family history of T2D also impact on the risk of developing T2D [World Health Organization 2016].

Genetics of type 2 diabetes

Family-based and twin studies have clearly demonstrated that heritability for T2D is important [Hemminki, et al. 2010; Medici, et al. 1999]. Today, more than 120 loci have been associated with T2D and diabetes-related traits, but together they still only account for a small portion (10-20%) of the total heritability of T2D [Prasad and Groop 2015]. Most of the genes associated with these loci are related to changes in beta cell function, while a few are associated with decreased insulin sensitivity and obesity. The strongest association with risk of T2D comes from an intronic variant in the *TCF7L2* gene, resulting in increased expression of *TCF7L2* and impaired insulin secretion [Lyssenko, et al. 2007]. Another variant located in an intron of melatonin receptor 1B (*MTNR1B*) gene is associated with impaired insulin release, fasting glucose and T2D risk [Lyssenko, et al. 2009]. It was further shown that human islets from risk G-allele carriers had increased *MTNR1B* expression and that the inhibition of insulin secretion by melatonin was increased by *MTNR1B* overexpression in INS-1 (832/13) cells. In addition, melatonin treatment in a human recall-by-genotype study reduced insulin secretion and raised glucose levels more extensively in risk G-allele carriers [Tuomi, et al. 2016]. These two examples, the variants in the *TCF7L2* and *MTNR1B* gene, clearly show the link between genotype and phenotype, via altered gene expression.

Insulin resistance and adipocyte dysfunction

Insulin sensitivity in peripheral target tissues is crucial for maintaining normal glucose homeostasis. Important and well-studied target tissues for insulin are skeletal muscle, liver and adipose tissue, where insulin binds to the insulin receptor and triggers several different signalling cascades in the cell. The insulin receptor is composed of two extracellular alpha subunits and two transmembrane beta subunits. When insulin binds, this leads to a conformational change resulting in auto-phosphorylation on tyrosine residues on the beta subunit. This enables members of the insulin receptor substrate family (IRS) to bind. The IRS proteins are phosphorylated at tyrosine residues, and can thus be recognised for binding by the lipid kinase phosphatidylinositol-3 (PI3). PI3-kinase phosphorylates the lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) resulting in the formation of the second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP₃). This leads to the recruitment and activation of Akt/PKB [Saltiel and Kahn 2001] (**Fig 6**).

In adipose tissue and muscle, insulin signalling results in relocation of the insulin sensitive glucose transporter 4 (GLUT4) to the plasma membrane, via Akt/PKB phosphorylation of the Rab GTPase-activating protein AS160 [Sano, et al. 2003] (**Fig 6**). In the liver, on the other hand, GLUT4 is not expressed. Hepatocytes express GLUT2, which, in contrast to GLUT4, are constantly located to the plasma membrane and has a lower affinity for glucose, thus enabling facilitated uptake of glucose. In muscle tissue and liver, excess glucose is stored as glycogen that can be used as an energy source when blood glucose levels decrease. Glycogen formation is initiated via insulin signalling, through Akt/PKB phosphorylation and inactivation of glycogen synthase kinase 3 (GSK-3) [Cross, et al. 1995] (**Fig 6**). In adipose tissue, excess glucose is stored as TAGs, via Akt/PKB activation [Saltiel and Kahn 2001]. Up to 75% of insulin-dependent glucose uptake occurs in skeletal muscles, and only a fraction in the adipose tissue [Klip and Paquet 1990]. However, mice with a muscle-specific knockout of the insulin receptor have normal glucose tolerance [Bruning, et al. 1998], whereas mice that lacks the GLUT4 in adipose tissue have impaired glucose tolerance [Abel, et al. 2001].

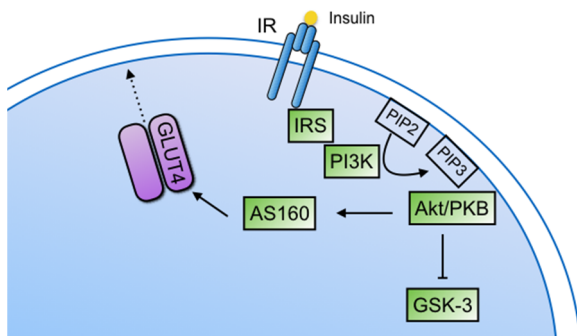


Figure 6. Insulin signalling cascade resulting in translocation of GLUT4 to the plasma membrane and glycogen formation. AS160: Akt substrate of 160; GLUT4: glucose transporter 4; GSK-3: glycogen synthase kinase 3; IR: insulin receptor; IRS: insulin receptor substrate; PI3K: phosphatidylinositol-3 kinase; PIP2: phosphatidylinositol 4,5-bisphosphate; PIP3: phosphatidylinositol 3,4,5-trisphosphate; PKB: protein kinase B (also known as Akt).

When insulin resistance develops in the adipose tissue, insulin is no longer able to inhibit lipolysis and the release of FFAs becomes dysregulated. As a consequence, circulating FFAs are often elevated in patients with insulin resistance and has been suggested to contribute to the insulin resistant state by inhibiting glucose uptake and glycogen synthesis, while increasing hepatic glucose output [Bergman and Ader 2000]. Another feature of dysregulated adipocyte metabolism is an imbalance in adipokines secreted from the adipose tissue that can contribute to the development of insulin resistance and T2D [Arner 2005]. Circulating levels of leptin are often increased in obese patients, which, at the same time, are leptin resistant and thus resistant to its satiating effects [Maffei, et al. 1995]. Obesity, which often precedes T2D, is frequently associated with low grade inflammation, characterised by higher circulating levels of pro-inflammatory cytokines and FFAs; these can interfere with insulin signalling and also induce beta cell dysfunction [van Greevenbroek, et al. 2013]. Increased macrophage infiltration in the adipose tissue is another feature of obesity that can contribute to the pro-inflammatory state in T2D patients [Xu, et al. 2003]. Thus, the adipose tissue is an important regulator of glucose homeostasis and deranged WAT function is a culprit in T2D.

Impaired insulin secretion

The preclinical stage of T2D is characterised by insulin resistance and compensatory hyperinsulinemia. The mechanisms underlying hyperinsulinemia are not completely understood; it most likely arises as a compensation for insulin resistance. It has been observed in humans that beta cell mass was increased in obese compared with lean nondiabetic subjects [Butler, et al. 2003], and obese mice fed a high fat diet displayed increased beta cell mass [Ahren, et al. 2010]. Insufficient insulin secretion

is the final culprit for overt T2D to develop. Insufficient insulin secretion can be caused by either reduced beta cell mass or by decreased function of remaining beta cells. Whereas insulin insufficiency due to beta cell death is well established in T1D, the picture is less clear for T2D. There are studies showing increased beta cell apoptosis and up to a 60% reduction in beta cell mass in patients with T2D [Butler et al. 2003; Rahier, et al. 2008]. It has, however, been suggested that this reduction cannot fully account for the total insulin deficiency [Rahier et al. 2008]. The other aspect, i.e., reduced beta cell function leading to insulin secretory defects, has been observed in T2D patients. Such secretory defects include reduction in oscillations of insulin secretion [O'Meara, et al. 1993], impaired first phase insulin secretion [Fujita, et al. 1975], reduced basal and stimulated plasma insulin concentrations [Temple, et al. 1989], and excess secretion of proinsulin, as a sign of deranged insulin processing [Temple, et al. 1990]. The secretory defect at the islet level has been demonstrated in isolated islets from T2D patients, which display impaired GSIS when comparing with islets from non-diabetic controls [Rosengren, et al. 2012]. It has also been shown that islets from T2D patients display reduced expression of GLUT1, GLUT2 and glucokinase [Del Guerra, et al. 2005] and that T2D islets display reduced oxidative glucose metabolism [Doliba, et al. 2012], indicating a defect that could be linked to the very first steps in GSIS. A recent study comparing islets from normoglycaemic and hyperglycaemic donors using DNA and RNA sequencing, revealed 35 different genetic variants regulating gene expression (eQTL)s, whose gene expression was differentially regulated by hyperglycaemia. Furthermore, silencing of four of these genes (TSPAN33, NT5E, TMED6 and PAK7) in INS-1 (832/13) cells resulted in reduced GSIS. Hence, it was shown that genetic variations can influence gene expression in islets, thereby altering glucose metabolism [Fadista, et al. 2014]. However, the molecular basis for defective insulin secretion in T2D patients is still not fully understood and needs further investigation. Furthermore, studies of interactions between genotype and environmental factors resulting in insulin secretion deficiencies would increase the understanding of the underlying mechanisms that drives the progression towards T2D.

Dysregulated glucagon secretion

In addition to insufficient release of insulin, dysregulated glucagon secretion is an important feature of T2D pathophysiology. The dysregulation is dual with excessive glucagon secreted during hyperglycaemia and insufficient during hypoglycaemia. Recent studies of glucagon receptor deficient mice demonstrate that lack of glucagon signalling protects against streptozotocin-induced diabetes [Lee, et al. 2011]. This illustrates an important role of glucagon in diabetes development, at least in mice. On the same theme, it has recently been suggested that “glucagon excess, rather than insulin deficiency, is the *sine qua non* of diabetes” [Unger and Cherrington 2012]. As much focus in T2D research previously has been devoted to

study insulin secretion, this statement shows that the role of glucagon has become of higher priority.

The inability of T2D patients to suppress glucagon secretion during a glucose challenge has been demonstrated in several *in vivo* studies [Dunning, et al. 2005]. It has also been shown that isolated islets from T2D patients respond to high glucose with increased glucagon secretion whereas islets from healthy donors respond with a decrease [Walker, et al. 2011]. The new diabetes therapies, GLP-1 receptor agonists and dipeptidyl peptidase (DPP)-4 inhibitors, that both augments insulin secretion and reduce glucagon secretion, are proven to be effective in reducing blood glucose, however it is not clear how much of the glucose-lowering effect that is mediated via reduced glucagon secretion [Holst 2007]. Nevertheless, it emphasises the role of the alpha cell as an important drug target, and new drugs targeting the alpha cells specifically could be future therapeutic avenues for T2D.

Treatment of type 2 diabetes

In addition to life style changes, metformin is the first drug of choice for patients with newly diagnosed T2D. Metformin belongs to the group of biguanides, and the primary effect is to lower blood glucose levels by decreasing hepatic glucose production. The exact cellular mechanisms are still not elucidated but the main effects of metformin are believed to be mediated through the activation of AMPK, a master regulator of cellular energy homeostasis [Zhou, et al. 2001]. The most serious side effect of metformin is the risk of lactic acidosis. If metformin alone is not sufficient to reduce blood glucose levels, a two-drug combination with metformin together with one of the following drugs is usually initiated; sulfonylureas, thiazolidinediones, DPP-4 inhibitors, GLP-1 receptor agonists or basal long-acting insulin [Inzucchi, et al. 2012].

Sulfonylureas (SU) are drugs that stimulate endogenous insulin secretion. SU binds to the regulatory subunit SUR1 of K_{ATP} -channels in the beta cells and hinders the efflux of K^+ , which results in increased insulin exocytosis [Schmid-Antomarchi, et al. 1987]. The major side effect of SU is risk of hypoglycaemia. Thiazolidinediones (TZD), or glitazones, activate peroxisome proliferator-activated receptors (PPARs). PPARs are nuclear receptors that mainly regulate adipocyte differentiation, and TZD has greatest specificity for PPAR-gamma receptors. The main output of activating these receptors is increased storage of fatty acids in adipocytes, via accelerated adipocyte FFA uptake and esterification, which leads to decreased levels of free fatty acids in the circulation. This results in increased insulin sensitivity in other tissues [Lehmann, et al. 1995]. The major side effects of TDZ are risk of heart failure, oedema and fractures.

GLP-1 receptor agonists and inhibitors of the protease DPP-4, the enzyme that catalyse the breakdown of GLP-1, are new classes of anti-diabetic agents

successfully introduced 10 years ago. The glucose-lowering effect of GLP-1, or DPP4-inhibitors, is mediated through increased insulin secretion, decreased glucagon secretion, and delayed gastric emptying [Lund, et al. 2014]. Unlike other T2D medications, GLP-1 receptor agonists have been shown to reduce weight, an effect probably mediated through its actions in the hypothalamus and other brain regions involved in food intake regulation [Meier 2012]. An important feature of GLP-1 receptor agonists and DPP4-inhibitors is that the insulinotropic effect is glucose-dependent. Therefore, the risk of hypoglycaemia when using GLP-1 receptor agonists or DPP-4 inhibitors is low, and the main side effects of GLP-1 receptor agonists are gastrointestinal problems and nausea. The latest T2D medication that was developed are the SGLT2-inhibitors. They act by inhibiting the reabsorption of glucose in the kidneys in the collecting ducts and tubules, which results in increased excretion of glucose through the urine, hence lowering blood glucose levels [Boldys and Okopien 2009]. One side effect of the SGLT2-inhibitors is urinary tract infections.

