Paracrine control of glucagon secretion in the pancreatic α-cell:

Studies involving optogenetic cell activation

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“Never stop seeking what seems unobtainable”

Star Trek

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ABSTRACT

The mechanisms controlling glucagon secretion by α-cells in islets of Langerhans were studied. We generated mice with the light-activated ion channel ChR2 specifically expressed in β-, α-, and δ-cells, and explored the spatio-temporal relationship between cell activation and glucagon release. In **paper I**, ChR2 was expressed in β-cells and photoactivation of these cells rapidly depolarized neighbouring δ-cell but produced a more delayed effect on α-cells. We showed that these effects were mediated via electrical signalling from the β- to δ-cells via gap-junction. Once activated, the δ-cells released somatostatin which repolarized the α-cells following its intercellular diffusion from the δ- to the α-cells. In **paper II** we used a novel antibody for detection of somatostatin, which showed great efficiency compared with commercially available antibodies. Immunostaining of intact islets showed an islet-wide network involving α- and δ-cells. Furthermore, we used immunostaining to compare the islet architecture as pertaining to δ-cell number, and morphology between islets from healthy human donors and type 2 diabetic donors and found that the number of δ-cells in type 2 diabetic islets is reduced. In **paper III** we expressed ChR2 in α- and δ-cells in two novel mouse models. We showed that photoactivation of α-cells depolarized the α-cells and evoked action potential firing, effects that were associated with stimulation of glucagon secretion regardless of the glucose concentration. In islets exposed to 1 mM glucose, photoactivation of δ-cells transiently hyperpolarized α-cells, produced a long-lasting inhibition of glucagon exocytosis and inhibited glucagon secretion at 1 mM glucose but had no additional inhibitory effect at 6 or 20 mM glucose. The effect of somatostatin was so strong that it was possible to suppress glucagon secretion by photoactivation of δ-cells even when measurements were performed using the perfused mouse pancreas.

Keywords: Glucagon, α-cell, somatostatin, δ-cell, optogenetics, secretion, type 2 diabetes

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SAMMANFATTNING PÅ SVENSKA

Insulin är kroppens viktigaste blodsockerreglerande hormon. Det verkar genom att stimulera upptaget av glykos (druvsocker) i fett, muskler och levern. Glukos lagras som fett i fettceller men som glykogen i muskler och i levern. Glykogen är koncentrerat druvsocker.

Vid sockersjuka (diabetes) är den normala blodsockerregleringen satt ur spel. Det orsakas av antingen bristande insulinproduktion eller minskad insulinkänslighet – ofta en kombination av bägge. I Sverige har ca 500 000 personer sockersjuka. Av dessa har 90% typ 2-diabetes (åldersdiabetes) och 10% typ 1-diabetes (ungdomsdiabetes). Mörkertalet är antagligen stort och många får diagnosen först efter flera år i samband med ett rutinbesök på vårdcentral eller hos en läkare. Typ 2-diabetes ansågs tidigare vara en så kallad ålderskrämpa, men drabbar nu allt yngre människor som en följd av minskad fysisk aktivitet och övervikt. En ytterligare bidragande faktor är ökad livslängd. Obehandlat högt blodsocker leder på sikt till blodkärlsskador som ökar risken för hjärt- och kärlsjukdomar, njursvikt och blindhet.

Insulin är dock bara en del av diabetsproblematiken. När blodsockerkoncentrationen sjunker (t.ex. vid fasta eller fysisk aktivitet) så återställs den normalt snabbt genom ökad frisättningen av ett annat hormon – glukagon. Glukagon verkar på levern och ökar blodsockret genom att stimulera nedbrytningen av glykogen.

Både insulin och glukagon produceras i bukspottkörtelns langerhanska öar. Insulin produceras av beta-cellerna och glukagon av alfa-cellerna. Dessa bägge celler sitter i omedelbar närhet till varandra inuti de langerhanska öarna. Varje langerhansk ö innehåller ungefär 300 celler och av dessa är 200 beta-celler och knappt 100 alfa-celler. Bukspottkörteln i en människa innehåller 1 miljon langerhanska öar som tillsammans inte väger mer än 1 gram. Beta- och alfa-cellerna är specialiserade för att kontinuerligt känna av små svängningar i blodsockerkoncentrationen. Genom att öka respektive minska frisättningen av insulin och glukagon håller de langerhanska öarna blodsockerhalten på en ganska konstant nivå som på ungefär 1 gram per liter. Det motsvarar ungefär en normal sockerbit löst i kroppens blod. Denna reglering störs i samband med diabetes och blodsockerhalten kan därvid öka så mycket att det skadar kroppens vävnader.

Vi vet nu att sockersjuka orsakas av att inte tillräckligt med insulin frisätts från beta-cellerna. Behandlingen av diabetes syftar till att i första hand minska insulinbehovet och i andra hand återställa beta-cellens förmåga att frisätta insulin men ofta måste till sist patienterna behandlas med insulin. Ett problem med detta är att insulin måste doseras mycket försiktigt så att blodsockerhalten inte sjunker för mycket. Det är en allvarlig – ibland dödlig - biverkan av insulinbehandling. Detta problem uppstår genom att inte bara beta-cellerna utan även alfa-cellerna påverkas vid sockersjuka. Vilka dessa störningar är och varför de uppstår är inte känt. Förhoppningen är att genom undersökningar av normal och sockersjuka langerhanska öar ta reda på om man med läkemedel kan återställa normal alfa- och betacellsfunktion och på så sätt bota diabetes.

I mitt arbete har jag använt en ny teknik för att studera beta- och alfa-cellernas reglering. Vi har modifierat dessa celler så att de bildar ett ljuskänsligt protein. När cellerna sitter inuti kroppen (t.ex. i en levande mus) är detta protein inte aktivt. Men när vi isolerat de langerhanska öarna kan vi belysa dem med blått ljus och på så sätt aktivera dem. Genom att placera proteinet i antigen beta- eller alfa-celler kan vi specifikt aktivera dessa celler. Vi har även placerat det ljuskänsliga proteinet i en tredje celltyp – de langerhanska öarnas delta-celler. Dessa celler producerar hormonet somatostatin. Med denna teknik har jag bokstavligen kunnat belysa den langerhanska öns funktion och se samspelet mellan de olika cellslagen. Mina studier har visat att det existerar en slags hierarki i den langerhanska ön: beta-cellerna reglerar delta-cellerna som i sin tur kontrollerar alfa-cellerna. Dessa fynd förklarar varför glukagonfrisättningen normalt är låg vid högt blodsocker. Vi har också fynd som tyder på att langerhanska cellöar i patienter med typ 2-diabetes innehåller färre delta-celler. Detta skulle kunna förklara varför dessa patienter trots att de har för högt blodsocker har för höga nivåer av det blodsockerhöjande hormonet glukagon.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Briant, L. Reinbothe, T. Spiliotis, J. Miranda, C. Rodriguez, B. Rorsman, P. δ-cells and β-cells are electrically coupled and regulate α-cell activity via somatostatin. J. Physiol. 2018, Jan 15: 596(2): 197-215

II. Miranda C, Kothegala L, Lundequist A, G Garcia, P Belekar, J-P Krieger, J Presto, Rorsman P, Gandasi NR. Structural correlations influencing regulation of somatostatin-releasing δ-cells *(Manuscript)*

III. Miranda, C. Tolö, J. Santos, C. Kothegala, L. Mellander, L. Hill, T. Briant, L. Tarasov, AI. Zhang, Q. Gandasi, NR. Rorsman, P. Dou, H. Intraislet paracrine crosstalk between islet cells unveiled by optogentic activation of α- and δ-cells. *(Manuscript)*

PUBLICATIONS NOT INCLUDED IN THIS THESIS

IV. Real J, Miranda C, Olofsson CS, Smith PA. (2018) Lipophilicity predicts the ability of nonsulphonylurea drugs to block pancreatic beta-cell KATP channels and stimulate insulin secretion; statins as a test case. *Endocrinol Diabetes Metab*. 30; 1(2)

V. Ramracheya R, Chapman C, Chibalina M, Dou H, Miranda C, González A, Moritoh Y, Shigeto M, Zhang Q, Braun M, Clark A, Johnson PR, Rorsman P, Briant LJB. (2018) GLP-1 suppresses glucagon secretion in human pancreatic alpha-cells by inhibition of P/Q-type Ca^{2+} channels. *Physiol Rep*. Sep;6(17)

VI. Hamilton A, Vergari C, Miranda C, Tarasov. AI. (2919) Imaging Calcium Dynamics in Subpopulations of Mouse Pancreatic Islet Cells *Jove-Journal of Visualized Experiments.*

VII. Guida C, Miranda C, Asterholm IW, Basco D, Benrick A, Chanclon B, Chibalina MV, Harris M, Kellard J, McCulloch J, Real J, Rorsman NJG, Yeung HY, Reimann F, Shigeto M, Clark A, Thorens B, Rorsman P, Ramracheya R. (2020). Promiscuous receptor activation mediates glucagonostatic effects of GLP-1(9-36) and GLP-1(7-36) *(Submitted)*

VIII. Kim A, Knudsen JG, Madara C, Benrick A, Hill T, Abdul Kadir L, Kellar JA, Melander L, Miranda C, Lin H, James T, Suba K, Spigelman F, Wu Y, MacDonald PE, Salem V, Knop FK, Rorsman P, Lowell BB, Briant L. (2020) Arginine-vasopressin evokes glucagon secretion during hypoglycaemia and maintains plasma glucose during dehydration. *(Submitted)*

CONTENT

ABBREVIATIONS

ADP	Adenosine diphosphate
APs	Action potentials
ATP	Adenosine triphosphate
ChR2	Channelrhodopsin-2
GJs	Gap junctions
GLUT2	Glucose transporter 2
iCre	Improved Cre recombinase
K_{ATP}	ATP-sensitive potassium channels
LED	Light-emitting diode
Na_v	Voltage-gated sodium channels
RFP	Red fluorescent protein
RIP	Rat insulin promoter
SSTR2	Somatostatin receptor 2
T2D	Type 2 Diabetes Mellitus
TTX	Tetrodotoxin
VGCC	Voltage-gated calcium channels
YFP	Yellow fluorescent protein

INTRODUCTION

In 1912, when A.J. Hodgson published his article on Diabetes Mellitus, he already highlighted the role of excessive consumption of glucose or "corn sirup", and poor diet that includes "the excessive use of starches" as well as a sedentary life style as probable causes of the disease[1]. So why are we, more than a hundred years later, still battling with lack of knowledge about it? We have come a long way since then. We know much more about the pathophysiology of the disease and we now have access to medications to treat – but not cure – diabetes. Thanks to these advances, diabetes is now regarded as a chronic rather than acutely fatal disease. However, the number of people suffering from diabetes in 2013, is estimated to be 56 million in Europe alone, with an overall estimated prevalence of 8.5%[2]. Before 1990, less than 2% of the children with diabetes would have type-2 diabetes (T2D). However, over the years due to increased obesity, the incidence of T2D cases has increased to 25-45% in children and young adults[3]. The world health organization (WHO) has ranked T2D as number 7 in the top 10 global causes of death in 2016.