Although there are many available anti-diabetic drugs on the market, there is still a need for new drugs that more efficiently reduce blood glucose levels, without the risk of hypoglycaemia or other serious side-effects. In addition, the ultimate goal for a new drug would be to reduce the occurrence of late diabetes complications, thus prolonging and improving quality of life for diabetes patients.

CART

Cocaine- and amphetamine-regulated transcript (CART) was first discovered as a transcript upregulated in rat striatum in response to administration of cocaine or amphetamine [Douglass, et al. 1995]. Later it became evident that a peptide fragment matching this mRNA had been isolated from ovine hypothalamus already in 1981 [Spiess, et al. 1981]. Since then, CART expression has been reported in many different vertebrate species including humans, rats, mouse, sheep, fish, and pig. CART is expressed in the central [Couceyro, et al. 1997; Koylu, et al. 1998] and peripheral nervous systems [Koylu, et al. 1997], including the enteric nervous system [Couceyro, et al. 1998; Ekblad, et al. 2003; Gunnarsdottir, et al. 2007]. CART is also expressed in endocrine cells in the adrenal medulla [Koylu et al. 1997], pituitary [Koylu et al. 1997], thyroid [Wierup, et al. 2007], as well as in enteroendocrine cells in the gastro intestinal (GI)-tract [Ekblad et al. 2003; Wierup et al. 2007] and in endocrine cells in the pancreas [Jensen, et al. 1999; Wierup, et al. 2006; Wierup, et al. 2004a; Wierup and Sundler 2006].

***CARTPT* gene structure and regulation**

Human CART is encoded by the gene *CARTPT* consisting of three exons and two introns, spanning approximately 2.0 kb [Douglass and Daoud 1996] (**Fig 7**). There are differences in the rat and mouse versus human *CARTPT* gene structure. In rat and mouse, alternative splicing results in two different propeptides: proCART 1-89 and proCART 1-102 [Douglass et al. 1995]. In humans, however, only the shorter variant, proCART 1-89, is found [Douglass and Daoud 1996]. Post-translational tissue-specific processing of pro-CART by prohormone convertases results in two biological active peptides, CART 55-102 and CART 62-102 from the longer variant in rodents and CART 42-89 and CART 49-89 from the shorter variant in humans [Dey, et al. 2003; Thim, et al. 1999] (**Fig 7**). The *CARTPT* gene is conserved across species, with 91% homology between rat and human exons and 98% between mouse and rat. This results in 100% amino acid identity at the carboxy-terminus (exon 3) [Douglass and Daoud 1996]. The conservation of CART across species suggests that CART has important biological functions.

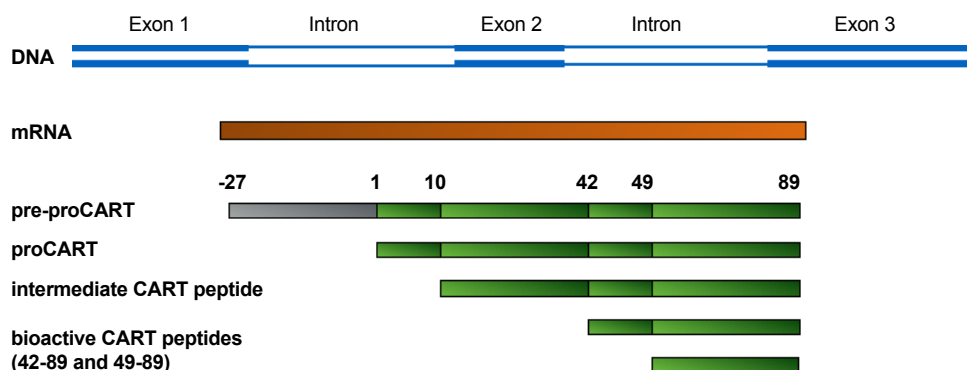


Figure 7. The human *CARTPT* gene and the pre-pro- and propeptides resulting in biological active CART peptides.

A rat pituitary adenoma cell line, GH3, which expresses high levels of CART has been used to study CART gene regulation. In this cell line, *CART* mRNA levels have been shown to increase in response to activation of adenylyl cyclase and PKA [Barrett, et al. 2001; Dominguez, et al. 2002]. The proximal promotor in the *CARTPT* gene contains a cAMP response element (CRE) [Dominguez et al. 2002]. It has been shown that overexpression of CRE-binding proteins (CREB) in the nucleus accumbens increases *CART* mRNA and protein [Rogge, et al. 2009] and that CREB and phospho-CREB directly binds to the CRE-site in the *CARTPT* promotor [Rogge, et al. 2010]. Thus, the available data, demonstrates that CART expression is positively regulated by the cAMP/PKA/CREB-dependent pathway. A negative regulator of *CART* mRNA expression in INS-1 (832/13) cells and PC12 cells is neuron-restrictive silencer factor (NRSF), also known as REST [Li, et al.

2008]. NRSF is expressed during neuronal as well as pancreatic development, but not in mature neurons or islet cells [Martin, et al. 2015]. NRSF binds to a neuron-restrictive silencer element (NRSE) that is conserved in the human and rodent CART promotor [Li et al. 2008].

The adipokine leptin is an important regulator of *CARTPT* gene expression in the central nervous system (CNS). *CART* mRNA levels are reduced in the arcuate nucleus (ARC) of hypothalamus in mice lacking the leptin gene (ob/ob mice), and leptin injections restored CART expression [Kristensen, et al. 1998]. Interestingly, Zucker diabetic fatty (ZDF)-rats that lack the leptin receptor have increased beta cell CART expression [Wierup et al. 2006], suggesting leptin-independent regulation of cart in islets. It has further been shown that acute administration of corticosterone results in increased expression of CART in the nucleus accumbens [Hunter, et al. 2005] and that adrenalectomised rats exhibit decreased levels of *CART* mRNA in the hypothalamic paraventricular (PVN) and ARC, which can be restored by hormone replacement [Balkan, et al. 2003; Vrang, et al. 2003]. Furthermore, subcutaneous injections of estradiol increased CART expression in the frontal cortices in rat [Sarvari, et al. 2010] and prolactin treatment decreased *CART* mRNA levels in INS-1 (832/13) cells [Arumugam, et al. 2007]. Taken together, the expression of CART in different cell lines and tissues is highly dynamic and influenced by many factors, sometimes with diverging results in expression levels. Thus, further studies are needed to fully understand how CART expression is regulated.

CART in CNS and the HPA axis

CART is highly expressed in the hypothalamus [Douglass and Daoud 1996; Gautvik, et al. 1996], especially in the PVN and ARC [Couceyro et al. 1997]. These nuclei are both critical for the regulation of energy homeostasis. Intracerebroventricular (ICV) administration of CART results in increased neuronal activity in the ARC and PVN [Vrang, et al. 1999]. Several studies have shown that ICV administration of CART results in decreased food intake and weight loss [Broberger 2000; Kristensen et al. 1998; Lau and Herzog 2014]. However, other studies using intra-ARC administration or overexpression of CART, have demonstrated orexigenic effects [Abbott, et al. 2001; Smith, et al. 2008]. An important role for CART in the regulation of energy homeostasis in humans is supported by the findings that mutations in the *CARTPT* gene are linked to obesity [del Giudice, et al. 2001; Guerardel, et al. 2005; Rigoli, et al. 2010]. Thus, although CART is often described as an anorexigenic peptide, the available data concerning the role of CART in energy homeostasis are far from consistent.

CART has also been reported to regulate behavioural and physiological responses to stress. In the hypothalamic-pituitary-adrenal (HPA) axis complex routes of direct

influences and feedback mechanisms between three endocrine glands are at hand: the hypothalamus secreting corticotropin releasing hormone (CRH), the anterior pituitary gland secreting adrenocorticotrophic hormone (ACTH), prolactin (PRL) and growth hormone (GH), and the adrenal cortex secreting corticosteroids. This is a central part of the neuroendocrine system that controls reactions to stress and regulates many processes in the body, such as the immune system, digestion, mood and emotions, sexual behaviour, as well as energy storage and expenditure. ICV administration of CART results in increased circulating levels of PRL, GH, ACTH and corticosterone [Stanley, et al. 2001]. Another study confirmed that central administration of CART increased circulating levels of ACTH and corticosterone, whereas intravenous injection of CART did not [Smith, et al. 2004]. CART has also been found to trigger anxiety-like behaviour and to reduce social interaction after central administration in mice [Chaki, et al. 2003], and adolescents carrying a missense mutation in the *CARTPT* gene exhibit increased anxiety and depression [Miraglia del Giudice, et al. 2006].

The initial observation that CART expression was increased by cocaine or amphetamine administration [Douglass et al. 1995] suggested that CART could be involved in the reward and addiction system. Release of dopamine from the ventral tegmental area (VTA) in the midbrain into the nucleus accumbens in the ventral striatum is central in the reward system. CART expression has been described in the nucleus accumbens in rat, and its expression was upregulated by cocaine treatment [Hubert and Kuhar 2008]. Furthermore, and suggesting a role in humans, cocaine abusers showed upregulation of CART in the nucleus accumbens [Albertson, et al. 2004]. It has also been shown that CART expression is increased in the nucleus accumbens by acute intraperitoneal administration of ethanol [Salinas, et al. 2006] and CART KO mice consume less alcohol compared with WT mice [Salinas, et al. 2014]. Taken together, these data imply that CART-containing neurons in the nucleus accumbens are activated by psychostimulants. In addition, a role for CART in promoting natural reward has been suggested: CART ICV injections and injections in the nucleus accumbens increased self-administration of sweet pellets in rats. Furthermore, morphine induced self-administration of sweet pellets was antagonised by CART antibody administration [Upadhyay, et al. 2012]. The positive regulatory role of CART in natural reward is interesting since it might interfere with its anorexigenic properties and could therefore explain some of the diverging results in food intake.

CART in adipose tissue

Expression of CART has been shown in subcutaneous and visceral WAT in rats and humans [Banke, et al. 2013]. Stimulation of primary rat adipocytes with CART potentiated isoprenaline-induced lipolysis and activated HSL. At the same time, CART potentiated the inhibitory effect of insulin on isoprenaline-induced lipolysis.

By inhibiting PKB activation, CART reduced insulin-induced glucose uptake and lipogenesis. Thus, the effect of CART on adipocytes seems to be dependent on the surrounding conditions, and CART can act either to promote or to reduce the effect of insulin [Banke et al. 2013]. This study examined only the acute effect of CART *in vitro*, and the impact of chronically elevated CART levels on adipocyte metabolism *in vivo* remains unknown.

CART in the islets

The expression of CART in islet cells and in nerves fibres innervating the islets has been studied in pigs, rats, and mice. In normal adult rats, CART expression is confined to delta cells, pancreatic sensory and parasympathetic nerve fibres emanating from pancreatic ganglia [Jensen et al. 1999; Wierup et al. 2004a]. However, during development CART is highly expressed also in alpha, beta, and PP-cells, but not in ghrelin cells [Wierup et al. 2004a]. In adult mice, CART is highly expressed in parasympathetic fibres and in a subpopulation of sensory pancreatic nerve fibres [Gilon 2016; Wierup, et al. 2005]. During development CART is upregulated in sub-population of the alpha cells [Wierup and Sundler 2006]. Islet cells in the pancreas of adult pigs are devoid of CART, however, CART expression was found in parasympathetic nerve fibres innervating the islets [Wierup et al. 2007].

Interestingly, CART expression was found to be upregulated in beta cells of three rat models of type 2 diabetes: GK-rats, dexamethasone (DEX)-treated rats and ZDF-rats [Wierup et al. 2006]. In DEX-treated rats, islet CART mRNA expression, and the number of CART-immunoreactive beta cells were 10-fold higher compared with control rats. The upregulation of CART was reversible and reverted back to normal levels after termination of DEX treatment. In GK rats, the relative number of CART-expressing beta cells was 30-fold higher than in Wistar control rats [Wierup et al. 2006]. CART has also been shown to be expressed in INS-1 (832/13) cells and the expression levels of CART was regulated by glucose; treatment with high glucose reduced CART expression at both mRNA and protein level [Sathanoori, et al. 2013]. The regulation of CART by diabetes in three different rat models together with the expression changes during development suggest that CART expression is highly dynamic, especially considering the return to normal CART levels after termination of treatment in DEX-rats. The collective data also imply that CART expression could be of importance for beta cell function.

The effect of CART on islet hormone secretion has been studied in INS-1 (832/13) cells and rat islets [Colombo, et al. 2003; Wierup et al. 2006]. In INS-1 (832/13) cells, at high glucose, CART potentiates cAMP-enhanced insulin secretion induced by isobutylmethylxanthine (IBMX), forskolin or GLP-1, but is without effect in the absence of cAMP-elevating agents. CART increases cAMP levels in INS-1 (832/13)

cells and the effect of CART on GLP-1-enhanced insulin secretion is abolished by the PKA inhibitor H89 [Wierup et al. 2006]. In one study, CART was without effect on insulin secretion from rat islets [Colombo et al. 2003]. However, our group has shown that CART decreases insulin secretion induced by glucose alone, while increasing GLP-1-enhanced insulin secretion in rat islets. Furthermore, somatostatin and glucagon secretion from rat islets is reduced by CART treatment at high glucose [Gilon 2016; Wierup et al. 2006]. Despite evidence for a role of CART as regulator of islet hormone secretion *in vitro* in rodents it is not known how administration of exogenous CART affects islet hormone secretion *in vivo* and, more importantly, the effect of CART in human islets remains unknown.