TYPE 2 DIABETES MELLITUS

T2D is the metabolic malady that results as the failure of the body to maintain normal blood glucose concentrations. This is a consequence of an imbalance between the body's requirements for insulin and the β-cells capacity to supply the hormone[4,5,6]. The inability to lower blood glucose ultimately results in peripheral tissue insulin resistance and continued hyperglycaemia[7,8], which is followed by many health issues causing organ dysfunction[9]. T2D has been redefined as a multi-hormonal disease[10,11,12]. It is increasingly evident that not only is insulin not being appropriately released, glucagon is hyper-secreted and this exacerbates the metabolic consequences of insulinopaenia[13,14]. Conversely, deficient glucagon secretion when blood glucose levels are too low, may lead to severe (potentially fatal) hypoglycaemia in insulin-treated patients[15,16,17].

Although the contribution of aberrant glucagon secretion was first described almost 50 years ago[14,18], our understanding of the underlying causes remain poorly understood. Indeed, our knowledge of how glucagon secretion is regulated physiologically is also fragmentary and this makes it more difficult to pinpoint the defects associated with T1D and T2D. A third hormone,

somatostatin, may be involved in disease pathogenesis[11,19] but many aspects of its physiological and pathophysiological roles are unclear[20,21,22].

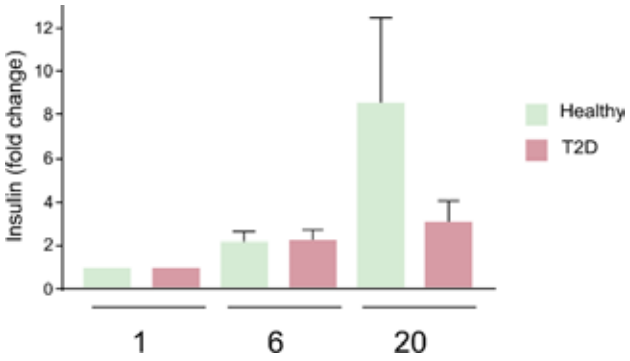

Figure 1: Insulin levels secreted by islets from a healthy human donor (green bars) and donor with T2D (pink bars). At high glucose (20mM) islets do not secrete significantly more than at 6mM, showing impaired ability to respond to elevated glucose.

THE ISLET OF LANGERHANS

BACKGROUND

Discovered in 1869 by the German pathologist Paul Langerhans, the islets of Langerhans are the structures responsible for sensing changes of and controlling blood glucose in order to uphold glucose homeostasis. They are multicellular micro-organs consisting of (on average) ~300 endocrine cells. They reside embedded in the exocrine tissue of the pancreas[23]. A human pancreas contains ~1 million islets and weight about 1g (1% of the whole pancreas). Islets of Langerhans are essentially composed of β, α and δ cells[24] that produce the hormones insulin, glucagon and somatostatin, respectively. Together, these endocrine cells help orchestrate glucose homeostasis.

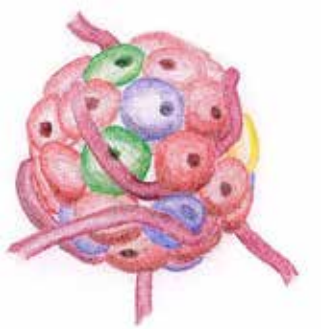

Figure 2: Illustration. The islet of Langerhans.

Insulin is the only hormone that is able to lower blood glucose levels[25]. In the fall of 1920, Frederick Banting, a young surgeon working at the University of Western Ontario, read an article by Moses Barron entitled "*The relation of islets of Langerhans to Diabetes with a special reference to cases of pancreatic Lithiasis*". It described a procedure to the endocrine factor of the pancreas after ligature of the pancreatic duct, thus producing pancreatitis and destruction of the

exocrine pancreas. Previous attempts to treat diabetes patients had been of very limited success. The article by Moses spiked Banting's inspiration and provided the motivation to perform his experiments. In the following months, he approached Toronto University Professor John McLeod, who allowed him to use the facilities at the University of Toronto to extract the juices of dog pancreases and inject the crude extract into pancreatectomized dogs. With the help of Charles Best, Banting demonstrated a few months later that glucose could be lowered after injection of the pancreas extract into the dogs whose pancreases had been removed surgically. This progress was arduous and slow, as extraction of dog pancreas usually failed and McLeod felt a need for more biochemical expertise. They were joined by James Collip, Professor of Biochemistry, at the end of 1921. In January 1922, Leonard Thompson, a 14-year-old boy with diabetes, was the first patient ever to receive the extract treatment. Banting and McLeod received the Nobel Prize in Physiology or Medicine in 1923, they shared the prize money with Best and Collip[26].

Glucagon was named after its action as a 'glucose agonist'. When Banting and Best started treatment of humans and dogs with pancreas extracts, they observed an initial transient period of hypergycaemia that preceded the subsequent fall in plasma glucose. At that time, this was attributed to the effect of adrenaline but in 1923, C.P. Kimball and John R. Murlin, upon studying extracts from pancreas, isolated the new substance that possessed hyperglycaemic properties[27].

The cells of the Islets of Langerhans received their names based on their chronological identification during staining with alcohol and fixation. A- and B-cells were the first to be identified; a third type remained 'clear' during staining and was therefore named C-cell. Later on, a fourth type was identified and received the name D-cells. It was later established that C- and D-cells were the same cells, namely δ-cells. In current terminology, the islet cells are referred to by the corresponding Greek rather than Latin letters: α for A, β for B and δ for D[28].

ARRANGEMENT OF ENDOCRINE CELLS WITHIN THE ISLETS

The localization of the different types of cells within the islets of Langerhans is species-specific. In mouse islets, the α- and δ-cells are localized to the mantle of the islet, while β-cells congregate in the core and are thus surrounded by the non-β cells. Their abundances vary in different species[29] but 60-80% are β-cells, 10-20% α-cells and 5-10% are δ-cells[20,30]. In humans, the arrangement of the cells is slightly different and consist of "sub clusters" of β-cells surrounded by non-β-cells, these non-β-cell units are then associated with islet capillaries[31]. This arrangement would clearly facilitate

paracrine communication in the intra-islet milieu. The δ-cells have received relatively little attention in studies, but it is now increasingly evident that somatostatin plays a role in the hormonal disturbances linked to diabetes. A role of the δ-cells in pancreatic islet function is further suggested by their architecture: whereas α- and β-cells have a rounded or diamond-shaped morphology, δ-cells have a more intricate morphology and present up to 20μm long filopodia-like projections[32].

GLUCOSE HOMEOSTASIS

Glucose homeostasis is the regulation that keeps blood glucose levels within healthy ranges[33,34]. In humans, normal blood glucose concentration is 80-100 mg/dl, corresponding to 4.5-5.5 mM. The brain depends highly on glucose for oxidative metabolism and function: if glucose levels in the body fall too low, the brain is deprived of energy within minutes (as little as 5 minutes), and this can cause severe cognitive impairment and can culminate in coma and death[35,36]. If glucose levels are chronically elevated beyond the normal range, glucotoxic effects occur in β-cells, neurons and endothelial cells. This leads to the progression of diabetes and ultimately cause microvascular complications and neuropathic disorders[37,38]. Collectively, these biochemical changes predispose to cardiovascular and renal disease and increase the risk of blindness and amputations[39].

Insulin has as main role in glucose homeostasis storing excess energy provided in the form of lipids, proteins and carbohydrates[40,41]. Experiments using knockout models suggest that most of insulin's hypoglycaemic effects occur in the liver. However, it is important that insulin's functions are not limited to blood glucose control and it is a master regulator of systemic fuel homeostasis[42]. Insulin exerts a myriad of functions across the body, acting in different tissues and stimulating different processes related to ATP production (cellular respiration) and energy storage (including lipogenesis, protein synthesis)[43,40].

After a meal, all the carbohydrates and proteins are broken down by enzymes of the digestive system into their monomers (glucose and amino acids). All of these molecules will enter the blood stream, and blood glucose levels will increase. They are sensed by the β-cells of the pancreas and the release of insulin into the blood is stimulated.

Glucagon's main role is to mobilize stored 'depots' to raise plasma glucose when it falls into the low range during exercise, fasting or starvation. Together, the hormones of the endocrine pancreas assure that blood glucose levels are always within the healthy range: between 4 and 5.5 mM when in fasted state and not exceeding 7.8 mM 2 hours after a meal[44].

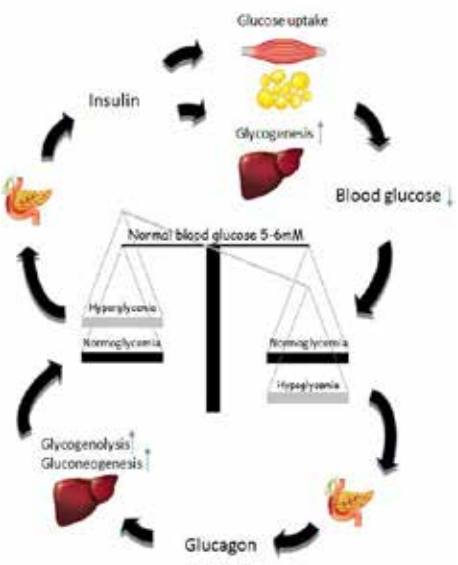

Figure 3:Physiological regulation of plasma glucose. At any given moment, a healthy individual has between 5-7.8mM glucose in the blood (normoglycaemia). The hormones insulin and glucagon secreted by the β- and α-cells, respectively, ensure that the processes that induce glucose uptake or break-down are in balance with the intake of nutrients. In T2D, this balance is perturbed.

ELECTRICAL ACTIVITY & HORMONE SECRETION

The patch-clamp technique was first applied to islet cells in the 1980s. This allowed the electrical activity of the β-cells to be analysed in much greater detail than had been possible with sharp intracellular electrodes[45]. This technological breakthrough enabled the identification of the ATP-sensitive potassium (K^+) channels (K_{ATP} channels) as the β-cell's resting conductance. These channels were subsequently found to be the molecular target of the hypoglycaemic sulphonylureas, compounds that at the time had been used to treat T2D for 30 years. The discovery of the K_{ATP} channels led to the formulation of a consensus model for glucose-induced insulin secretion[46].

The β-cells are equipped with the insulin independent glucose transporter 2 (GLUT2)[47] in mice and GLUT1 in humans[25]. Once in the cell, glucose is rapidly phosphorylated by glucokinase. Glucokinase is considered to be the 'glucose sensor' of β-cells. Phosphorylation of glucose into glucose-6-phosphate determines the rate of glycolysis. This first step of glucose metabolism produces pyruvate; pyruvate, a 3-carbon molecule is sent into the mitochondria via active transport and fed into the citric acid cycle or tricarboxylic cycle (TCA). In the mitochondria, the "powerhouses" of the

cell, the last step of cellular respiration takes place, namely electron transport chain, with a net yield of ATP of ~30 ATP per glucose molecule[48,49]. Insulin secretion in β-cells is tightly regulated by the activity of ATP sensitive potassium channels (K_{ATP} channels). K_{ATP} channels provide the β-cells with the means to link metabolism to electrical activity and insulin secretion[50]. At low glucose concentrations (>5.5 mM), the β-cell membrane potential is kept at -70 mV[45]. This is because of the activity of the K_{ATP} channels. At low glucose, when glucose metabolism proceeds at a low rate, the K_{ATP} channels are open and K^+ distributes freely across the plasma membrane. This drives the membrane potential towards the K^+ equilibrium potential. In β-cells, with the transmembrane K^+ gradient this potential is ~-70 mV. This negative membrane potential keeps the voltage gated Ca^{2+} channels (VGCC) shut. As cytoplasmic Ca^{2+}($[Ca^{2+}]_i$) must be elevated for insulin to be released, this means that insulin secretion is kept low at low glucose[51]. However, when blood glucose increases, the acceleration of glucose metabolism and the resulting increase in the cytoplasmic ATP/ADP ratio leads to closure of the K_{ATP} channels[45]. The fall in K^+ permeability unveils the depolarizing influence of other membrane conductance that are too small to affect the β-cell membrane potential when K_{ATP} channel activity is high. This results in membrane depolarization and initialization of Ca^{2+}-dependent action potential (AP) firing (Figure 4). This culminates in elevation of the cytoplasmic $[Ca^{2+}]_i$ and triggers exocytosis of insulin-containing secretory granules[52]. Using the same molecular machinery involved in neurotransmitter release[25].

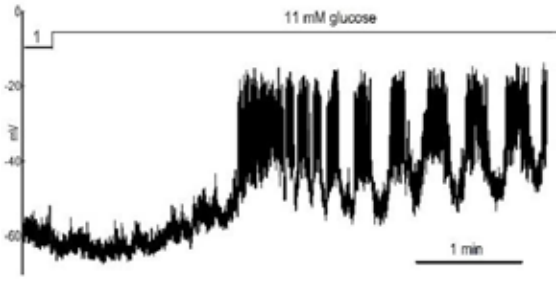

Figure 4: A β-cell was initially superfused with extra-cellular solution containing 1 mM glucose. Under these conditions, the resting potential is around -70mV, and the cell electrically silent. When glucose is elevated to 11mM, the β-cell depolarizes and starts generating oscillatory electrical activity consisting of Ca^{2+} - dependent action potentials.

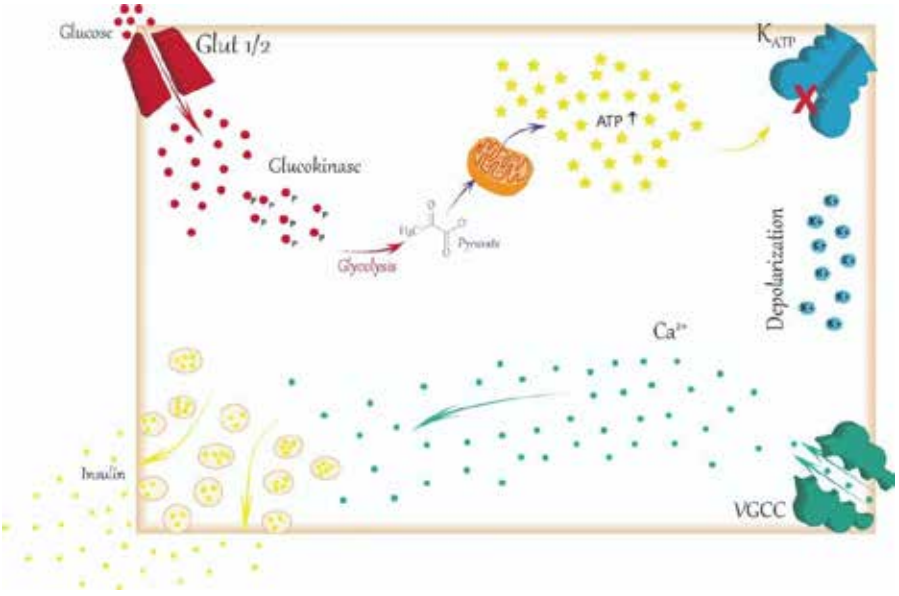

Figure 5: Stimulus secretion coupling in β-cell.

The regulation of glucagon secretion by glucose is more controversial and a consensus model for the α-cell remains to be formulated.

One popular concept is that the α-cells are under paracrine control by insulin (from the β-cells) or somatostatin (from δ-cells; Figure 6)[53]. However, one caveat with this idea, is that glucagon secretion is maximally inhibited at glucose concentrations (6 mM) with little stimulatory effect on the release of either of these hormones[54,55]. indicating that α-cells must also rely on other (intrinsic) signals for metabolic control of glucagon secretion[56]. Indeed, glucose retains the capacity to regulate glucagon secretion in the presence of insulin and somatostatin receptor antagonists[57] (and own unpublished). Moreover, high glucose concentrations that maximally stimulate insulin and somatostatin secretion, do not produce stronger inhibition of glucagon secretion[58]; if anything, glucagon secretion is less suppressed under these conditions[59,60].

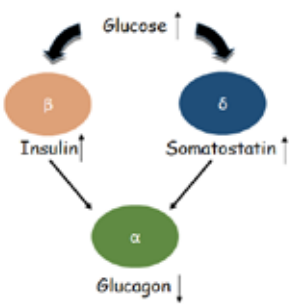

Figure 6: Paracrine regulation within pancreatic islets.

Taken together, these observations strongly suggest that α-cells have their own glucose sensing machinery.

Nevertheless, it is quite clear (as will be discussed below) that both insulin and somatostatin can act as paracrine regulators to suppress glucagon secretion[61]. Clearly, the interactions between the paracrine and intrinsic mechanisms are complex and require advanced technology to be disentangled.

INTRINSIC REGULATION OF δ-CELL GLUCAGON SECRETION

There is much evidence suggesting the involvement of glucagon in hyperglycaemia in patients with uncontrolled diabetes[62]. In recent studies, mice that lack glucagon receptors ($Gcgr^{-/-}$mice) do not develop hyperglycaemia even after complete destruction of their β-cells induced by the β- cytotoxic streptozotocin[63]. However, when glucagon signalling was restored in the liver by adenoviral infection, hyperglycaemia promptly developed[64]. Moreover, addition of exogenous somatostatin lowered glucagon secretion and decreased hepatic glucose production in the clamped pancreas[65]. Collectively, these data illustrate the pivotal role glucagon plays in the pathogenesis of diabetes. Thus, normalizing glucagon secretion in T1D and T2D may represent a means to ameliorate consequences of these disorders.

Several models have been proposed to explain the intrinsic regulation of glucagon secretion in α-cells. According to one model, glucose inhibits glucagon secretion by promoting intracellular Ca^{2+} uptake into intracellular Ca^{2+} stores (like the sER), which was postulated to switch off depolarizing plasmalemmal store-operated channels[66,67]. Another model (proposed by the same team of investigators) instead postulates that high glucose inhibits glucagon secretion by reducing intracellular cAMP[68]. A third model, backed up by experimental and clinical evidence, highlights a role of K_{ATP} channels. These channels are expressed at very high levels in α-cells. At the molecular level, the K_{ATP} channels in α-cells are identical to those found in the β-cells[69].

Like β-cells, α-cells are electrically excitable. They generate action potentials (APs) and electrical activity regulates release of glucagon containing vesicles. However, unlike β-cells, they generate APs at low glucose concentrations[70]. These are large-amplitude voltage APs[71], which elicit opening of high-voltage VGCC[72]. The α-cells are equipped with both L-type and P/Q type Ca^{2+} channels[46]. Some observations suggest that the P/Q-type Ca^{2+} channels are particularly important for glucagon secretion induced by low glucose[73,72].

Like β-cells, α-cells are electrically excitable and electrical activity is associated with the release of glucagon containing vesicles. Unlike β-cells, α-cells generate APs at low glucose concentrations[70]. These are large-

amplitude voltage APs[71] and culminate in the activation of high-voltage VGCC[72]. The α-cells are equipped with both L-type and P/Q type Ca^{2+} channels[46]. Some observations suggest that the P/Q-type Ca^{2+} channels are particularly important for glucagon secretion induced by low glucose[73,72].

Islets cells also contain voltage-gated Na^+ channels (Na_v)[74,75]. These channels are TTX-sensitive, and blocking these channels results in strong glucagon release inhibition[76,77]. Interestingly, the functional properties of the Na_v channels differ between the islet cell types. Part of this variability arises because of the relative expression of different Na_v subtypes. Whereas β-cells predominantly express *Scn9a* ($Na_V1.7$), α-cells express more *Scn3a* ($Na_V1.3$)[75,78]. The two channels exhibit slightly different biophysical properties and $Na_V1.3$ channels are more active at physiological membrane potentials than $Na_V1.7$ channels. Because of the different expression pattern, electrical activity in α-cells is more dependent on Na^+ channels than is the case in β-cells. The higher Na^+ conductance explains why action potentials in α-cells are of greater amplitude than those in β-cells: in β-cells, action potentials peak between -20 to -10 mV whereas they peak between 0 and +10 mV in α-cells[72]. This difference is functionally important, as will be discussed below[76].