CART receptor and CART KO mice

More than 20 years after the discovery of CART and despite evidence for many biological functions, the receptor for CART has not yet been identified. Specific binding for various CART peptides has been reported. *In vitro* studies suggest that CART acts via yet unidentified GPCR. There are also indications that several CART receptors exist [Vicentic, et al. 2006; Zhang, et al. 2012b]. There is evidence suggesting that CART peptides may act via a G_i GPCR since voltage gated calcium signalling is reduced in rat hippocampal neurons [Yermolaieva, et al. 2001] and the activation of CART peptides on ERK activation is blocked by pertussis toxin in a pituitary-derived cell line (AtT20 cells) [Lakatos, et al. 2005]. However, the activation of ERK by CART seems to be tissue- and cell-specific, which further supports the idea of several CART receptors [Lakatos et al. 2005]. In beta cells, CART has been shown to induce phosphorylation of CREB, insulin receptor substrate (IRS), PKB, and mitogen-activated protein kinases (MAPK) as well as to increase the levels of cyclic AMP, which indicates that CART signals through a G_s GPCR [Sathanoori et al. 2013]. Our lab has recently identified a putative CART receptor, but further studies remains before we have definitive evidence.

Although the CART receptor is not yet identified, CART KO mice have enabled studies of the impact and function of CART *in vivo*. Two different CART KO strains have been generated. The first strain had two exons removed and was backcrossed on a C57Bl/6J background [Asnicar, et al. 2001]. The second strain, studied in our lab, had all three exons removed and was maintained on an outbred, black swiss x 129SvJ background [Wierup et al. 2005]. Both strains were reported to have no gross anatomical differences and to be healthy into adulthood. In the first strain CART KO mice displayed increased food intake and gained more weight when fed a high fat diet. However, in this study no effect was observed on body weight in WT mice after 14 weeks of high fat diet compared with control diet [Asnicar et al. 2001]. A later study using the same strain, showed differences in body weight after 20 weeks of age on regular diet [Moffett, et al. 2006]. The second strain was reported to gain more weight on regular diet after 40 weeks of age [Wierup et al. 2005]. The

second strain was also characterised with respect to islet function, revealing that CART KO mice displayed impaired GSIS both *in vivo* and *in vitro* in isolated islets. *In vivo*, the impairment in first-phase insulin secretion was associated with reduced glucose elimination, and was apparent both in young mice (with no difference in body weight) as well as in old mice. This study also revealed that CART KO mice had reduced expression of GLUT-2 and PDX-1 in beta cells [Wierup et al. 2005]. Thus, the available data obtained in CART KO mice suggests that CART is necessary for maintaining normal islet function.

Aims of this thesis

- I. To examine CART expression in human islet cells and assess whether CART expression is altered in T2D or by glucose and/or insulin therapy in mouse, rat and/or human beta and alpha cells (Paper I).
- II. To study the effect of CART on insulin secretion *in vitro* in mouse islets, *in vivo* in mice, and *in vitro* in human islets (Paper I).
- III. To study the effect of CART on glucagon secretion *in vitro* in mouse islets, *in vivo* in mice, and *in vitro* in human islets (Paper I).
- IV. To study how overexpression of CART in beta cells affects insulin secretion and glucose homeostasis (Paper II).
- V. To study how overexpression of CART in beta cells affects insulin sensitivity and adipocyte metabolism (Paper III).

Experimental methods

In this chapter I will briefly discuss the main methods and models that I have used to generate data for the papers included in this thesis. Details about methods used by myself and co-workers can also be found in the original papers.

Animal models in research

The earliest references to animal research can be found in writings from the Greeks in the 2nd and 4th centuries BCE. The use of animals in research has been essential for the development of modern medicine. A broad variety of model organisms, such as *Drosophila melanogaster*, *Xenopus*, mouse, rat, pig, dog, and nonhuman primate is used in medical research to study physiological and pathophysiological events. Although an extensive amount of research has been devoted to develop alternatives to animal models, there is still a need for medical research to be performed in living organisms. In order to decrease the amount of animals used for research, the three Rs (replacement, reduction, refinement), described already in 1959 [Russell and Burch 1959], are used as guiding principles for improved animals ethics in research. *Replacement* refers to the use of non-animal models, such as cell systems, whenever possible. This could be very useful when studying cell signalling in a particular cell, e.g. the use of INS-1 (832/13) cells for the study of beta cells. The cellular response to different stimuli, however, often differ substantially between differentiated cell lines and primary cells or to the *in vivo* situation and therefore requires the use of confirmatory studies in animals. *Reduction* refers to methods that use the minimum amount of animals sufficient to answer a specific scientific question. This is always a fine balance, since an experimental design with few animals might result in low statistical power and, hence, cannot be properly evaluated. *Refinement* refers to development of techniques to reduce the suffering for the animals used for research.

Mouse models of diabetes

The C57Bl/6J mouse

The C57Bl/6J mouse is a widely used inbred laboratory mouse in metabolic research. They are easy to breed, they live long and are prone to develop diet-induced insulin resistance, all of which are features that make them suitable for diabetes research. C57Bl/6J mice can be metabolically challenged by several methods and protocols. In this thesis, I have used two different models: high fat diet (HFD) feeding and streptozotocin (STZ)-induced diabetes.

The high fat diet fed mouse

Feeding C57Bl/6J mice a HFD results in marked obesity, stable hyperglycaemia, progressively increasing hyperinsulinemia, insulin resistance and glucose intolerance [Surwit, et al. 1988; Winzell and Ahren 2004]. These are all metabolic features of pre-diabetes, which makes the HFD fed mouse a good model for the study of impaired glucose tolerance and early type 2 diabetes. Increased weight gain, hyperglycaemia and reduced glucose tolerance are apparent already after one week on HFD and is evident up to at least one year [Winzell and Ahren 2004]. Increased beta cell mass compared with mice fed control diet is manifested after 3 months, and evident up to at least 12 months [Ahren et al. 2010]. In contrast to chemically induced diabetes, there are less problems with adverse effects, such as toxicity in organs other than the islets. One disadvantage of the HFD model is the high variability in development of obesity between individual mice [Zhang, et al. 2012a].

The streptozotocin mouse

STZ is an antibiotic shown to have diabetogenic effects in dogs and rats already in 1963 [Rakieten, et al. 1963]. STZ is taken up by GLUT2 in beta cells and exerts its cytotoxic effects by inducing DNA damage [Szkudelski 2001]. I have used a multiple low dose streptozotocin protocol, which leaves the mice with some residual insulin secretion capacity. The advantages of this model include selective partial loss of beta cells, and residual insulin secretion enabling the animals to survive without insulin therapy [Like and Rossini 1976]. In contrast, a high single dose of STZ results in total beta cell death and severe hyperglycaemia leading to death unless insulin therapy is given [Deeds, et al. 2011]. Other advantages with the STZ models include diabetes and hyperglycaemia being rapidly and easily induced. Hyperglycaemia in STZ-mice is a result of diminished insulin secretion due to beta cell death and not of altered insulin sensitivity. Therefore, the STZ-model may not

be suitable for the study of T2D but could be useful for the study of beta cell survival and T1D. Also here, great variability in the susceptibility to STZ and hence the efficacy and resulting residual insulin secretion could be a problem [Deeds et al. 2011]. I have used male mice in our STZ-studies since they are more susceptible to STZ-induced diabetes compared with female mice [Deeds et al. 2011; Egerod, et al. 2011].

In vivo glucose and insulin tolerance tests

I have studied the effect of exogenous CART on insulin and glucagon secretion *in vivo* in female C57Bl/6J mice. The reason for choosing female mice is mainly because they are less aggressive and therefore easier to handle [Van Loo, et al. 2003]. To enable tolerance tests with tight intervals of blood sampling in many mice simultaneously, we used anaesthetised mice (fentanyl/fluanisone; Hypnorm and midazolam; Dormicum). Mice were fasted 1-4 h before starting the tolerance test to achieve uniform basal blood values, but without inducing starvation. We have evaluated different fasting times for the intravenous glucose tolerance test (IVGTT) and concluded that 1 h fasting yielded the same results in insulin response as 4 h fasting [Fischer, et al. 2014]. I performed IVGTT with or without additional secretagogues to assess the direct effect on islets and hormone secretion without influence from e.g. incretin hormones. To study absorption of glucose from the intestine and the contribution of incretins to insulin secretion, I performed oral glucose tolerance test (OGTT). In addition to study the effect of CART in C57Bl/6J mice, I have also studied insulin and glucagon secretion in mice with beta cell overexpression of CART (CARTtg) mice generated in our lab.

Mouse islet isolation and hormone secretion

To study the direct effect on hormone secretion by glucose or other secretagogues (e.g. CART) pancreatic islets from mice were isolated. The islet yield when isolating islets from one mouse is on average between 200-300 islets [Li, et al. 2009]. This enables studies with many different conditions, e.g. addition of multiple different insulin secretagogues in the same experiment. I cultured the islets overnight, before starting the secretion experiments, in order to allow the islets to recover from the insult of collagenase digestion. Overnight culture, however, could alter gene expression and islet function [Carter, et al. 2009]. For the experiments in this thesis, I have used 1 h static incubations of the islets. Other alternatives could be to stimulate for 15 min to study first phase insulin secretion, or to perform perfusion of islets to study the dynamics of GSIS. For the papers included in this thesis, I have

used mouse islets to study hormone secretion from CARTtg mouse and to study the effect of CART on insulin secretion from C57Bl/6J mouse islets.

Human islets

Mouse islets are, thanks to its availability and low degree of variability, the first option to use when studying islet function and secretion. However, for research questions concerning human islet hormone secretion, human islets are the golden standard. At Lund University Diabetes Centre, we have the privilege of having frequent access to islets from human cadaver donors from the Nordic Network for Clinical Islet Transplantation, co-ordinated by Uppsala University. As mentioned in the background, there are many similarities as well as differences between mouse and human islets. An important difference, likely to have implications for function of the islets, is islet cytoarchitecture (**Fig 1**) [Brissova et al. 2005]. Therefore, studies in human islets are necessary to understand human islet biology. Some of the limitations using human islets are related to the high degree of variability between islets preparations from different donors as opposed to islets from inbred mice. Age, gender, health status, and BMI are examples of variables differing between donors. The purity of the islet preparation and how many days they have been in culture could also affect the parameters studied. I have used human islets to study the effect of CART on insulin and glucagon secretion.

INS-1 (832/13) cells

The clonal rat insulinoma cell line INS-1 displays many important characteristics of the pancreatic beta cell, including a high insulin content and responsiveness to physiological concentrations of glucose [Asfari, et al. 1992]. The original cell line created in 1992, was later stably transfected with the human proinsulin gene, followed by selection of clones based on glucose-responsiveness. One of the most responsive clones was INS-1 (832/13), which I have used for my studies [Hohmeier, et al. 2000]. An advantage of using INS-1(832/13) cells is that gene targeting using siRNA or viral techniques have a higher success rate compared with islets. Another advantage is that, since it is a pure beta cell system, a direct effect on beta cells, without influence from other cell types, can be studied. I have used INS-1 (832/13) cells to study the impact of adenoviral overexpression of CART on insulin secretion.

Immunohistochemistry

Immunohistochemistry enables studies of protein expression from tissue level, where protein expression in different cell types can be assessed, down to subcellular level to assess cellular localisation of a protein. This is in contrast to Western blot, which is limited to protein expression in tissue homogenates and does not allow for assessment of cellular localisation, unless subcellular homogenates are prepared. The specificity, and hence the reliability, of immunohistochemistry is dependent on two criteria: the specificity of the primary antibody and specificity of the protocol used [Burry 2000]. Ways of ensuring antibody specificity include pre-absorption with recombinant protein, testing the antibody in tissue from knockout animals or in cells where the gene is silenced, and testing the antibody with Western blot to confirm that the antibody binds to a protein of correct molecular weight. The specificity of an antibody can also be confirmed by performing *in situ* hybridisation against the corresponding mRNA, in parallel with immunohistochemistry. Ways to ensure specificity of the method include use of negative control (without primary antibody and reaction in tissues known to lack the protein of interest) and using a positive control (with tissue where the protein is known to be highly expressed). For the work in this thesis I have used immunohistochemistry to study islet CART expression and to measure beta cell mass.

Main findings

Aim I: CART expression in islet cells

- CART is expressed in human alpha and beta cells.
- T2D patients have increased CART expression in alpha and beta cells.
- CART expression is increased in beta cells in three mouse models of T2D.
- CART expression is regulated by glucose in rat islets and is normalised by insulin therapy in DEX-rats.