Although α-cells express K_{ATP} channels at a density which is 5-fold higher than in β-cells[79], K_{ATP} channel activity at low glucose is much lower in α-cells than in β-cells. The underlying mechanisms remain to be elucidated. At 1 mM glucose, K_{ATP} channel activity has been measured as 0.07 nS and 3 nS in α- and β-cells, respectively. It is important to notice that K_{ATP} channel activity in α-cells - although low - is greater than zero. This keeps the α-cell sufficiently depolarized to allow AP and yet prevents excessive membrane depolarization. Generation of large-amplitude APs leads to opening of P/Q-type VGCC and the associated influx of Ca^{2+} triggers exocytosis of glucagon-containing secretory vesicles (Figure 7a).

When glucose is elevated, the associated acceleration of glucose metabolism raises the ATP/ADP ratio, leading to complete closure of the K_{ATP} channels. As in β-cells, the depolarization increases action potential firing in α-cells but more importantly produces a decrease in their amplitude such that they peak at -15 mV rather than +5 mV. This has been attributed to a reduction of the voltage-gated Na^+ current resulting from membrane potential-dependent inactivation of the channels. The significance of this is that the APs no longer lead to the opening of the P/Q-type VGCCs (which only open when the membrane potential exceeds -10 mV). As a result, exocytosis of glucagon-

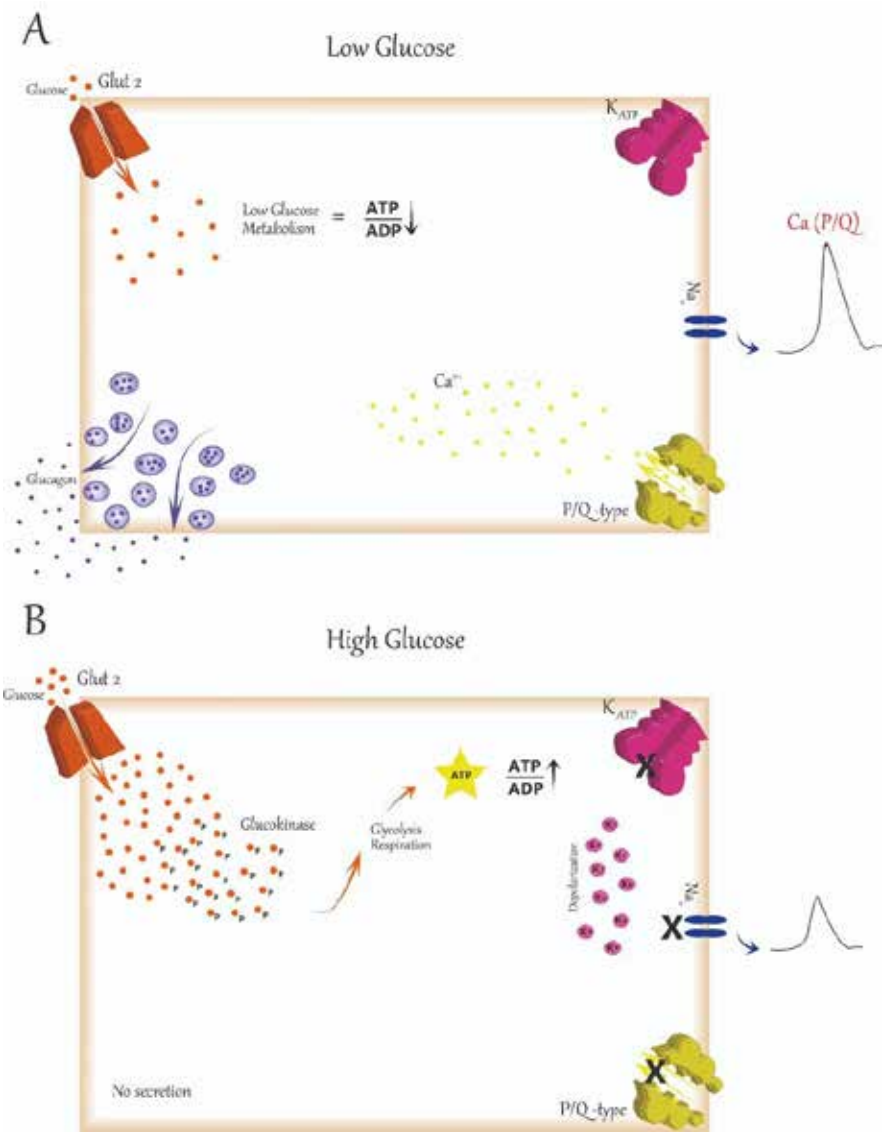

Figure 7: The α-cell. KATP model of intrinsic regulation of glucagon secretion. See main text for details.

containing secretory vesicles is reduced by 80-90% that cannot be compensated for by increased action potential firing (+150%) and as a result glucagon secretion is reduced by 50-70% (Figure 7b).

It is important to emphasize that there is compelling evidence that glucagon secretion is also influenced by paracrine factors[80,81]; these include γ-aminobutyric acid (GABA)[82,83], Zn^+ [84], somatostatin and insulin[85]. It should

be emphasized that the two models (paracrine vs intrinsic regulation) are certainly not mutually exclusive. By contrast, it seems highly likely that they are complementary and operate in parallel to ensure precise (minute-by-minute) regulation of glucagon (and insulin) secretion, which in turn is required for optimal glucose homeostasis[46,86].

OPTOGENETICS

Optogenetics is a technology that uses light to control biological systems[87]. Optogenetic techniques have been implemented in a wide range of organisms such as bacteria, eukaryote cell-lines, nematode worms, fruit flies and mice. Examples of applications of optogenetics include: to guide cell movement towards a specific path; to control cell signalling by regulating activity of receptors in the cell membrane, or downstream proteins in the signalling pathway[88]; to induce cell death by triggering apoptosis; to control cell fate[89], by enforcing cell differentiation mediated by light[90].

There are presently three main molecular systems of optogenetics: Light-induced dimerization, photocaging and photo-sensitive ion channels[91,92]. Systems based on light-induced dimerization and photocaging are known as non-neuronal optogenetics. Systems based on photo-sensitive ion channels are referred to as 'neuronal optogenetics' but this is arguably a misnomer and these techniques can in fact be applied to any electrically excitable cell. Amongst the many advantages of optogenetics, the spatio-temporal accuracy it affords stands out as it provides a means of cell-specific but non-invasive cell activation in more intact preparations (such as brain slices, embryos or islets of Langerhans)[93].

Optogenetics has been used in neuroscience for more than 10 years in order to study connections between excitable neurons[94]. Recently, the whole concept of optogenetics has been broadening to involve more cell types, and other applications, including β- and α-cell physiology[95,96].

Chlamydomonas reinhardtii are aquatic organisms that measure ~10μm in length. They have one eye and two flagella, which together work as the sensory machinery that allows the algae to respond to light. A pseudo eye focuses incident light onto a membrane that contains circa 10^5 pigment receptor molecules. While the organism rotates when swimming, the pseudo eye scans the light pattern thereby controlling changes in movement, driving the flagella[97]. Two rhodopsins were identified in *C. reinhardtii* as their means to behavioural light responses (phototaxis)[98]. They were named channelrhodopsin-1 and 2 (ChR1, ChR2). Rhodopsins are seven-

transmembrane (7-TM) proteins[99] that have operated as photosensors throughout evolution of eyes in vertebrates and invertebrates[97]. These 7-TM proteins regulate activity of ion channels[100].

Studies regarding the electromagnetic spectrum response in *C. reinhardtii* in nature identified all-*trans* retinal as the natural chromophore i.e. they require all-*trans* retinal as co-factor to absorb photons[101,102] also showing a response spectra of maximum 505 nm in wavelength (blue light). Studies on ChR2 reveals that illumination results in a large cationic conductance, showing ChR2's great potential to depolarize cells. Other rhodopsins have the ability to allow conductance of other ions, such as halorhodopsins, which transport Cl^- and archaerhodopsins that transport H^+ [103].

In Paper I and III, we used optogenetics-based tools to explore hormone secretion regulated by paracrine and intrinsic mechanisms in islets of Langerhans in transgenic mice to allow cell-specific expression of ChR2 (Figure 8) in islet α- β- and δ-cells.

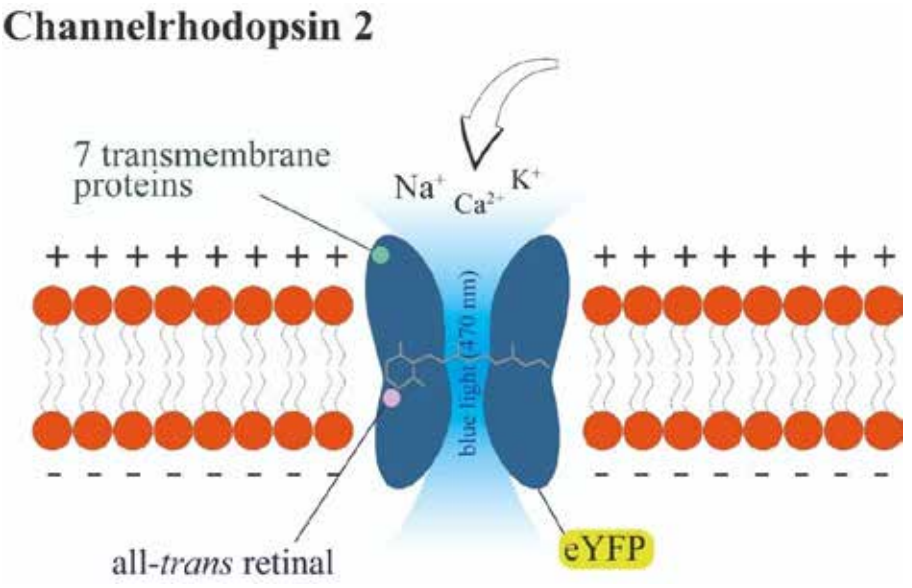

Figure 8: Channelrhodopsin is a 7 transmembrane protein found in green algae. Fusion of the ChR2 with an eYFP allows ChR2 positive cells to be identified.

AIM

T1D and T2D are multi-faceted diseases that involves the loss of/aberrant secretion of not only insulin but also the other pancreatic islet hormones.

The overall aim of this thesis was to define the physiological regulation of glucagon secretion and to define the respective roles of intrinsic and paracrine modulation.

The specific aims were to use optogenetics to:

i. elucidate how β-cells control α- and δ-cell function (Paper I);

ii. investigate the microanatomical basis for paracrine regulation of α-cells by δ-cells (Paper II);

and

iii. establish the paracrine crosstalk between α- and δ-cells (Paper III)

METHODS

Papers I-III contain detailed descriptions of the methods used for the separate studies. A short summary of key methodologies is provided below.