Aim II: The effect of CART on insulin secretion

- CART increases glucose-stimulated insulin secretion from mouse and human islets.
- CART increases insulin secretion and improves glucose homeostasis *in vivo* in mice.
- CART augments mouse beta cell exocytosis.
- CART promotes slow wave intracellular beta cell Ca^{2+} oscillations and synchronises Ca^{2+} oscillations within mouse islets.

Aim III: The effect of CART on glucagon secretion

- CART decreases glucagon secretion from mouse and human islets.
- CART decreases glucagon secretion *in vivo* in mice.
- CART attenuates mouse alpha cell exocytosis.

Aim IV: The effect of overexpressed beta cell CART

- Compared with WT mice, CARTtg mice have improved glucose control and higher residual insulin secreting capacity after STZ-treatment.
- Old CARTtg mice have improved insulin secretion and glucose homeostasis.
- CARTtg mice have improved insulin secretion after a HFD but are also more insulin resistant.
- Overexpression of CART in INS-1 (832/13) cells results in increased insulin secretion.

Aim V: The effect on insulin sensitivity and adipocyte metabolism in mice with overexpressed beta cell CART

- CARTtg mice have increased rate of weight gain and adiposity when fed a HFD.
- CARTtg mice have altered adipocyte metabolism and increased liver triglycerides.

Results and discussion

Aim I: CART expression in islet cells

The expression of CART in islet cells has been studied in mice, rats and pigs [Wierup et al. 2007; Wierup et al. 2004a; Wierup et al. 2005; Wierup and Sundler 2006], but previously not in humans. Therefore, our first aim was to assess whether CART is expressed in human islet cells. By use of immunohistochemistry, we showed that CART was expressed in a substantial subpopulation of both alpha and beta cells (**Fig 8a**), as well as in nerve fibres innervating the islets (unpublished observations). CART expression in human islets was confirmed by *in situ* hybridisation (**Fig 8d**), qPCR and Western blot. Thus, the localisation of CART in humans differed from that of the adult rat, where CART is expressed in a subpopulation of delta cells [Wierup et al. 2004a], and the mouse where CART is expressed in a small subpopulation of beta cells. Importantly, we could also confirm that it was the biologically active form CART 55-102 that is expressed in human islets. Based on previous findings of upregulated CART expression in beta cells in three diabetic rat models, we examined whether CART was differentially regulated by T2D also in humans. *CART* mRNA expression was three times higher in islets from T2D patients (**Fig 8e**). In addition, T2D patients had three- and two-fold more CART positive beta- and alpha cells compared with controls (**Fig 8b, c**).

Differential expression of *CARTPT* mRNA in T2D islets compared with control islets was first confirmed with microarray (performed by the Human Tissue Lab at LUDC) from the first 63 donors analysed. However, when microarray data from the next batch of donors were merged with the first data set, resulting in a total of 130 donors, the effect of T2D on *CARTPT* mRNA expression was no longer statistically significant. A potential explanation for this could be batch effects, since the islets were analysed at different time points and using different protocols. Expression data from RNA sequencing of human islets at LUDC could, however, not confirm any correlation between *CARTPT* expression and HbA1c, but there was a trend ($p=0.052$) towards decreased *CARTPT* expression in islets from T2D patients. The diverging results from microarray and RNA sequencing compared with our published results might be explained by the variation between the donors in terms of e.g. gender, culture days, and handling. Another important aspect is that the islets analysed by microarray and RNA sequencing were not picked, hence the resulting mRNA expression emanates from a mix of islet cells and exocrine cells.

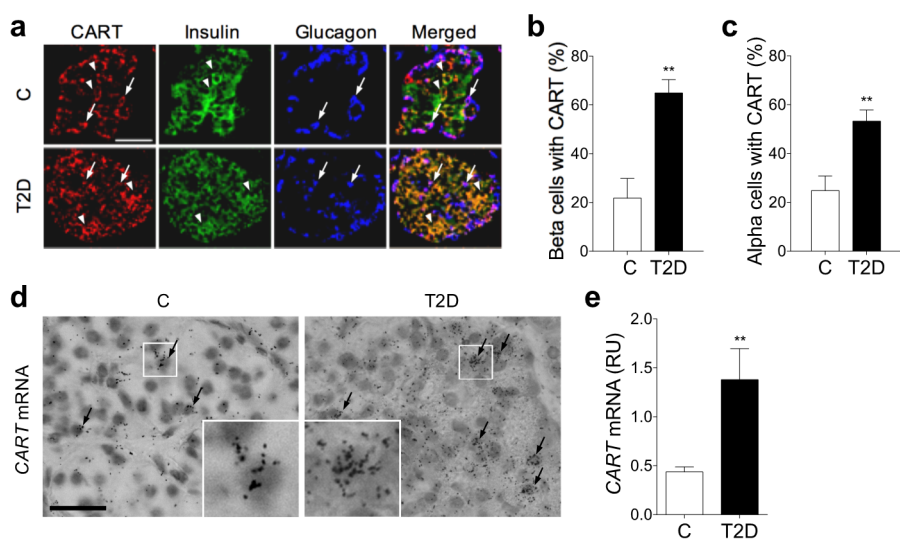


Figure 8. CART protein (a-c) and mRNA (d, e) are expressed in human alpha and beta cells and upregulated in T2D patients (a-e).

Our findings that CART was upregulated in beta cells of three different diabetic mouse models (**Fig 9**), but not in alpha cells, further strengthen the notion of a role for CART in beta cell function and T2D pathogenesis. It also emphasises the differential expression pattern of regulatory peptides between species.

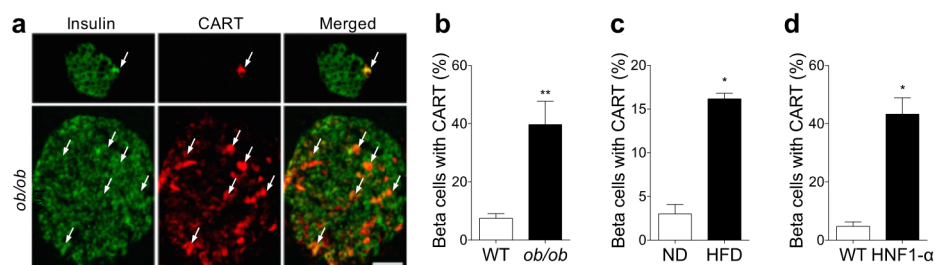


Figure 9. CART is upregulated in beta cells of ob/ob mice (a, b), HFD fed mice (c) and HNF1-alpha mice.

Based on our findings that *Cart* mRNA expression was increased in rat islets cultured in high glucose (**Fig 10a**) and that insulin therapy of DEX-rats normalises beta cell CART expression (**Fig 10b-d**), I believe that the upregulation of CART is a compensatory mechanism trying to protect the beta cells against hyperglycaemia. In fact, it has already been shown that exogenous CART protects against glucotoxicity in primary rat beta cells and INS-1 (832/13) cells [Sathanoori et al. 2013], and that CART has neuroprotective effects [Bharne, et al. 2013; Mao, et al.

2007; Wu, et al. 2006]. In contrast to isolated rat islets, CART expression in INS-1 (832/13) cells is reduced after culture in high glucose [Sathanoori et al. 2013]. Together, this suggest that islet cell CART expression is highly dynamic and species-specific, both during development and during diabetes development. It should also be kept in mind that the influence of other islet cell types is absent in a culture of clonal beta cells. Moreover, a diabetic milieu is complex and not limited to hyperglycaemia. Although well established in rats and mice, the expression pattern of CART in human islet cells during foetal development is still not examined, and could be a key to understand the dynamics of CART expression in humans.

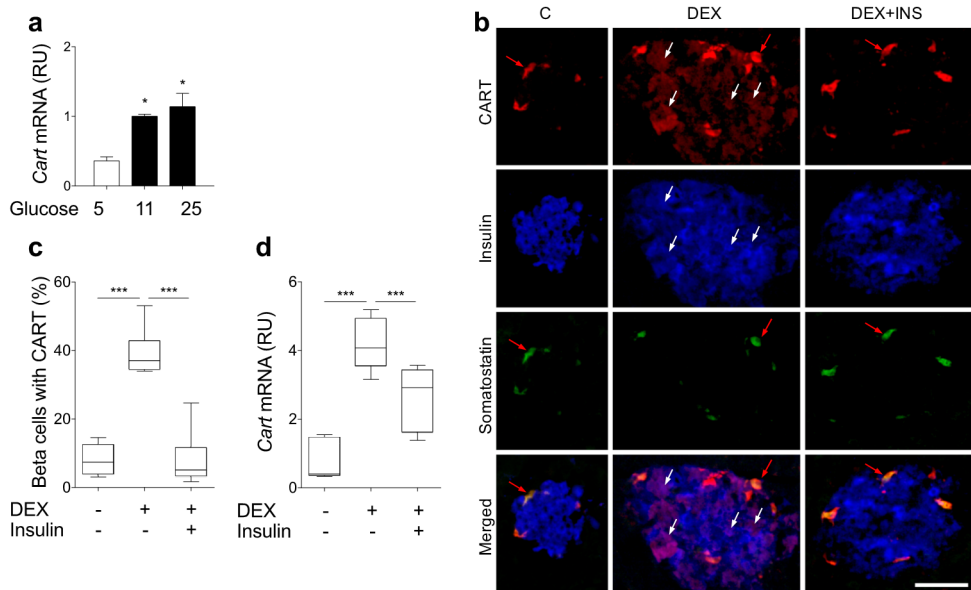


Figure 10. CART is upregulated rat islets cultured in high glucose (a) and CART expression is upregulated in beta cells of DEX-treated rats but is normalised by insulin therapy (b-d).

The fact that CART KO mice are viable and healthy into adulthood shows that CART is not necessary for survival in mice. However, CART KO mice exhibited reduced insulin secretion, deranged glucose tolerance and reduced beta cell expression of GLUT2 and PDX1 [Wierup et al. 2005], demonstrating that CART is necessary for proper beta cell function. A missense mutation in the *CARTPT* gene resulting in an amino acid substitution has been linked to obesity in humans [del Giudice et al. 2001; Yanik, et al. 2006], but not to insulin secretion or T2D. To test if genetic variants in or in near proximity to the *CARTPT* gene associates with diabetes risk or metabolic traits, we genotyped single nucleotide polymorphisms (SNPs) covering +/- 50 kb in the *CARTPT* gene in the Malmö Preventive Project

(MPP) with 17,491 subjects (2,212 of whom developed T2D) and in the population-based Prevalence Prediction and Prevention (PPP)-Botnia study with 4,641 non-diabetic participants. We found two SNPs to be linked to obesity, and one of them was found to affect insulin and glucagon secretion in the PPP-Botnia study. This variant, rs770310, was further evaluated: while there was no significant effect of the SNP on risk of T2D in the whole MPP cohort, there was an interaction between rs770310 and BMI for the risk of T2D, as manifested by a stepwise increased risk of developing future T2D with increasing BMI. This result could, however, not be replicated in larger cohorts, hence it still remains to be elucidated whether *CARTPT* genetic variants could influence the risk of T2D.

Aim II: The effect of CART on insulin secretion

CART has previously been shown to affect insulin secretion in pancreatic rat islets and INS-1 (832/13) cells in one study (Wierup et al., 2006), whereas another study reported no effect of CART in rat islets [Colombo et al. 2003]. We now aimed to examine whether CART affects insulin secretion in human and mouse islets. We found that CART increased insulin secretion from human islets at high glucose (16.7 mM) and that there was a trend towards increased insulin secretion at intermediate (8.3 mM), but not at low glucose (2.8 mM) (**Fig 11a**). CART has been shown to increase insulin secretion in diabetic (GK) rat islets [Wierup et al. 2006] and importantly, as a proof-of-concept, we demonstrated an insulinitropic effect of CART also in islets from T2D patients (**Fig 11b**). Interestingly, we observed a gender-dependent effect of CART on insulin secretion; islets from female donors had much stronger response to CART stimulation compared with islets from male donors (unpublished observations). The gender-dependent observation is supported by reports showing that CART has gender-specific expression and effects in the CNS [Kappeler, et al. 2006]. On the other hand, other effects in the CNS has been shown to be gender neutral [Job, et al. 2014]. We next investigated the effect of CART on insulin secretion in mouse islets, and found that CART exerted glucose-dependent stimulatory effects on insulin secretion also in mouse islets (**Fig 11c**). We also assessed the effect of CART in combination with the key insulin secretagogues potassium, carbachol, and alpha-KIC. Potassium depolarises the plasma membrane, thus opening VDCCs to increase intracellular Ca^{2+} , the cholinergic agonist carbachol binds to and activates acetylcholine receptors, and alpha-KIC enters the Krebs cycle to increase the ATP:ADP ratio. This revealed that CART was unable to further increase insulin secretion stimulated by these secretagogues. However, CART potentiated GLP-1 enhanced insulin secretion. Since GLP-1 increases the levels of cAMP, the results from mouse islets agrees with previous results with INS-1 (832/13) cells and rat islets where CART potentiated

cAMP-enhanced (by forskolin, IBMX and GLP-1) insulin secretion [Wierup et al. 2006].