MOUSE MODELS

This Thesis is based on optogentic activation of α-, β- and δ-cells. To this end, we generated mice that express ChR2 specifically in these cell types.

The Ai32 mouse expresses ChR2 under the Lox system. Designed to result in larger photocurrents, the ChR2 was modified with a substitution H134R (CAC to CGC). The gene for the mutant channel is fused with an enhanced YFP (eYFP) sequence. Crossing these mice with cre harbouring mice, excises a STOP cassette upstream of the ChR2-YFP gene, allowing CAG promoter driven expression. The offspring from the ChR2-YFP x Cre cross can be used in studies of optoactivation of excitable cells using blue light illumination. Furthermore, by using light to control biological systems, we uphold spatio-temporal precision[101], keeping islet integrity while avoiding side effects when using pharmacologic drugs to control biological processes.

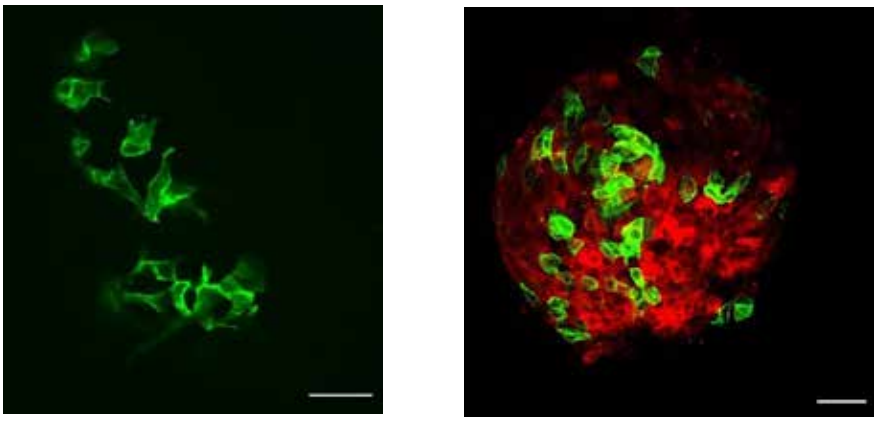

Figure 9: (left) SST-ChR2 islet (viewed as YFP fluorescence). (right) Glu-ChR2 islet stained with an insulin primary antibody and alexa fluor 594 secondary antibody. Note that there is no overlap between ChR2 and insulin. Images were acquired on a 2-photon microscope. Scale bars are 20 µm.

All animal experiments were approved by the local ethics committee at the Sahlgrenska Academy, Gothenburg University (approval number: 948/17). Ai32 Channelrhodopsin-2 (H134R)-YFP harbouring mice (Jackson

laboratories #012569) were bred with RipCre, GluiCre or SSTiCre mice, to obtain ChR2+/- RipCre+, ChR2+/- GluiCre+/- or ChR2+/- SSTiCre+/- mice.

MOUSE ISLETS

Mice were anesthetized prior to killing, which was effected by cervical dislocation. Liberase solution (Liberase TL Roche TM) was injected through the bile duct to inflate the pancreas. The pancreas was excised and digested in a water bath at 37°C for 12 minutes. Islets were handpicked under a stereo microscope in Hanks' balanced salts buffer (HBS) containing 5 mM glucose. Islets were cultured for 1 hour in RPMI containing 10% foetal bovine serum (FBS), 1% antibiotics and 10 mM glucose.

HUMAN ISLETS

Pancreatic islets were obtained from human cadaveric donors by the Nordic Network for Clinical Islet Transplantation (ethical approval by Uppsala Regional Ethics Board)[104]or the ADI Isletcore at the University of Alberta (ethical approval by Alberta Human Research Ethics Board) (Lyon et al., 2016), with written donor and family consent for use in research. Work with human tissue complied with all relevant ethical regulations for use of human tissue in research and the study was approved by the Gothenburg Regional Ethics Board. Isolated islets were cultured free-floating in sterile dishes in RPMI 1640 culture medium containing 5.5 mM glucose, 10% foetal calf serum (FCS), 2 mM L-glutamine, streptomycin (100 U/ml), and penicillin (100 U/ml) at 37 °C in an atmosphere of 5% CO_2 up to a week.

ANTIBODY STAINING

Islets were prepared by tissue fixing with 4% formalin, followed by permeabilization with 0.3% Triton X in PBS on ice for 30 minutes. Blocking of the tissue was done in PBS containing 5% FBS for 30 minutes.

Somatostatin staining was done with similar Alexa antibody but with ImmunoStar SST14/28 (Cat# 20067) or AbCam (ab8903) or novel antibodies that we developed. The dilutions of these antibodies vary and are specified under each experiment in the figure legends.

Glucagon staining was done by incubating the islets with mouse monoclonal antibody to glucagon from AbCam (cat# K79bB10; 1:200 dilution) for 2 hours and incubation with Alexa Fluor 594 Donkey anti mouse from Jackson immune laboratories (cat# 715-587-003; 1:200 dilution) for 1 hour.

Insulin staining was done by incubating the islets with guinea-pig antibody to insulin from Europroxima (cat# 2263B65-1; 1:200 dilution) for 2 hours and incubation with goat anti guinea-pig Alexa Fluor 594 (cat# ab150188; dilution: 1:200) for one hour.

Two-photon microscopy

Imaging of the stained tissue was performed in a TrimScope II, LavisionBiotec 2-photon microscope using a Ti:Sa laser (MaiTai, Spectra-physics) tuned to 990 nm. Islets were imaged by acquiring z-stacks with 1-µm step-size. Emitted light was separated using an T585LP dichroic mirror into green and red channels and recorded with PMT's H6780 Hamamatsu.

Confocal microscopy

Confocal microscopy was performed with a Zeiss LSM780 using a 20X or 40X objective (Zeiss) with sequential scanning of the red (excitation 561 nm, emission 578–696 nm) and green channel (excitation 488 nm, emission 493–574 nm). Pinhole size was 0.61 µm, corresponding to 1 Airy unit. Images were acquired in 16-bit at gain settings 750 for both channels.

STATIC INCUBATION FROM FRESHLY ISOLATED ISLETS

Hormone secretion is essential to the maintenance of glucose homeostasis[105]. Static islet incubations affords fast and robust dose-response hormone measurement in response to glucose and other stimulus[106].

Isolated islets were transferred into RPMI medium supplemented with 10% FBS, 1% penicillin/streptomycin and 10 mM glucose for 2 hours for recovery. The islets were then transferred to a dish containing Krebs Ringer buffer (KRB) composed of (mM) NaCl 140, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4,1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25, HEPES 10 (pH 7.4 with NaOH), supplemented with 7 mM glucose, 0.1% BSA and kept therein for 30-45 minutes.

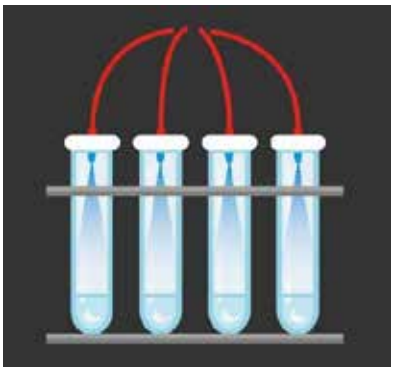

Figure 10: Each tube was capped with a lid fitted with a blue LED.

The islets were divided into groups of 10 size-matched islets and placed into round bottom test tubes containing KRB. The tubes were capped and incubated in the water bath at 37°C for one hour.

For the lights ON experiments, a blue LED (470 nm) was coupled to the tube cap as illustrated in Figure 10.

Supernatant was collected into 300 ul tubes containing aprotinin. Glucagon and insulin were measured by ELISA (Mercodia, Uppsala, Sweden). Somatostatin was measured by radioimmunoassay (Eurodiagnostica, Malmö, Sweden)

REAL-TIME WHOLE PANCREAS PERFUSION

While isolated islet perfusion is a useful means of exploring kinetics of hormone secretion, hormone measurement in the perfused pancreas is even more so, as it accounts for cellular interactions that occur in the microenvironment the islets are situated.

Dynamic measurements of glucagon secretion were performed using *in situ* pancreas perfusion as previously described[75]. Throughout the experiment, the pancreas was maintained at 37ºC and the solutions infused are kept at the same temperature. The pancreas was first perfused in the dark for 20 min with KRB containing 1 mM glucose. Glucose was then varied as indicated. Light-stimulation was effected by 2-min exposure to blue LED (162 super bright 470 nm LEDs in a 9 by 18 cm LED array; iorodeo, (Pasadena, CA, USA) as indicated. At all other times the pancreases were kept in the dark to prevent activation of ChR2. The infusion rate was calculated and adjusted individually for each animal according to body weight[107]. The perfusate was collected from the portal vein with a frequency of 1 min^{-1} and stored on ice during the experiment and then frozen at -80ºC pending analysis.

ELECTROPHYSIOLOGY

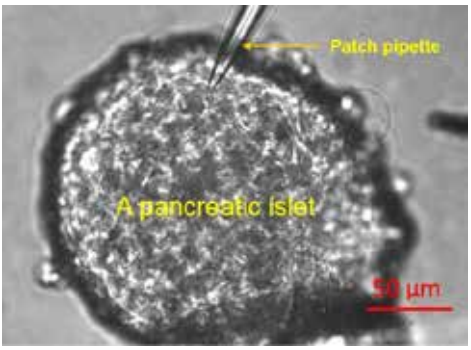

Figure 11: Patch-clamp recordings from cells in an intact pancreatic islet. The islet is held in place by suction pipette. The recording electrode (patch pipette) approaches islet from the top.

We applied the patch-clamp technique to intact pancreatic islets (Figure 11) to measure electrical activity and membrane currents in β-, α- and δ-cells [108,109]. In most cases, cell identity was established by expression of ChR2-YFP under cell-specific promoters. In other cases, cell identification was based on electrophysiologial fingerprinting as previously described[110]. Membrane potential recordings was performed by using an EPC-10 USB amplifier and Patch Master Software (HEKA Electronic, Germany). The perforated patch technique was used with 60 µg/ml amphotericin B added to the intracellular (pipette-filling) solution to achieve perforation. The extracellular solution contained (mM) NaCl 140, KCl 3.6, $CaCl_2$ 1.3, MgSO4 0.5, HEPES10, $NaHCO_3$ 5, NaH_2PO_4 0.5 (pH 7.4 with NaOH), and glucose as indicated. While the pipette solution contained (mM) K_2SO_4 76, KCl 10, NaCl 10, $MgCl_2$ 1, HEPES 5 (pH 7.35 with KOH).