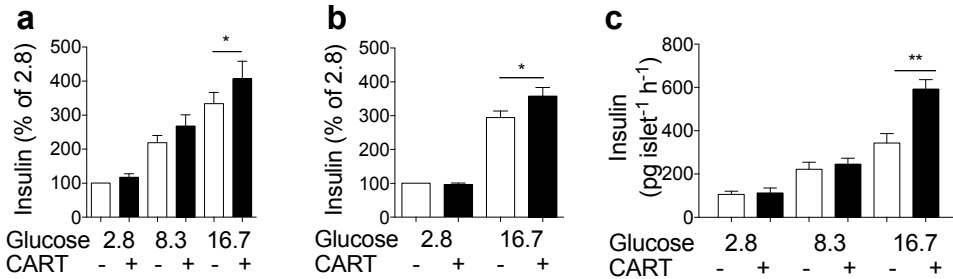


Figure 11. CART increases insulin secretion in a glucose-dependent fashion in human islets (a) and in islets from donors with T2D (b). CART increases insulin secretion from mouse islets (c).

Having established that CART increases insulin secretion in mouse islets, we next performed an intravenous glucose tolerance test (IVGTT) with or without intravenous injections of CART and/or GLP-1 in mice to assess the effect of CART on insulin secretion *in vivo*. This revealed that CART increased the acute insulin response (AIR) two-fold (**Fig 12a, b**) and improved the glucose elimination rate (**Fig 12c, d**). Also *in vivo*, CART further potentiated GLP-1-enhanced insulin secretion.

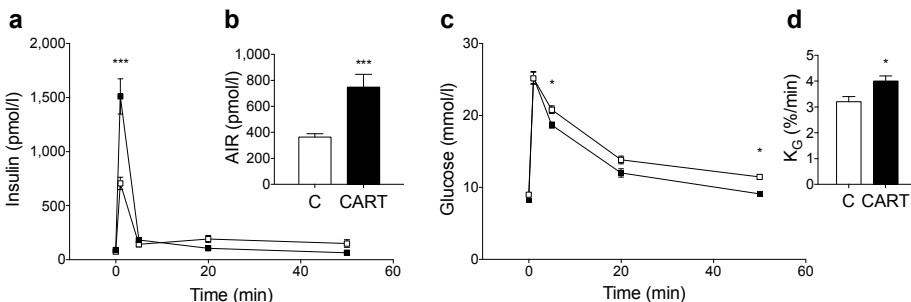


Figure 12. CART increases insulin secretion (a, b) and improves glucose elimination (c, d) during an IVGTT in mice.

Based on these and previously published data we conclude that CART improves GSIS from human and mouse islets, but has no stimulatory effect alone on GSIS from rat islets and INS-1 (832/13) cells, where the effect was evident only during cAMP-enhanced insulin secretion. The differences may be linked to species differences in second phase insulin secretion, since isolated mouse and human islets display a sustained low suprabasal second phase and isolated rat islets have an increasing second phase that reaches a plateau (**Fig 4**) [Henquin et al. 2002]. Our

data suggest that CART mainly affects first phase insulin secretion, and since we have measured insulin secretion during 1 h static incubations, this effect could have been “diluted” in rat islets that secrete more insulin during the second phase. The *in vivo* data also showed an effect of CART primarily on the first phase of insulin secretion, and unpublished data from our lab where CART is silenced in INS-1 (832/13) cells further support this (Shcherbina, Abels et al. submitted manuscript). Studies with perfused islets or shorter static incubations are needed to answer how CART affects the dynamics of GSIS.

Since the insulinotropic effect of CART *in vivo* and *in vitro*, we next wanted to assess if CART directly affects beta cell exocytosis in intact mouse islets. Exocytosis was monitored as increases in membrane capacitance using the whole-cell patch-clamp technique; exocytosis was evoked by trains of ten 500-ms depolarisations from -70 to 0 mV. There was a trend towards an increase in beta cell exocytosis after 1 h CART treatment, and 24 h treatment with CART augmented the exocytotic responses in beta cells (**Fig 13a-d**). Since CART increased beta cell exocytosis after 24 h, we performed static incubations of mouse islets after 24h CART treatment. We could then confirm that long-term treatment of CART increased insulin secretion at 16.7 mM glucose (**Fig 13e**). Increased insulin secretion after long-term treatment suggests that CART may alter the expression of genes important for beta cell function. There are only a few studies reporting changes in gene expression after CART treatment [Lv, et al. 2009] and most studies instead focus on short-term effects, such as protein phosphorylation. Interestingly therefore, we have unpublished evidence for that silencing of CART in INS-1 (832/13) cells downregulates the expression of genes important for beta cell function, including *Pdx1* and *MafA*.

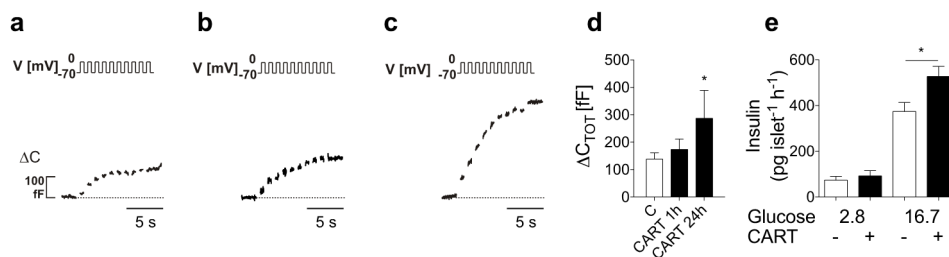


Figure 13. Effects of CART on single cell exocytosis in mouse islet cells. Representative cell recordings in control beta cells (a), beta cells treated with 10 nmol/l CART for 1 h (b), and for 24 h (c). Average total increase in capacitance evoked by the trains (ΔC_{TOT}) (d). Static incubation with mouse islets cultured 24 h with CART (e).

To further understand the insulinotropic effects of CART we measured intracellular Ca^{2+} oscillations, which are essential for optimally synchronised insulin secretion from the islets. Spreading of beta cell membrane depolarisation by gap junctional coupling is likely the most important mechanism for synchronisation of pulsatile

insulin release between beta cells in islets [Tengholm and Gylfe 2009]. We did not observe any effects of CART on intracellular Ca^{2+} concentrations per se. However, Ca^{2+} oscillations within the beta cells stimulated with high glucose and forskolin (i.e. the conditions where CART had the strongest effect in mouse islets) changed from fast, or mixed fast and slow, to slow oscillations (**Fig 14a**) when CART was added. Interestingly, the CART-induced change in intracellular Ca^{2+} oscillations was also associated with improved synchronisation of Ca^{2+} oscillations in different regions within the islet (**Fig 14b-d**). Intercellular coupling between beta cells within the islets are essential for glucose regulation of the insulin pulse amplitudes [Tengholm and Gylfe 2009]. The effects on Ca^{2+} oscillations were fast and evident within a few minutes after the addition of CART, once again suggesting that CART affects first phase insulin secretion. It has been shown that PKA-activity is essential for Ca^{2+} oscillations in MIN-6 beta cells [Ni, et al. 2011], and that CART activates PKA and its downstream signals in INS-1 (832/13) cells and rat islets. Furthermore, the effect of CART on proliferation in INS-1 (832/13) cells was blocked by PKA-inhibitors [Sathanoori et al. 2013]. Therefore, the acute effects of CART could be mediated via PKA, which both modulates Ca^{2+} oscillations and directly stimulates insulin secretion. Supporting this notion, CART stimulation increases cAMP levels in INS-1 (832/13) cells and the effect of CART on insulin secretion is blocked by inhibition of PKA [Sathanoori et al. 2013; Wierup et al. 2006].

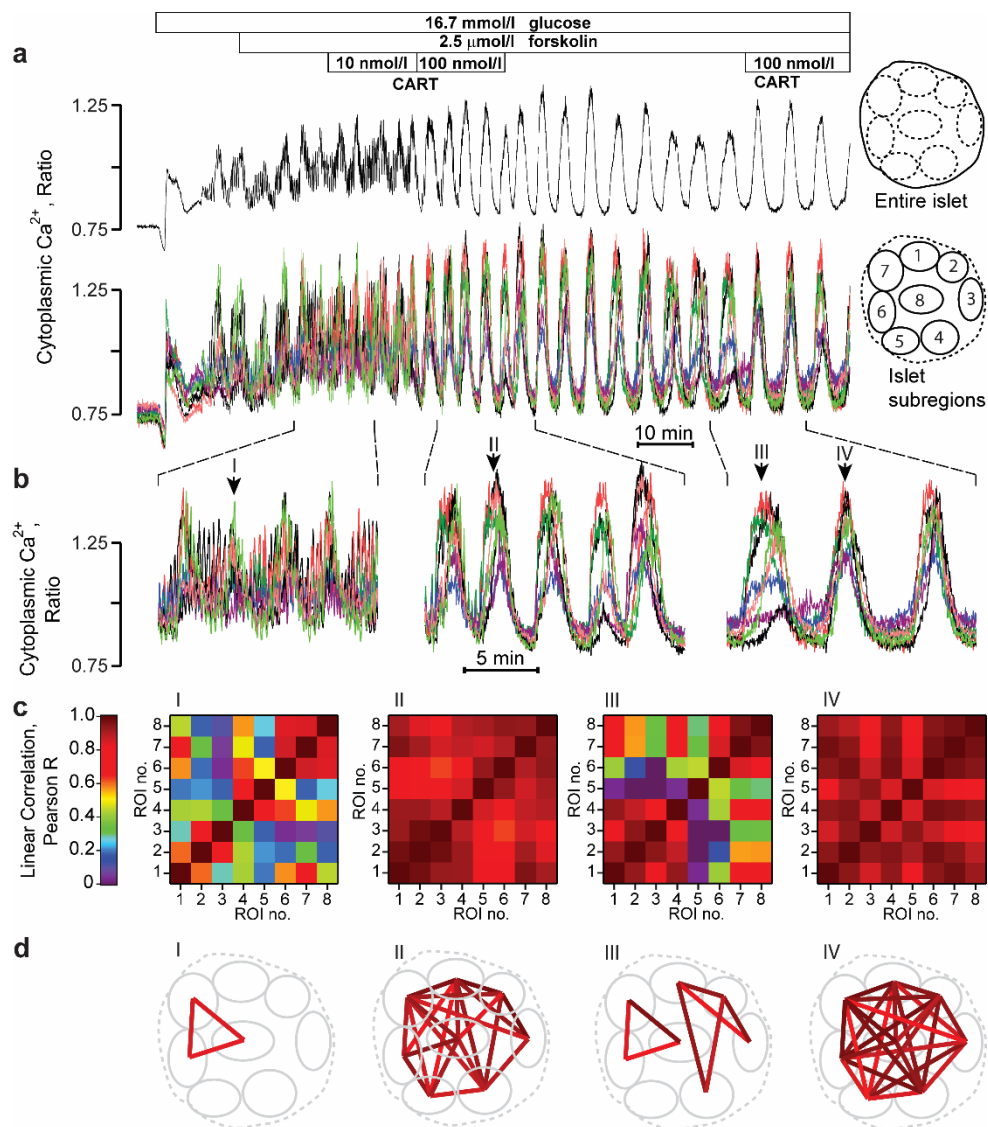


Figure 14. Effects of CART on $[\text{Ca}^{2+}]_i$ in mouse islet during high glucose followed by stimulation with forskolin. (a): $[\text{Ca}^{2+}]_i$ response pattern recorded from the entire islet (black trace) and from indicated smaller regions-of-interest (ROI) (coloured traces). CART transforms 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 in (c). (c): Correlation of $[\text{Ca}^{2+}]_i$ responses in different islet ROI. 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 display color-coded Pearson R coefficients for each ROI pair. CART improves synchronisation between islet sub-regions. (d): Connectivity maps highlighting the ROI pairs with correlation coefficients exceeding 0.8.

Aim III: The effect of CART on glucagon secretion

It is not well studied how CART affects glucagon secretion, and previous observations suggest that CART has modest inhibitory effects on glucagon secretion from rat islets at 16.7 mM glucose [Wierup et al. 2006], but that CART does not affect glucagon secretion in InR1G9 hamster cells [Chen, et al. 2011]. Starting with human islets, we found that CART decreased glucagon secretion at stimulatory low (1 mM) glucose concentrations. At 8.3 mM glucose, glucagon secretion was maximally inhibited and CART was without effect (**Fig 15a**). In mouse islets, CART decreased glucagon secretion at 2.8, 8.3 and 16.7 mM glucose (**Fig 15b**). To investigate the effect of CART on glucagon secretion *in vivo*, we performed intravenous injections of CART. Two minutes after injection, glucagon levels were 25% lower in mice injected with CART compared with mice injected with vehicle (**Fig 15c**).

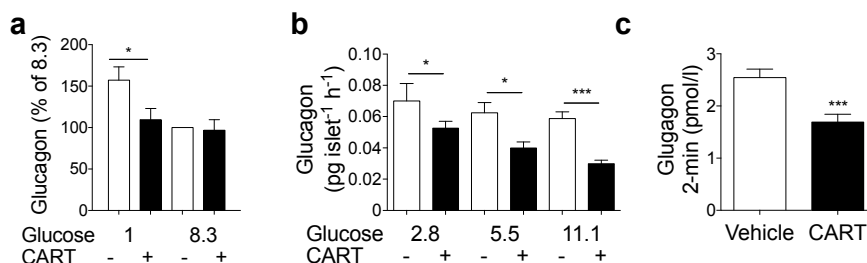


Figure 15. CART decreases glucagon secretion from human islets (a) and mouse islets (b) during 1h static incubations. Intravenous injection of CART results in decreased glucagon levels after 2 min (c).

To study if CART had direct effects on alpha cells we examined alpha cell exocytosis using the whole-cell patch-clamp technique. This revealed that CART reduced alpha cell exocytosis already after 1 h of treatment compared with untreated cells (**Fig 16a-c**).

When studying isolated islets, it is difficult to dissect whether the observed effect on hormone secretion is a direct effect of the secretagogue on the particular endocrine cell of interest, or secondary due to effects on hormone secretion from other islet cells. Insulin and glucagon regulate the secretion of each other and CART affects the secretion of both hormones. Somatostatin is another regulator of both insulin and glucagon secretion, and the role of CART in delta cells and somatostatin secretion has only been assessed in rat islets at high glucose concentrations (16.7 mM), where somatostatin secretion was decreased by CART [Wierup et al. 2006]. Hence, from data obtained in whole islets it is difficult to conclude whether CART has direct effects on specific islet cell types. It has been shown that CART exerts direct effects in INS-1 (832/13) cells [Sathanoori et al. 2013; Wierup et al. 2006].

However, cell lines are model systems, which do not perfectly reflect the situation in primary cells. One way to circumvent this problem, and get cell-specific information, would be to test the effect of CART in sorted alpha and beta cells from dispersed islet cells [Lernmark 1974]. Unfortunately, this method requires harsh treatment of the cells which is thought to affect the beta cells more than the alpha cells [Barg, et al. 2000], hence, the method itself could skew the results.

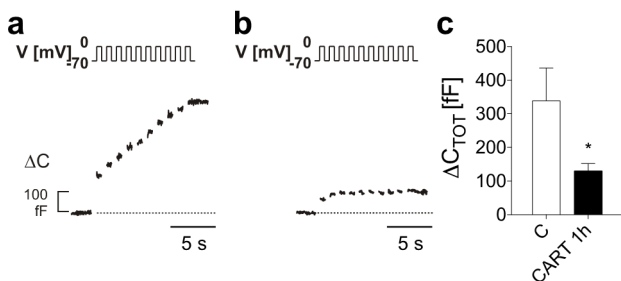


Figure 16. Effects of CART on single cell exocytosis in mouse islet cells. Representative cell recordings in control alpha cells (a), and alpha cells treated with 10 nmol/l CART for 1h (b). Average total increase in capacitance evoked by the trains (ΔC_{TOT}) (c).

Aim IV: The effect of overexpressed beta cell CART

We hypothesise that beta cell CART is upregulated in T2D as a compensatory mechanism trying to reduce plasma glucose levels by promoting beta cell survival, increasing insulin secretion and reducing glucagon secretion. To test this hypothesis, we developed transgenic mice with overexpression of human CART coupled to enhanced green fluorescence protein (EGFP) under the control of the pancreas duodenal homeobox (PDX1) promotor (CARTtg mice). PDX1 is one of the earliest genes expressed in the developing pancreas and PDX1 KO mice selectively lack the pancreas [Jonsson, et al. 1994]. After birth, PDX1 expression is restricted to beta cells, as well as to a few epithelial cells in the dorsal side of the stomach and in the duodenal mucosa [Offield, et al. 1996]. In a study using a mouse reporter model, PDX1 promotor activity was detected in transgenic pancreas and marginally in the transgenic duodenum, but not in the liver or brain [Shiraiwa, et al. 2007]. The use of the PDX1 promotor would ensure close to beta cell-specific expression, and at the same time avoid expression in the brain that can be the result when using the INS2 promotor (also known as RIP) [Johnson 2014]. A potential drawback could be that CART will be overexpressed in islet progenitor cells during development. We could confirm beta cell-specific expression of CART by immunohistochemistry and EGFP-fluorescence, and by Western blot we showed that CARTtg mice had approximately 20 times higher CART expression in isolated

islet compared with WT mice. Starting with young female mice, we examined insulin secretion *in vivo* by OGTT and IVGTT, but found no differences in insulin secreting capacity or glucose tolerance. We also examined insulin secretion *in vitro* from isolated islets, however, no differences between islets from CARTtg and WT mice were detected. Supporting these results, insulin content and beta cell mass were similar in the two genotypes. Glucagon secretion, as well as insulin tolerance, was also found to be similar in both genotypes (**Fig 17**). Collectively, the data generated in young female mice demonstrate that CARTtg mice have normal islet function and glucose tolerance.

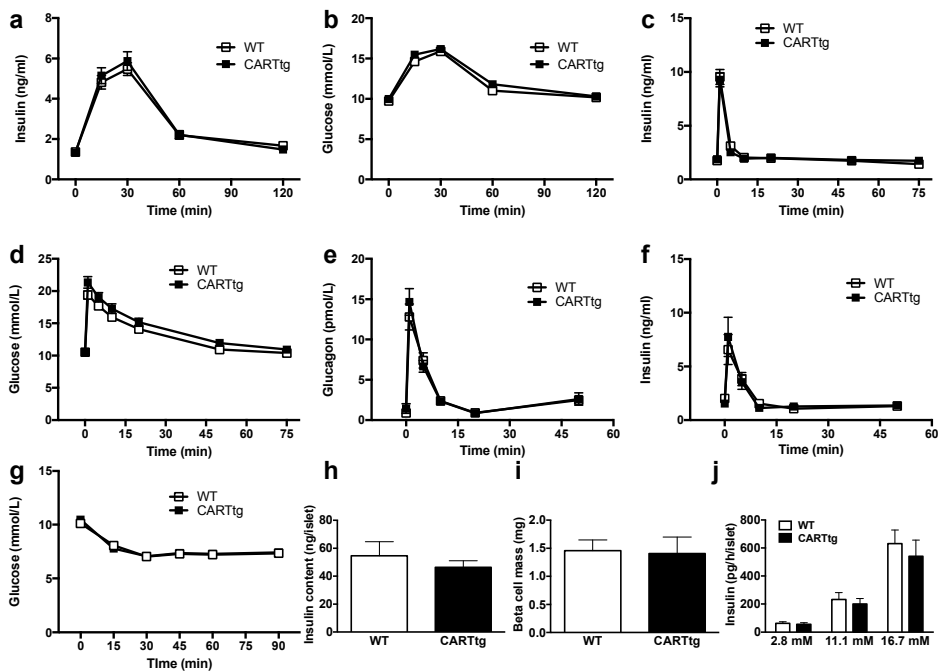


Figure 17. Young (<6 months) female CARTtg mice fed control diet display similar insulin secretion capacity and glucose homeostasis during an OGTT (a, b) and an IVGTT (c, d) as WT mice. Glucagon (e) and insulin secretion (f) during arginine tolerance test is normal and insulin sensitivity is normal during ITT (g). Insulin content (h), beta cell mass (i) and *in vitro* insulin secretion (j) are not different compared with WT mice.

Aging is associated with decreased insulin sensitivity in both man and mouse [Fink, et al. 1983; Houtkooper, et al. 2011], thereby increasing the demand for insulin and the burden on the beta cells. Therefore, we tested the impact of ageing on insulin secretion in CARTtg mice. We found that old CARTtg mice had improved insulin secretion capacity (AIR) and improved glucose elimination compared with WT littermates during an IVGTT (**Fig 18a-e**). When further studying the mice at different ages we found an age-dependent increase in AIR from 6 months to 12

months (**Fig 18f**). The increased insulin secretion observed *in vivo* was, however, not evident *in vitro* in isolated islets from old mice (<12 months). I believe that a potential explanation for this could be the 1 h static incubations of the islets where we might have overlooked an effect on first phase insulin secretion. Hence, we cannot exclude that CARTtg islet have affected insulin secretion dynamics and experiments with shorter incubation times or studies in perfused islets are warranted. Another potential explanation for the divergent data could be that the increased insulin secretion seen *in vivo* involves the autonomic nervous system. To address this, we performed intravenous tolerance tests using 2-deoxy-glucose in both young and old mice, but the two genotypes responded in a similar fashion.

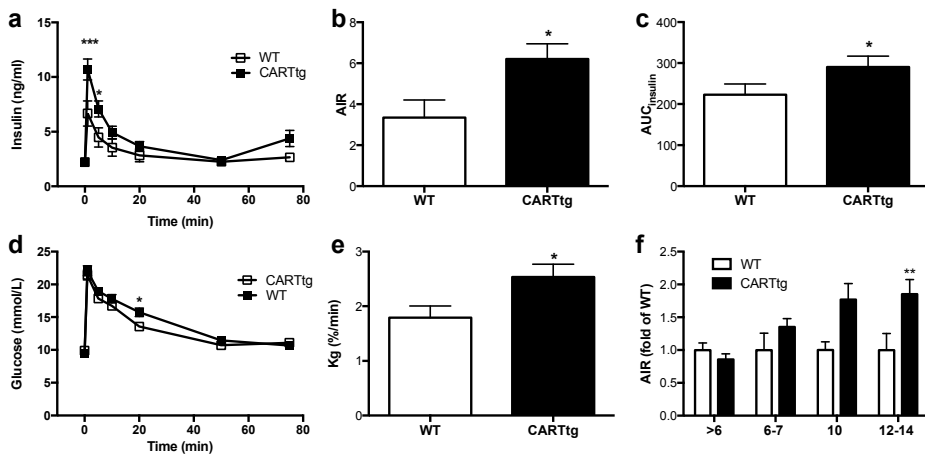


Figure 18. a-e: IvGTT in 12 months old CARTtg and WT mice. CARTtg mice display increased insulin secretion at 1 and 5 min (a), increased acute insulin response (AIR; b), and increased AUC_{insulin} (c). 20 min glucose levels were lower in CARTtg mice (d) and glucose elimination rate (Kg) was improved (e). AIR increases with age in CARTtg mice compared with WT age-matched mice (f).

Another commonly used model of insulin resistance and the metabolic syndrome is the HFD-fed mouse [Surwit et al. 1988; Winzell and Ahren 2004]. Young CARTtg mice fed HFD displayed elevated fasting insulin levels and increased insulin secretion during an OGTT (**Fig 19a-c**). However, glucose homeostasis was deteriorated with decreased glucose elimination (**Fig 19d, e**). Also, glucose levels during an IVGTT and an insulin tolerance test were higher, thus confirming reduced insulin sensitivity in CARTtg mice.

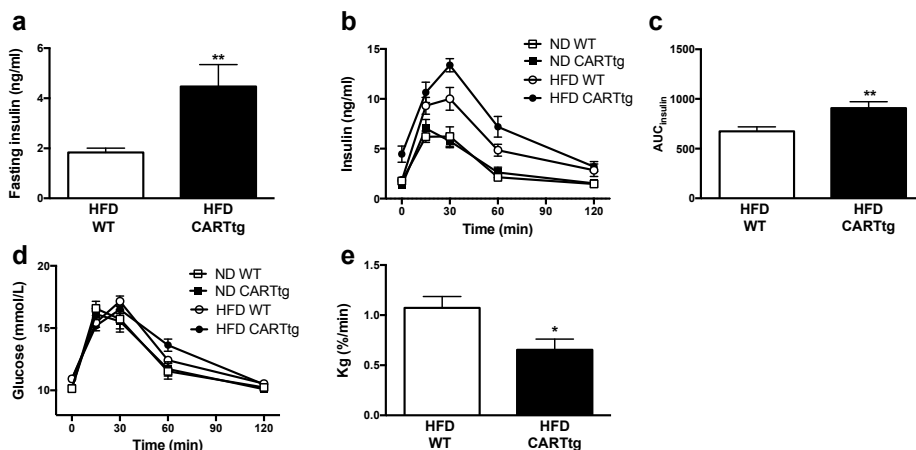


Figure 19. OGTT in CARTtg and WT mice after 8-12 weeks of HFD (a-e). Fasting insulin (a) and insulin levels (b, c) were increased during OGTT in CARTtg mice. Glucose clearance was decreased in CARTtg mice (d, e).

To study the direct effect of glucose on insulin secretion in HFD-fed CARTtg mice, we isolated islets and found that insulin secretion was increased at 11.1 mM glucose (**Fig 20a**). Furthermore, and in line with increased insulin secretion *in vivo*, there was a trend towards increased beta cell mass in CARTtg mice (**Fig 20b**).