Membrane potential recordings were performed within intact mouse islets, as described previously[111]. The data were analysed with Clampfit 9 (Molecular Devices, Sunnyvale, CA). All electrophysiological recordings were performed at 31-34ºC. A 470 nm LED was used to stimulate ChR2-Expressing α-cells.

CALCIUM MEASUREMENTS

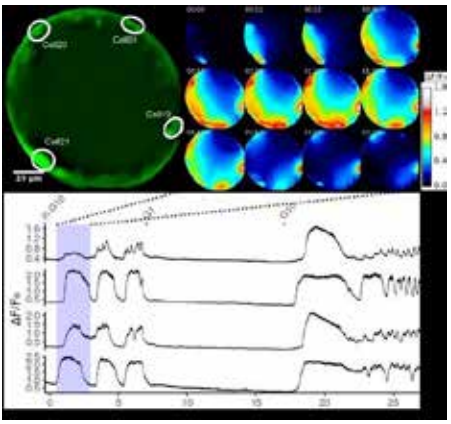

Figure 12: Calcium measured from an islet loaded with calcium dye Fluo-4. Glucose was varied from 10 mM (G10) to 1 mM (G1) and back to 10 mM (G10). Responses in 4 different cells shown (taken as indicated in image top left). The images top right show changes in [Ca2+]$_i$ displayed in pseudocolours for the period highlighted by blue rectangle superimposed on the traces (lower).

Intracellular free-Ca^{2+}([Ca^{2+}]$_i$) was recorded from intact isolated islets using the red organic calcium indicator cal-630 AM (20530, AAT Bioquest). Recordings were done on a custom microscope using a 40x, 1.15 NA, water immersion objective. A LED with peak intensity at 565 nm (M565D2, Thorlabs) served as excitation light source for cal-630. For activation of ChR2, a 473 nm fibre-coupled laser (Rapp OptoElectronics) was used.

Islets isolated from transgenic mice harbouring ChR2-YFP (Lox) and GluiCre$^+$ or SSTiCre$^+$ (iCre) were loaded with cal-630 by incubating around 10 islets for 60 minutes at room temperature in Extracellular Solution (ES) supplemented with 5.5 mM glucose, 1 mg/mL BSA (Sigma-Aldrich), 0.04% pluronic acid (F-127, Invitrogen) and 5 µmol/L cal-630 (20530, AAT Bioquest). For imaging, one islet was transferred to the centre of a poly-L-lysine (P4707, Sigma-Aldrich) coated 12-mm coverslip mounted in an imaging chamber (RC25, Warner Instruments) on the microscope stage. The remaining islets were stored in fresh ES at room temperature for up to 3 h. The chamber was pre-filled with ES (with 5.5 mM glucose). The islet was superfused for 20 min before the experiment commenced. The influent medium was pre-heated to keep the centre of the coverslip at +34°C. YFP fluorescence was recorded using 488 nm laser in order to identify ChR2 expressing cells. Cal-630 time-lapse imaging was then started with an exposure time of 10 ms and a framerate of 2-10 frames per second (fps). Acquisition was controlled using the software, Micro-Manager.

The stimulation laser was triggered using an arduino microcontroller running in-house custom-designed software. The laser irradiance was measured above the microscope objective to be 3.3 mW/mm^2.

RESULTS & DISCUSSION

PAPER I

In Paper I, we explored the importance of paracrine regulation of glucagon secretion using an optogenetic model[94] on a mouse harbouring ChR2 under the rat insulin promoter (RIP). This allows an exploration of α- and δ-cell function in response to activation of β-cells.

INHIBITION OF α-CELLS BY β-CELLS

Optoactivation of β-cells evoked electrical activity and $[Ca^{2+}]_i$ oscillations in the β-cells. The amplitude of the electrical response was glucose-dependent with very little electrical activity being measured in response to optoactivation in low glucose concentrations. Optoactivation did not evoke electrical activity in β-cells from littermate control islets.

To understand the paracrine regulation of α-cells by β-cells, we optoactivated β-cells in the ChR2-YFP-RIPCre islets whilst simultaneously recording electrical activity in α-cells. We observed that optoactivation of β-cells resulted in hyperpolarization and a reduction of α-cell firing frequency. Moreover, this response was delayed by ~10 s after optoactivation of the β-cell. This effect was not observed in α-cells from littermate control islets. Finally, optoactivating β-cells resulted in a ~25% reduction in glucagon secretion from islets.

STIMULATION OF β-CELLS ACTIVATE δ-CELLS

Optoactivation of β-cells rapidly activated the δ-cells as measured by perforated patch whole-cell measurements. There was also an increase in SST secretion in response to optoactivation of β-cells.

When surveying the delay in δ- and α-cell responses to optoactivation of β-cells, we observed that the response in δ-cells was very rapid (~30 ms) whereas the result in α-cells was slower (on the timescale of 10s of seconds; see above). We postulated that the rapidity of activation of δ-cells may be due to gap-junction (GJ) coupling with the β-cell.

GJS COUPLE δ-CELLS AND β-CELLS

To determine whether the coupling of optoactivated β-cells to activation of δ-cells was indeed via GJ, we validated GJ conductance in δ-cells by patch-clamp in mice expressing RFP in δ-cells (SST-RFP mice). Exposing the islets from the transgenic mice to high glucose concentrations evoked spontaneous inward currents in (RFP^+) δ-cells under voltage-clamp. Such currents represent firing of APs in nearby β-cells, which spread to the δ-cell via the GJs, analogous to what has been described for electrical coupling between β-cells. This was verified when the currents ceased after addition of the GJ inhibitor carbenoxolone.

Our data support that GJs functionally connect β- and δ-cells. Comparing the β-cell action potential amplitude to the current waveforms observed in δ-cell suggests a GJ conductance of 1.5 nS, similar to the 1.2 nS found for the electrical coupling between β-cells.

δ-CELL MEDIATES α-CELL INHIBITION VIA β-CELL

The delay from optoactivation of the β-cell and repolarization of the α-cell (400-fold longer than the time required for stimulation of δ-cells) and the slow reversal thereof is a strong indication of a paracrine mechanism mediated by a diffusible intercellular factor. We hypothesized that the inhibitory effect of β-cell on α-cell is attributable to a factor released from δ-cells activated via the GJs. As to the identity of this factor, somatostatin is an obvious candidate. Indeed, when we applied the SSTR2 antagonist CYN 154806 to patch-clamped α-cells, optoactivation of β-cells did not inhibit the α-cell electrical activity.

Moreover, addition of CYN 154806 increased firing frequency in α-cells more than twofold when compared with control experiments, an effect that was associated with a slight membrane depolarization. These results suggested a tonic inhibition at basal conditions that affects the α-cells membrane potential as well as electrical activity.

MATHEMATICAL SIMULATIONS OF HUMAN ISLETS

To investigate the validity of these findings in human islets, we opted for a computational approach. We constructed mathematical models of islets based on detailed morphological data from human islets (the morphology was determined from serial immunofluorescence of pancreatic sections from

human donors, conducted by Prof. Manami Hara, U. Chicago). We endowed the δ-cells in the model with GJ connections to their "nearest" β-cell (if the distance was less than a specified threshold). Simulations of these models suggested that this coupling of β- to δ-cells explains approximately ~25% of the suppression of glucagon by glucose, consistent with our secretion data.

MODEL

Figure 13 provides a model to explain these results. Our data suggest the existence of a complex crosstalk between islet cells that involves intercellular communication both by electrical (gap junction-mediated) and paracrine mechanisms.

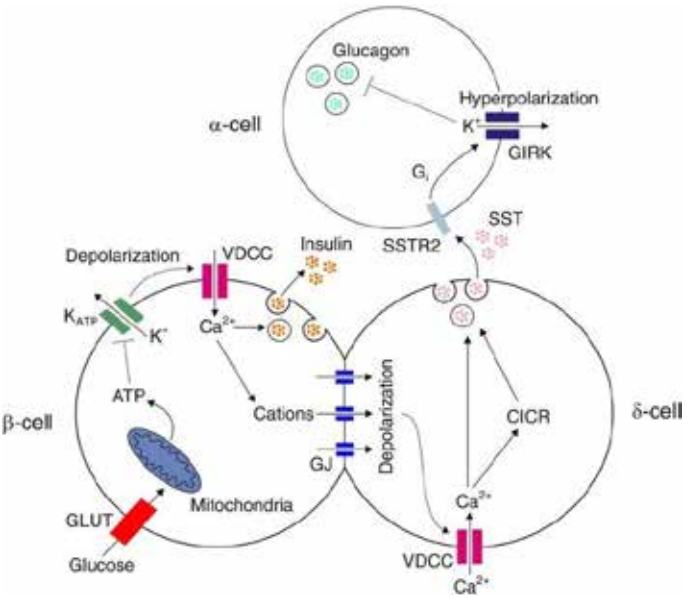

Figure 13: **Schematic summary of β- to δ- GJ pathway regulating α-cell activity.** *At high glucose, glucose enters the β-cell via GLUT. After being metabolized in the mitochondria there is a resulting increase in ATP, which closes the K_{ATP} channels, depolarizing the cell membrane. Membrane depolarization opens Ca^{2+} channels allowing Ca^{2+} entry resulting in insulin secretion. We have shown that the depolarization incites activation of the gap junctions with coupled δ-cells. This current in turn depolarizes the δ-cell and Ca^{2+} entry via Ca^{2+} channels, driving somatostatin release.*

Whether electrical signalling is mediated by pannexins (which are thought to exist in δ-cells[112]) or connexins remains to be determined. Another interesting aspect is whether electrical coupling proceeds exclusively from β- to δ-cells or whether it also goes the opposite direction. It is important to notice that whereas all δ-cells are likely to be connected to at least one β-cell, most β-cells are probably not in direct electrical contact with δ-cells (as they are only surrounded by β-cells).

PAPER II

Paper I focussed on electrical coupling of δ-cells and β-cells and how the secreted somatostatin regulated α-cells. We next attempted to develop an efficient method for detecting somatostatin secreted from δ-cells. A secondary objective was to study the arrangement of somatostatin-secreting δ-cells in mouse and human islets and whether it is altered in T2D. To investigate these objectives, we sought to develop a highly specific antibody as a tool for detecting δ-cells in the pancreas.