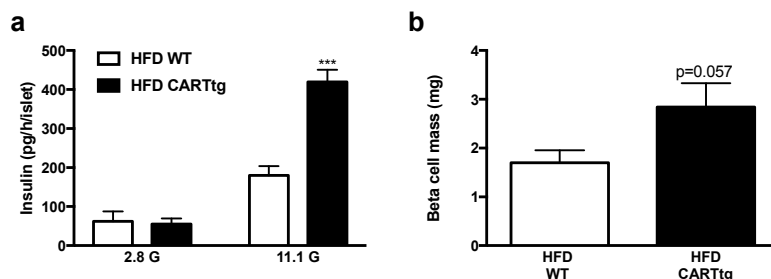


Figure 20. Insulin secretion is increased in CARTtg isolated islets (a) and there is a trend towards increased beta cell mass after 4 months of HFD (b).

Although insulin secretion was increased, CARTtg mice had reduced glucose elimination and were less insulin sensitive compared with WT mice. High levels of plasma insulin may desensitise insulin target tissues [Shanik, et al. 2008]. It is therefore possible that the observed insulin resistance is secondary to the hyperinsulinemia. This has been shown in mice transfected with multiple copies of the insulin gene. These mice have elevated basal levels of insulin [Shanik et al. 2008], similar to what we observed in CARTtg mice fed HFD, along with impaired glucose elimination and decreased insulin sensitivity. The authors conclude that

basal hyperinsulinemia can result in insulin resistance [Shanik et al. 2008]. The opposite phenomena, that impaired insulin secretion would protect against insulin resistance, has been suggested in transgenic mice lacking beta cell ATGL, the enzyme catalysing the first step in the breakdown of triacylglycerol. ATGL KO mice displayed impaired insulin secretion but were protected against obesity-induced diabetes and had improved insulin sensitivity [Attane, et al. 2016]. Therefore, I believe that in CARTtg mice, improved insulin secretion may not be beneficial during conditions when insulin secretion is already elevated and insulin sensitivity is reduced.

To study the effect of overexpressed beta cell CART in mice with hypoinsulinemia instead of hyperinsulinemia, we used mice subjected to streptozotocin (STZ) treatment. We used a multiple low dose STZ treatment, which results in partial beta cell death, insulin deficiency and hyperglycaemia [Leiter 1982]. As expected, glucose monitoring confirmed markedly elevated plasma glucose in STZ mice compared with vehicle-treated mice one week after the last injection of STZ (**Fig 21a**). Further, STZ-treated CARTtg mice displayed improved glucose homeostasis compared with STZ-treated WT mice three weeks after the treatment (**Fig 21a**). IVGTT revealed that CARTtg mice had higher residual insulin secretion capacity and improved glucose elimination compared with WT mice (**Fig 21b-f**). STZ is taken up by the beta cells via the GLUT2 receptor [Schnedl, et al. 1994], however, we could not find any differences in beta cell GLUT2 expression between CARTtg and WT using immunohistochemistry (unpublished observations). Furthermore, both genotypes had similar beta cell mass and we believe that overexpression of CART in the beta cells resulted in improved insulin secretion that is independent of beta cell mass. This notion gains support from the fact that CART KO mice [Wierup et al. 2005] and old CARTtg mice have normal beta cell mass, as well as by observations that silencing of CART in INS-1 (832/13) cells does not affect cell survival (Shcherbina, Abels et al. submitted manuscript).

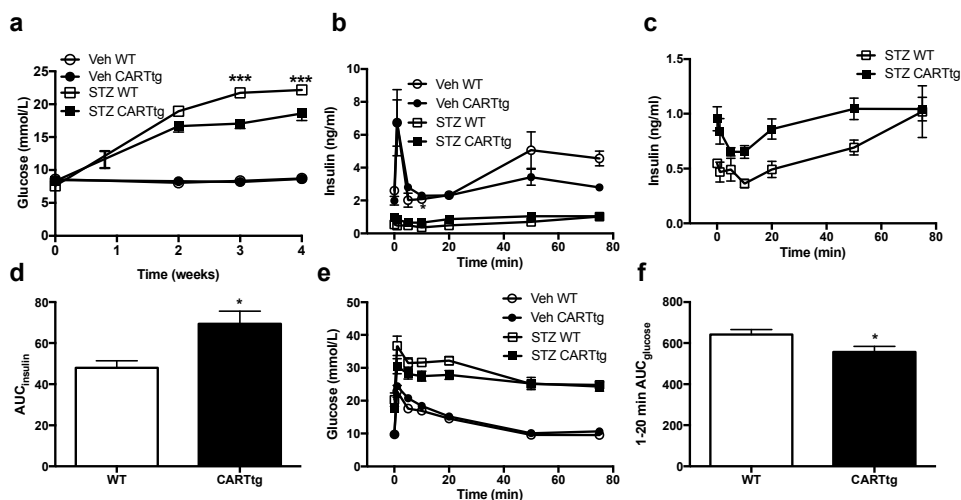


Figure 21. CARTtg mice treated with a multiple low dose STZ have lower glucose levels after 3 and 4 weeks compared with STZ-treated WT mice (a). IVGTT on STZ- and vehicle-treated CARTtg and WT mice (b-f). Insulin secretion all treatment groups (b). Insulin secretion from STZ-treated mice (c). AUC_{insulin} is increased in STZ-treated CARTtg mice compared with STZ-treated WT mice (d). Glucose levels in all treatment groups (e). Glucose levels are lower from 1-20 min in STZ CARTtg mice compared with STZ WT mice (f).

Overexpressed beta cell CART results in increased circulating CART levels (presented in paper III, see below). CART can cross the blood-brain-barrier [Kastin and Akerstrom 1999] and might therefore influence glucose homeostasis via effects mediated by the hypothalamus. Circulating CART levels could also potentially affect the adrenal glands and alter secretion of corticosteroids, which could influence on insulin secretion and insulin signalling. To exclude that increased insulin secretion in CARTtg mice was a consequence of non-beta cell effects, we overexpressed CART in a pure beta cell system. To this end, INS-1 (832/13) cells were transiently transfected with adenovirus containing CART (or green fluorescence protein (GFP) used as a control). Overexpression was confirmed by qPCR and Western blot. We assessed insulin secretion during 15 min static incubation, to study first phase insulin secretion, 72 h after transfection. Indeed, CART overexpressing cells displayed moderately, but consistently increased insulin secretion capacity at 16.7 mM glucose (**Fig 22**).

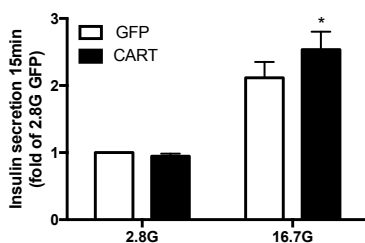


Figure 22. Adenoviral overexpression of CART in INS-1 (832/13) cells increases GSIS during 15 min static incubation.

Aim V: The effect on insulin sensitivity and adipocyte metabolism in mice overexpressing beta cell CART

To understand the basis for reduced insulin sensitivity seen in CARTtg mice fed HFD, we assessed if this could be explained by an effect in the target tissues of insulin. We have previously shown that CART potentiates isoprenaline-induced lipolysis and activates HSL in isolated rat adipocytes. CART also potentiates the inhibitory effect of insulin on isoprenaline-induced lipolysis, by inhibition of PKB phosphorylation. In addition, CART reduces insulin-induced glucose uptake and lipogenesis. This, together with our observations that CARTtg mice fed HFD had a higher weight gain rate, larger gonadal fat pads and larger visceral adipocytes (**Fig 23a-c**), prompted the assessment of adipocyte metabolism in CARTtg mice. Insulin has anti-lipolytic actions and promotes fat storage in the adipocytes. The increased weight gain rate and fat storage in CARTtg fed HFD could hence be a consequence of the increased circulating insulin levels observed. In a diabetes perspective, increased fat storage could be considered both beneficial and disadvantageous. If the increased fat storage is linked to insulin resistance and hepatic steatosis, this is considered to be a risk factor. However, the capability of the adipose tissue to store lipids and protect other tissues from increased circulating levels of fatty acids, altered levels of cytokines and inflammation are considered to be beneficial. As an example, the anti-diabetic drugs TZDs improve insulin sensitivity by reducing circulating levels of FFAs, via accelerated adipocyte FFA uptake and esterification, leading to increased fat storage in adipocytes [Yki-Jarvinen 2004].

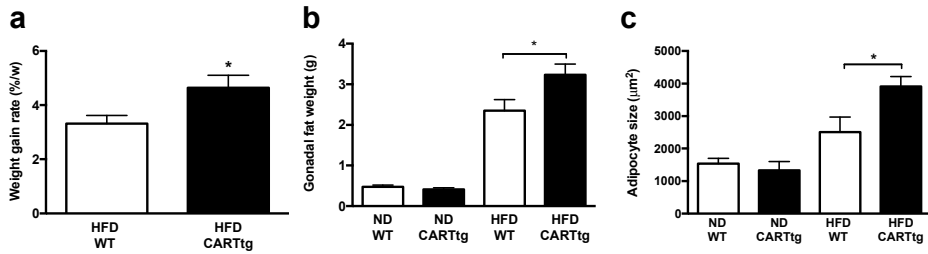


Figure 23. Weight gain rate (a), gonadal fat weight (b) and adipocyte cell size (c) are increased in CARTtg mice fed HFD.

Signs of dysfunctional adipocytes include increased release of FFAs (lipolysis), altered adipokine release and low grade adipocyte tissue inflammation, which could result in peripheral insulin resistance and T2D [Cusi 2010]. In CARTtg mice fed HFD lipolysis was reduced (**Fig 24a, b**) and the total expression of HSL was lower compared with WT mice. The observed reduced basal and isoprenaline-stimulated lipolysis in adipocytes from CARTtg mice most likely contributes to the increased fat storage seen in HFD fed CARTtg mice as compared with WT mice. In adipocytes from mice fed HFD, the effect of insulin on anti-lipolysis was abolished, both in WT and CARTtg mice, which reflected the insulin resistance induced by HFD. *De novo* lipogenesis, which is the metabolic process that converts glucose into fatty acids and subsequently triglycerides for storage, is positively regulated by insulin [Goldman and Cahill 1964]. Basal and insulin-stimulated *de novo* lipogenesis was unchanged in CARTtg mice compared with WT mice on the same diet. And when normalising the results against basal lipogenesis, there was no difference in the effect of insulin on *de novo* lipogenesis. Together, these data suggest that the increased insulin resistance in CARTtg mice at the whole body level is most likely not explained by reduced insulin sensitivity in the adipose tissue. However, the effect of insulin on specific mechanisms *in vitro* in isolated adipocytes may not always reflect the situation *in vivo*. To further understand the basis for the observed insulin resistance we next studied whether this could be explained by defects in the liver. To this end, we measured levels of liver triglycerides which is often associated with hepatic insulin resistance [Nagle, et al. 2009]. We could confirm that liver triglyceride content was increased in CARTtg mice fed HFD compared with WT mice, but on control diet (CD) no differences was found between CARTtg and WT mice. Thus, it is possible that reduced insulin sensitivity in the liver, caused by increased liver triglycerides, is responsible for the insulin resistant phenotype of CARTtg mice fed HFD.

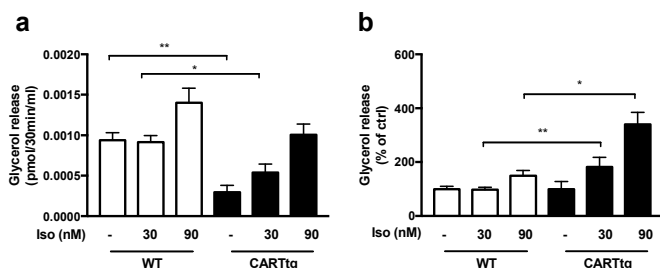


Figure 24. Basal and isoprenaline (iso) stimulated lipolysis in mice fed HFD is decreased in adipocytes from CARTtg compared with WT (a). However the response to insulin, when normalising to basal lipolysis, is increased (b) in CARTtg.

When fed a CD, there was no difference in lipolysis between CARTtg and WT mice. However, the inhibitory effect of insulin on lipolysis induced by 30 nM and 90 nM isoprenaline was significant in CARTtg mice, whereas the effect on lipolysis induced by 30 nM in WT mice was absent. Hence, these results do not favour the idea that increased insulin resistance in CARTtg mice is explained by reduced insulin sensitivity in the adipose tissue. On CD, basal and insulin induced *de novo* lipogenesis was reduced in CARTtg mice compared with WT mice (**Fig 25a**). The effect of insulin on lipogenesis was, however, similar in both genotypes (**Fig 25b**), which indicates that the reduced lipogenesis is not an effect of decreased adipocyte insulin sensitivity.

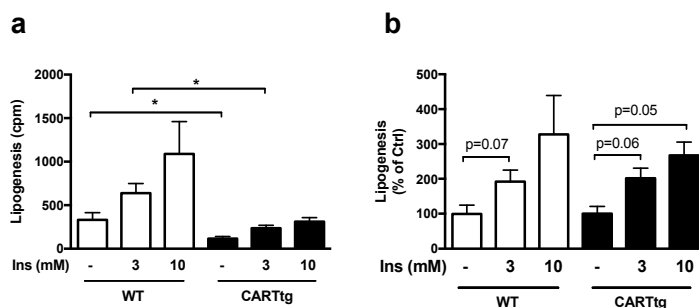


Figure 25. Basal and stimulated *de novo* lipogenesis is reduced in adipocytes from CARTtg mice fed CD compared with WT mice (a). The response to insulin, when normalising to basal lipogenesis, is similar between the genotypes (b).

Since CARTtg mice displayed altered adipocyte metabolism, we hypothesised that this could be linked to elevated circulating CART levels originating from the beta cells. Therefore, we examined whether CART-EGFP was secreted into the circulation in CARTtg mice. Since CART may be secreted from multiple sources e.g. adrenal glands, GI-tract and pituitary, we took advantage of the EGFP-tag to specifically quantify secretion of beta cell CART. Indeed, we could confirm that

CART-EGFP was secreted from the beta cells into the circulation in CARTtg mice. Therefore, I suggest that the observed effects in adipocytes results from long-term elevated CART plasma levels. So far, only acute effects of CART on isolated rat adipocytes have been investigated [Banke et al. 2013]. It is therefore difficult to compare earlier findings with the data obtained in the present study. CART has been shown to have effects that could be associated both with insulin sensitivity and insulin resistance, depending on the surrounding conditions [Banke et al. 2013]. In this study we found that adipocyte metabolism was differently altered in CARTtg mice on CD, with normal insulin levels, compared with mice on HFD, with increased basal insulin levels. Some effects were insulin-like, e.g. decreased lipolysis, and some were opposing those of insulin, e.g. decreased *de novo* lipogenesis. We cannot rule out the possibility of secondary effects of overexpressing CART in the beta cells that could affect adipocyte metabolism. Increased levels of circulating CART could for example affect the adrenal glands, thereby altering the levels of corticosteroids that can alter adipocyte metabolism and differentiation [Peckett, et al. 2011]. Insulin strongly regulates adipocyte metabolism, and the observations from adipocytes in mice fed HFD could be a consequence of increased basal insulin levels. However, the fact that CARTtg mice displayed altered adipocyte metabolism, but normal fasting insulin levels, when fed CD speaks in favour of a direct effect of CART.

Anti-diabetic effects of CART

A long term goal of our research group is to evaluate the potential for CART as an anti-diabetic agent. We have now established that CART increases insulin secretion in human islets in a glucose-dependent fashion, meaning that CART does not increase insulin secretion at low glucose levels. This is important since the outcome of increasing insulin secretion when blood glucose is low is risk of hypoglycaemia. Importantly, we also show that CART has glucose-dependent insulinotropic effects in islets from diabetic donors, which is a proof-of-concept that CART could be used as a diabetes medication. We have also shown that CART decreases glucagon secretion in human islets in a glucose-dependent fashion. This has so far only been shown in islets from healthy donors. Further studies are needed to understand whether CART has glucagonostatic actions also in T2D islets. An important feature of an anti-diabetic agent would be to reduce the hypersecretion of glucagon at high blood glucose levels. The effects of CART on islet hormone secretion are reminiscent of those of GLP-1, one of the incretins secreted in response to a meal to decrease blood glucose levels. We have unpublished observations showing that CART is expressed in K- and L-cells in the human intestine, and therefore, we believe that CART could be a novel incretin. So far we have not been able to show that CART is secreted from the intestine. However, we have evidence that CART

plays important roles in L-cells by regulating GLP-1 secretion both *in vivo* and *in vitro* (Shcherbina et al. manuscript). Furthermore, CART potentiates the insulinotropic effects of GLP-1 both *in vivo* and *in vitro* in mice. This opens the opportunity for a combination therapy with CART and GLP-1-based drugs.

Another important aspect of an anti-diabetic agent would be its effects on insulin sensitivity in insulin target tissues. We have previously shown that the effects of CART on adipocyte metabolism act either in concert with or against the action of insulin [Banke et al. 2013]. The long-term effect of CART exposure *in vivo* in CARTtg mice suggests that CART affects adipocyte metabolism, but it is still not clear if CART affects insulin sensitivity specifically in adipose tissue. Hence, further studies are needed both *in vivo* and in adipose tissue from humans to address these questions. Another important tissue for insulin action and glucose uptake is the skeletal muscle. The effect of CART on muscle metabolism has not yet been studied, but we have preliminary observations suggesting that CART could promote translocation of GLUT4 to the plasma membrane in muscle. One of the most important insulin target tissue for regulation of blood glucose levels is the liver, but so far, studies addressing the effect of CART in hepatocytes are lacking. Whether CART affects glucose metabolism in kidney is not known. Kidneys have emerged as a clinically important site for regulation of blood glucose, since absorption of glucose in the collecting ducts and tubules can be blocked by interfering with glucose and sodium co-transport using SGLT2-inhibitors.

It would also be an advantage if an anti-diabetic agent could promote beta cell survival and reduce apoptosis, as evident for GLP-1 [Brubaker and Drucker 2004]. This has been shown for CART in INS-1 (832/13) cells and in isolated rat islets [Sathanoori et al. 2013], but information from *in vivo* studies and from human islets is missing. Collectively, our previous and present results establish an important role of CART in beta cell function and in regulation of glucose homeostasis. Therefore, the potential for CART-based therapies for T2D should be further evaluated.

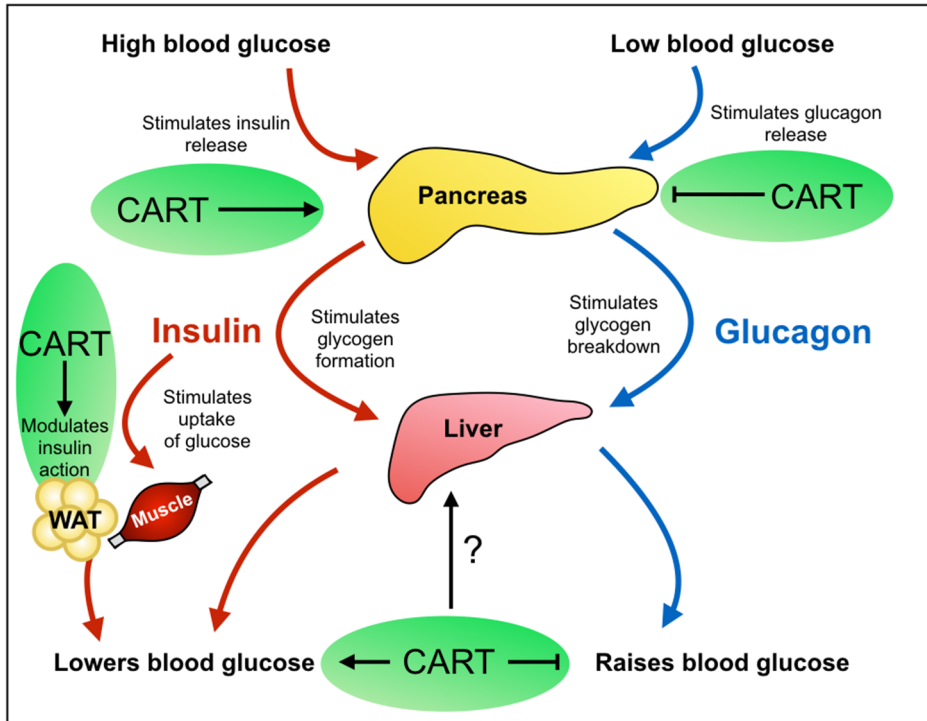


Figure 26. Schematic illustration showing how CART can regulate glucose homeostasis.

Concluding remarks and perspectives

The work presented in this thesis shows that CART increases insulin secretion and decreases glucagon secretion both *in vivo* in mice and in human islets. This can partly be explained by alterations in granule exocytosis and synchronisation of intracellular Ca^{2+} oscillations. However, the exact mechanism of how CART exerts its effect on hormone secretion in islet cells remains to be elucidated and one important key would be to identify the receptor for CART. Such work is in progress in our research group and we aim to describe a receptor that is either general, and hence transmit the effect of CART in many different cells, or that is specific to islet cells, within a near future. Another main finding is that the expression of CART is upregulated in beta cells in animal models of diabetes as well as in alpha and beta cells in patients with diabetes, likely as a consequence of hyperglycaemia. We show that overexpression of CART in beta cells results in increased insulin secretion and hence, we suggest that CART is upregulated as a compensatory mechanism trying to overcome hyperglycaemia. Another project that soon will be published from our research group describes how silencing of CART affects beta cell function in a negative direction. Together with the data presented in this thesis it suggests that CART has important roles in beta cell function and therefore could be critical during diabetes development. An islet cell- or beta cell-specific CART knockout mouse would be a useful tool to further study the role of endogenous CART in islet function. Finally, we show *in vivo* that long-term exposure of increased CART levels affects adipocyte lipolysis and lipogenesis; metabolic processes that are regulated by insulin. More studies on the effect of CART on insulin target tissues *in vivo* are warranted to understand the effect of CART on insulin action.

Although the exact mechanism for the effects mediated by CART in islets remains to be elucidated, we conclude that CART has an important role in islet function and as a regulator of glucose homeostasis. This conclusion is based on the glucose-dependent insulinotropic and glucagonostatic properties of CART. These are features that position CART as a potential candidate for future anti-diabetic drug development once the CART receptor has been identified.

Abbreviations

AC	acetylcholine
ACC	acetyl CoA carboxylase
ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AIR	acute insulin response
AMPK	AMP-activated protein kinase
ARC	arcuate nucleus
AS160	Akt substrate of 160
ATGL	adipocyte triglyceride lipase
ATP	adenosine triphosphate
BMI	body mass index
CART	cocaine- and amphetamine-regulated transcript
CARTtg	transgenic mouse with beta cell-specific CART overexpression
CD	control diet
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CO	carbon monoxide
CRE	cAMP response element
CREB	CRE-binding proteins
CRH	corticotropin releasing hormone
DAG	diacylglycerol
DEX	dexamethasone
DPP-4	dipeptidyl peptidase 4
EPAC2	exchange protein directly activated by cAMP 2
FADH2	reduced form of flavin adenine dinucleotide
FAS	fatty acid synthase
FFA	free fatty acid
G3P	glycerol-3-phosphate
GABA	gamma aminobutyric acid
GFP	green florescence protein
GH	growth hormone
GI	gastro intestinal
GIP	glucose-dependent insulintropic peptide
GK	Goto-Kakizaki
GLP-1	glucagon-like peptide-1

GLUT	glucose transporter
GRP	gastrin-releasing peptide
GSIS	glucose-stimulated insulin secretion
GSK-3	glycogen synthase kinase 3
HFD	high fat diet
HPA	hypothalamic-pituitary-adrenal
HSL	hormone sensitive lipase
IBMX	isobutylmethylxanthine
ICV	intracerebroventricular
IP3	inositol 1, 4, 5-triphosphate
IRS	insulin receptor substrate
IVGTT	intravenous glucose tolerance test
K_g	constant for glucose elimination rate
KO	knockout
LADA	latent autoimmune diabetes in the adult
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinases
MGL	monoacylglycerol lipase
MODY	maturity onset diabetes of the young
MPP	Malmö preventive project
MTNR1B	melatonin receptor 1B
NADH	nicotinamide adenine dinucleotide
NO	nitric oxide
NPY	neuropeptide Y
NRSE	neuron-restrictive silencer element
NRSF	neuron-restrictive silencer factor
OGTT	oral glucose tolerance test
PACAP	pituitary adenylate cyclase-activating polypeptide
PDE3B	phosphodiesterase 3B
PDX1	pancreatic and duodenal homeobox 1
PI_3	phosphatidylinositol-3
PIP_2	phosphatidylinositol 4,5-bisphosphate
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PKA	protein kinase A
PKB	protein kinase B (also known as Akt)
PKC	protein kinase C
PLC	phospholipase C
PMA	phorbol 12-myristate 13-acetate
PP	pancreatic polypeptide
PPAR	peroxisome proliferator-activated receptors
PPP	prevalence prediction and prevention

PRL	prolactin
PVN	paraventricular
RP	releasable pool
RRP	ready releasable pool
SNP	single nucleotide polymorphism
SP	substance P
SSTR	somatostatin receptor
STZ	streptozotocin
SU	sulfonylurea
T1D	type 1 diabetes
T2D	type 2 diabetes
TAG	triacylglycerol
TCF7L2	transcription factor 7-like 2
TZD	thiazolidinediones
VDCC	voltage-dependent calcium channel
VIP	vasoactive intestinal polypeptide
WAT	white adipose tissue
WT	wild type
ZDF	zucker diabetic fatty

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The role of CART in islet function

The focus of this thesis is the regulatory peptide cocaine- and amphetamine-regulated transcript (CART) and its role in islet function. It is described that CART is expressed in human islet cells and that its expression is upregulated in diabetic states. CART increases insulin secretion, decreases glucagon secretion and regulates glucose homeostasis. The effect of upregulated beta cell CART is studied both in vivo and in vitro. This thesis is written by Mia Abels, doctoral student in the research group Neuroendocrine Cell Biology at Lund University Diabetes Centre.



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