After synthesis, prosomatostatin undergoes post-translational modification resulting in somatostatin 14 (SST14) and somatostatin 28 (SST28), containing 14 and 28 amino acids, respectively (Figure 14)[113]. These peptides are very short-lived with a half-life of only a few minutes in blood[114]. Somatostatin-producing cells are distributed throughout the body. They occur in δ-cells of the pancreas and enteroendocrine cells in stomach and duodenum and neurons in the hypothalamus[115]. We developed 6 antibodies with high specificity for SST14 or SST28 to identify the entire somatostatin-producing cell population efficiently.

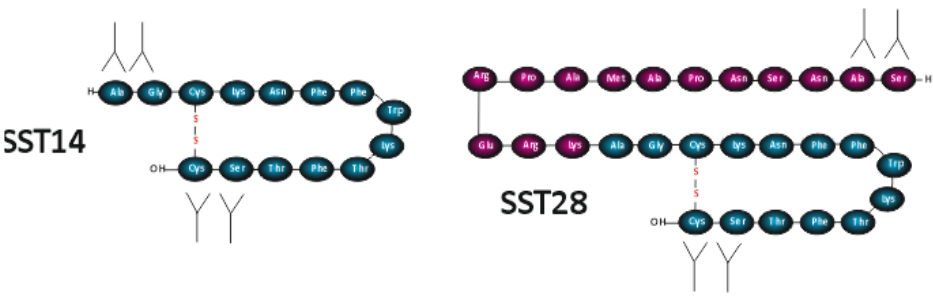

Figure 14: Depiction of SST14 and SST28 with antibodies detecting the N-terminal of C-terminal parts of the peptide.

NOVEL ANTIBODIES SHOW HIGH LEVEL OF CO-LOCALIZATION WITH δ-CELLS

We investigated expression of somatostatin using the antibodies we developed. For this purpose, mouse tissues such as islets from the pancreas, hypothalamus in the brain, stomach and duodenum were obtained. Hypothalamus, stomach and duodenum were cryo-sectioned whereas islets

were fixed using paraformaldehyde. We stained sections of mouse hypothalamus, longitudinal sections of stomach and duodenum using antibodies specific for SST14 (10G5) or SST28 (32A1). Overall, of the 6 antibodies that we developed only one of the antibodies displayed a high specificity for SST14 and the other displayed a high specificity for SST 28.

NEURITE-LIKE PROCESSES

Somatostatin-containing δ-cells were described as neurone-like structures that creates a network, potentially aiding intra-islet communication[113]. We observed similar neuron-like or neurite-like processes in the islets with δ-cell specific antibodies described earlier as δ-cell signatures[32]. Our assessment showed >80% of the stained δ-cells presented neurite-like structures.

δ- AND α-CELL INTERACTIONS IN PROCESSES IN MOUSE

The projecting δ-cells have been previously described to interact with α- and β-cells[30,32]. To confirm that, we used islets from ChR2-YFP expressing under the proglucagon promoter (Glu-iCre) (see Paper III) labelling α-cells with YFP. Islets from the transgenic mice stained with 10G5 showed a peripheral arrangement of α- and δ-cells. Both α- and δ-cells were in close vicinity to each other (Figure 15). Higher resolution images of these structures showed δ-cells that are arranged in "clusters" on the mantle of the islets. The mantle had spaces in the form of "holes". These holes/spaces were filled by α-cells ensuring the physical interaction of α- and δ-cells aided by projecting δ-cells surrounding the α-cells. Overall, physical interaction of α- and δ-cells of the mouse islets ensures regulation of paracrine secretions from these islets[32].

δ-CELLS IN HUMAN ISLETS

The arrangement of δ-cell distribution in the human islets is very different to the distribution seen in mouse islets. The mouse islets have a more peripheral distribution of δ-cells compared to the human islets[29,24]. We investigated the distribution with SST14-specific antibody (10G5). Our results confirmed approximately 60% of the δ-cells distributed in the periphery of mouse islets compared to less than 30% peripheral distribution δ-cells seen in human islets. The total number of δ-cells per islet was approximately the same in mouse and human islets. This number decreased significantly in human islets obtained from donors with T2D. As discussed below, this may be related to

the clinical observation that many patients with T2D hypersecrete glucagon despite the prevailing hyperglycaemia.

SIGNIFICANCE

There are currently, limited assays to reliably detect somatostatin. These limitations have driven researchers to resort to radio-labelled assays[116]. Our antibodies show a high degree of efficiency in detecting δ-cells setting the basis for future assay development for secreted somatostatin, potentially enabling the release of islet SST14 from the bulk somatostatin immunoreactivity. We point out that only a small fraction of circulating somatostatin originates from the pancreas[114].

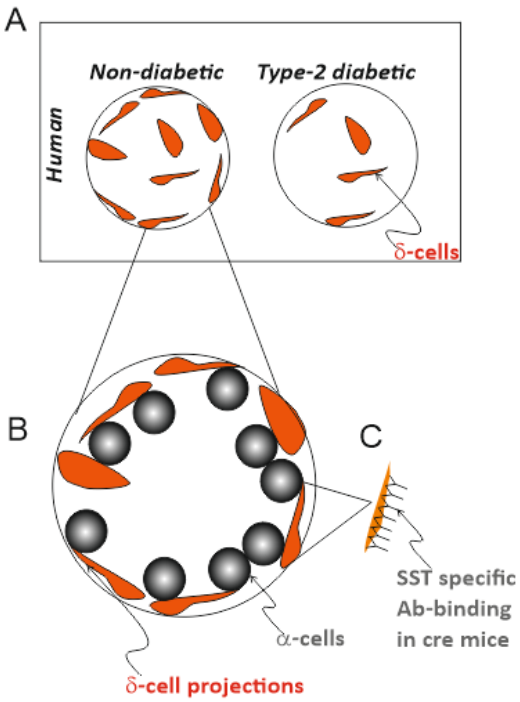

Figure 15: New insights from the study. A) Islets obtained from donors diagnosed or not with T2D show differences in number and distribution of δ-cells. B) Projecting δ-cells in the mouse islets which are peripherally distributed. The islets have δ-cells which are physically in contact with mostly peripherally distributed α-cells. C) Our antibodies specific for SST14 detect δ-cell specific somatostatin efficiently in the SST-ChR2 mouse islets.

PAPER III

After successfully recording the response in non-β-cells when optoactivating β-cells in the previous paper, we extended the observations to non-β cells. To this end we generated two mouse models that specifically express the light-sensitive ion channel channelrhodopsin in α- and δ-cells,

THE ROLE OF MEMBRANE POTENTIAL IN INTRINSIC REGULATION OF GLUCAGON RELEASE

Glucose regulates glucagon secretion from pancreatic α-cells, but the mechanisms involved remain hotly debated[55,105,117]. As discussed above, both intrinsic and paracrine mechanisms have been proposed to mediate the effects of glucose. There is also a debate whether the effects of glucose are mediated by changes in α-cell electrical activity and $[Ca^{2+}]_i$ or if they are exerted at the level of exocytosis itself. Using photoactivation of α-cells to induce 'electrical activity' our data confirm that electrical activity, via increases in $[Ca^{2+}]_i$, stimulates glucagon secretion. Importantly, the relative stimulatory effect of optoactivation was stronger at high glucose than at low glucose. Although this is the consequence of glucagon secretion being stimulated by low glucose, it is difficult to reconcile this observation with the idea that glucose exerts its inhibitory effect by an effect mediated distal to electrical activity/elevation of $[Ca^{2+}]_i$. In fact, the small inhibitory effect (20%) of glucose when comparing glucagon secretion at low and high glucose in light-stimulated Glu-ChR2 islets may result from paracrine signals (such as somatostatin).

TONIC INHIBITION OF α-CELLS BY ENDOGENOUS SOMATOSTATIN

We found that 40% of the α-cells (identified by expression of fluorescently tagged ChR2) were electrically silent at low glucose. This is in agreement with the earlier observation that 30% of α-cells identified by their responsiveness to β-adrenergic stimulation did not exhibit any spontaneous $[Ca^{2+}]_i$ oscillations at low glucose[118]. Such inactive cells could be activated by application of the somatostatin receptor 2 antagonist CYN154806, suggesting that they are under tonic inhibition by intraislet somatostatin. It is therefore of interest that 20% of δ-cells are spontaneously active at 1 mM glucose[119]. This observation might account for the strong stimulation of

glucagon secretion produced by CYN154806 in isolated islets incubated at 1 mM glucose[72].

ELECTRICAL ACTIVITY STIMULATES α-CELL METABOLISM

We also found that the α-cells in islets from Glu-ChR2 mice that were electrically silent at 1 mM glucose could be activated by light. This was seen as a gradual depolarization during light stimulation and the continuation of action potential firing even after the discontinuation of stimulation. In β-cells it has been reported that Ca^{2+} entry during electrical activity stimulates mitochondrial ATP production[120]. Thus, it is possible that electrical activity in α-cells stimulates further activity by a feed-forward mechanism. The ion channel underlying this effect remains to be established but the K_{ATP} channel is an obvious candidate. For reasons that are unknown, these channels may be slightly more active in electrically silent α-cells than in α-cells firing action potentials. This idea is not incompatible with the idea that electrically silent cells are under tonic inhibition by somatostatin. Somatostatin activates G protein-coupled inward rectifying K^+ (GIRK) channels[121]. The capacity of the α-cells to generate electrical activity is controlled by the total K^+ channel activity and for action potential firing to occur it must be reduced sufficiently for the membrane potential to exceed the threshold potential for action potential initiation. Conceivably, this can be achieved by closing GIRK (by CYN154806) or K_{ATP} channels.

OPTOGENETIC CONTROL OF δ-CELL ACTIVITY AND SOMATOSTATIN RELEASE

The finding that endogenous somatostatin leads to tonic inhibition of a subset of α-cells illustrates the importance of a better understanding of the crosstalk between α-and δ-cells. To explore this aspect further, we generated mice that express ChR2 in δ-cells.

In order to validate our ChR2-YFP x SSTiCre (SST-ChR2) mouse, we immunostained islets from transgenic mice with SST antibodies. Immunofluorescence confirmed that the vast majority ChR2-YFP-expressing cells were somatostatin-positive (see paper II). The ChR2-YFP cells show neurite-like projections (1 per cell), conforming with the known morphological characteristics of δ-cells[113].

Electrophysiology showed that 87% of the δ-cells were silent at 1 mM glucose but depolarized and started generating large overshooting action potentials when glucose was elevated to 10 mM. As expected, these ChR2-YFP-expressing cells exhibited the electrophysiological characteristics of δ-cells (including large voltage-gated Na^+ currents and transient A-type K^+ currents)[110].

Optoactivation of islets from Sst-Chr2 mice increased $[Ca^{2+}]_i$ in δ-cells but not in non- δ-cells (that did not express ChR2-YFP). The increase almost entirely reflected voltage-dependent 'recruitment' of the δ-cells' endogenous VGCCs. This is suggested by the finding that the light-induced increase in $[Ca^{2+}]_i$ was nearly abolished by isradipine, a blocker of L-type Ca^{2+} channels (one of the Ca^{2+} channels expressed in δ-cells). These observations argue that the Ca^{2+} influx observed upon light stimulation occurs via VGCCs and not through ChR2 itself.

Somatostatin secretion from δ-cells is Ca^{2+}-dependent[119,122]. We observed that optoactivation of δ-cells increased somatostatin release both at low and high glucose. The observation that glucose exerts an additive effect in excess of that produced by optoactivation alone is consistent with the observation that glucose stimulates somatostatin release in mice lacking functional K_{ATP} channels[123]. This suggest that part of the stimulatory effect of glucose is exerted downstream of action potential firing. Possible mechanisms include Ca^{2+}-induced Ca^{2+} release. In future experiments it will therefore be of interest to investigate the effects of thapsigargin (an inhibitor of SERCA, the Ca^{2+} pump in sER) and ryanodine (an inhibitor of Ca^{2+}-induced Ca^{2+} release) on the $[Ca^{2+}]_i$ increases induced by optoactivation in δ-cells.

REGULATION OF GLUCAGON SECRETION BY SOMATOSTATIN

Abundant evidence suggests that exogenous somatostatin inhibits insulin and glucagon secretion[119,124,125,126]. Exactly how endogenous somatostatin release from δ-cells impacts on insulin and glucagon secretion in the neighbouring β- and α-cells has only partially been resolved. In Paper I we show that optoactivation of β-cells leads to inhibition of glucagon secretion and that the effect is mediated by electrical activation of the δ-cells (via the gap junctions) and stimulation of somatostatin secretion[96]. With access to SST-ChR2 mice, we can directly activate the δ-cells without taking the 'detour' via the β-cell.

We found that optoactivation of δ-cells exerts dual inhibitory effects on the α-cells: it (i) repolarized the α-cell and suppressed action potential firing; and (ii) inhibited exocytosis of glucagon-containing secretory vesicles. Interestingly, the effects of optoactivating δ-cells had much clearer effects in α- than in β-cells. Why this is the case remains to be elucidated but it may be related to the fact that β-cells express a somatostatin receptor (SSTR3) with a lower affinity for somatostatin than the receptor subtype found in α-cells (SSTR2). Alternatively, the α-cells may be in closer physical proximity to the δ-cells than the β-cells. This idea is not necessarily in conflict with the finding that optoactivation of β-cells depolarize δ-cells via gap-junctions. Whilst it is clear that some δ-cells must be in close proximity to the β-cells, statistical considerations (for example, the β-cells outnumber the δ-cells by a factor of 10-15) argue that most β-cells must reside some distance away from the nearest δ-cell.

The inhibitory effects in α-cells produced by optoactivating the δ-cells developed with a 12 s delay, similar to that observed when optoactivating β-cells (paper I). We attribute this delay to the time it takes for somatostatin to diffuse within the islet interstitium from the δ-cell to the target α-cell and to activate the intracellular single transduction mechanisms.

Measurements of glucagon release indicated that the inhibitory effect of optoactivating the δ-cells was nearly as strong as the maximum glucagonostatic effect of glucose. It is possible that previous studies using SSTR antagonists have underestimated the true contribution of somatostatin signalling to the regulation of glucagon secretion *in vivo*. Given that we observe a reduction of the number of δ-cells in human islets from donors diagnosed with T2D, it is possible that the hypersecretion of glucagon seen in T2D[127] is a consequence of reduced paracrine suppression. In this context, it is notable that we were also able to observe a strong effect of δ-cell optoactivation on glucagon secretion in the intact *in situ* perfused pancreas. In this preparation, which involves the perfusion of the pancreas via the vascular network at the physiological rate, and in the correct direction, it took 4 min for the glucagonostatic effect of optoactivating the δ-cells to reverse. This suggests that somatostatin may be involved in the minute-by-minute regulation of glucagon secretion *in vivo*. Again, the effects of δ-cell activation were much stronger on glucagon than insulin secretion. Clearly, the opportunity to combine optogenetics with whole-pancreas measurements of islet hormone secretion opens exciting experimental opportunities.

CONCLUSION OF PAPER III

The studies in Paper III extend the observations made in Paper I and suggest the existence of a tight regulatory network within the pancreatic islets. This work illustrates the usefulness of optogenetics to explore the cell physiology of hormone secretion in the α- and δ-cells. A definitive advantage of this methodology is that it allows cell function to be modulated in a non-invasive fashion. In addition, it allows the synchronization of the activity of all cells in the islet (or even pancreas), which is clearly not feasible with traditional (electrophysiological) techniques. These studies provide a framework for future studies aiming to determine paracrine regulation of islet hormone secretion.

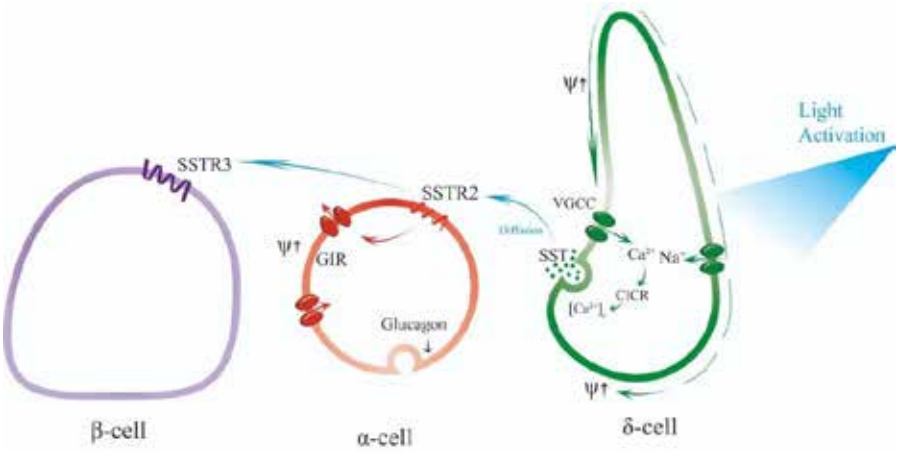

Figure 16: Photoactivation of ChR2 in δ-cells (lightening) leads to membrane depolarization (Ψ↓). The depolarization propagates across the δ-cell plasma membrane and activates voltage-gated Ca^{2+} channels (VGCC). Associated Ca^{2+} influx produces a small initial increase in $[Ca^{2+}]_i$ that triggers Ca^{2+}-induced Ca^{2+} release (CICR) and produces a much larger increase in $[Ca^{2+}]_i$ that amplifies exocytosis of somatostatin (Sst)-containing vesicles. Released somatostatin diffuses in the islet interstitium to neighbouring α-cells where it activates SSTR2. The short diffusion distance combined with the receptor's high affinity for somatostatin ensure strong paracrine effects, culminating in opening of G protein-coupled inwardly rectifying K^+ (GIRK) channels, membrane repolarization (Ψ↑) and inhibition of plasmalemmal VGCC, leading to suppression of glucagon exocytosis. The insulin-secreting β-cells are situated further away from the δ-cells and the longer diffusion (for the majority of the β-cells) means that the effective Sst concentration is lower when it reaches the β-cell. In addition, the SSTR subtype expressed in β-cells (SST3) has a 5-fold lower affinity for somatostatin than the SSTR2s present in α-cells. Collectively., these factors mean that somatostatin is a relatively weak paracrine regulator of the β-cell, which ensures that glucose-induced somatostatin secretion does not prevent glucose-induced insulin secretion.

CONCLUSION OF THE THESIS

The specific Aims have been addressed as follows:

i) β-cells control in a hierarchical fashion: electrical stimulation of β-cells spreads electrically to δ-cells via gap-junctions and the resultant stimulation of action potential firing and somatostatin secretion inhibit glucagon secretion by a paracrine mechanism mediated by diffusion of somatostatin;

ii) Using a novel and specific antibody against SST14, the δ-cell variety of somatostatin, we found that δ-cells concentrate to the islet periphery, where they form an extensive network that may ensure effective paracrine regulation of α-cell electrical activity/glucagon secretion, and that the number of δ-cells is reduced in islets from human donors with T2D;

and

iii) somatostatin released from δ-cells exerts a particularly strong paracrine suppression of α-cells whereas β-cells are much less affected – differences that may be attributable to the islet microanatomy as well as different somatostatin receptor subtypes.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Research on the causes of diabetes has traditionally focused on defects of insulin action and secretion. This β-cell-centric perspective has recently been replaced by a more nuanced 'multihormonal' viewpoint and there is increasing recognition of the roles played by islet glucagon and somatostatin in the aetiology of both T1D and T2D. This warrants further research into physiology of non-β-cells. For example, aberrant secretion of glucagon is a hallmark of both T1D and T2D. Clinically, this exacerbates hyperglycaemia due to the insulinopaenia and complicates insulin therapy because of counter-regulatory glucagon secretion in response to hypoglycaemia being compromised[8]. Our understanding of why these disturbances develop in the α-cell remains – at best – fragmentary. This lacuna in our knowledge is exacerbated by the rudimentary knowledge about the function and role of the somatostatin-secreting δ-cell in health and disease.

The three papers that constitute this thesis highlight the very important role of δ-cell in the regulation of glucagon secretion. Our finding that somatostatin mediates much of the glucagonostatic effect of high glucose combined with the discovery of a lower number of δ-cells in islets from organ donors diagnosed with T2D might be directly related to the hypersecretion of glucagon in T2D patients under hyperglycaemic conditions.

The studies also illustrate the usefulness of optogenetics to dissect the regulation of islet hormone secretion, both at the level of the individual endocrine cells but also at the level of the entire islet micro-organ (or even the entire pancreas).

Future efforts should focus on the application of these techniques to models of human T1D and T2D. Naturally, human islets are the best model. With improved methods for maintaining human islets in tissue culture it should be possible (by viral introduction of, for example, ChR2 under cell-specific promoters) to apply optogenetic approaches also to this preparation and thereby determine whether the information gleaned from the mouse models can be extended also to human islets.

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