Exploring alpha cell activation, inhibition, and heterogeneity using genetically-encoded biosensors

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ABSTRACT

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The α-cells of the pancreatic islets are defined by their hormone, glucagon, which they release in response to signals indicating increased demand for glucose in the body. They exist in dynamic co-regulation with their neighboring β-cells, which release insulin to lower blood glucose, and δ-cells, which secrete somatostatin to impose local inhibitory feedback in the islet. The activity of all three cells are largely reliant on circulating glucose levels, but α-cells are also activated by nutrients like amino acids and hormones like epinephrine and arginine vasopressin. This system is disrupted in diabetes, and glucagon dysfunction ultimately contributes greatly to diabetic hyperglycemia and impaired counterregulation. Understanding what goes wrong in diabetes is complicated by that fact that while many physiological signals are known to regulate glucagon secretion, α-cells are often viewed as a single population and thus individual α-cell responses are not well characterized.

In this dissertation, we have applied improved genetic tools for expressing fluorescent biosensors specifically in mouse α-cells in order to gain a population-level view of their activity in intact islets in real time. In doing so we are able to interrogate how intra-islet signaling and various glucagon secretagogues, as well as diabetes, shape α-cell behavior. Chapter 1 provides a comprehensive overview of islet biology, including the changes that occur in diabetes. Chapter 2 investigates the role of another β-cell hormone, Urocortin3, in inhibitory paracrine feedback on α-cells. Chapter 3 characterizes functional heterogeneity in α-cell activation by common physiological stimuli and how diabetes affects these responses. Chapter 4 provides a summary of the work performed, concluding remarks, and plans for future studies.

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Chapter 1

Integrating the inputs that shape pancreatic islet hormone release

1.1 Preface

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1.2 Abstract

The pancreatic islet is a complex mini organ composed of a variety of endocrine cells and their support cells that work in concert to tightly control blood glucose homeostasis. Changes in glucose concentration are commonly regarded as the chief signal controlling insulin-secreting β-cells, glucagon-secreting α-cells, and somatostatin-secreting δ-cells. However, each of these cell types is highly responsive to a multitude of endocrine, paracrine, nutritional, and neural inputs, which collectively shape the final endocrine

output of the islet. Here we review the principal inputs for each islet cell type and the physiological circumstances in which these signals arise through the prism of the insights generated by transcriptomes of each of the major endocrine cell types. A comprehensive integration of the factors that influence blood glucose homeostasis is essential if we are to succeed in improving therapeutic strategies to better manage diabetes.

1.3 Introduction

Over the past four decades, the number of adults with diabetes has nearly quadrupled with over 420 million individuals estimated to be affected by the disease worldwide[1]. As these numbers are expected to continue to rise, it is evident that improved therapeutic strategies to manage diabetes are necessary. Diabetes is a disease of chronically high blood glucose stemming principally from insulin impairment. However, defects in glucagon secretion – inappropriate hyperglucagonemia as well as impaired counterregulation – are also inextricably intertwined with the etiology of diabetes[2]. This places the source of insulin and glucagon – the pancreatic islets – in the crosshairs of researchers' attempts to understand and ameliorate the disease. A better appreciation for the mechanisms controlling islet hormone secretion is imperative to developing better strategies for dealing with diabetes.

The pancreatic islets are a heterogeneous mixture of endocrine cells and non-endocrine support cells that maintain homeostatic blood glucose levels via balanced hormone secretion. The β-cells make up (50-75%) of the islet cell mass in humans, and 60-80% in mice (Figure 1.1)[3-5], and are the sole source of insulin in the body[6]. Insulin release, triggered by increased blood glucose[7,8], lowers glycemia through the net effect of decreased glycogenolysis and gluconeogenesis at the liver and skeletal muscle and increased uptake of glucose in the liver, skeletal muscle, and adipose tissue[9,10] (Figure 1.2). Insulin further stimulates nutrient uptake and triglyceride (TG) synthesis in adipocytes.

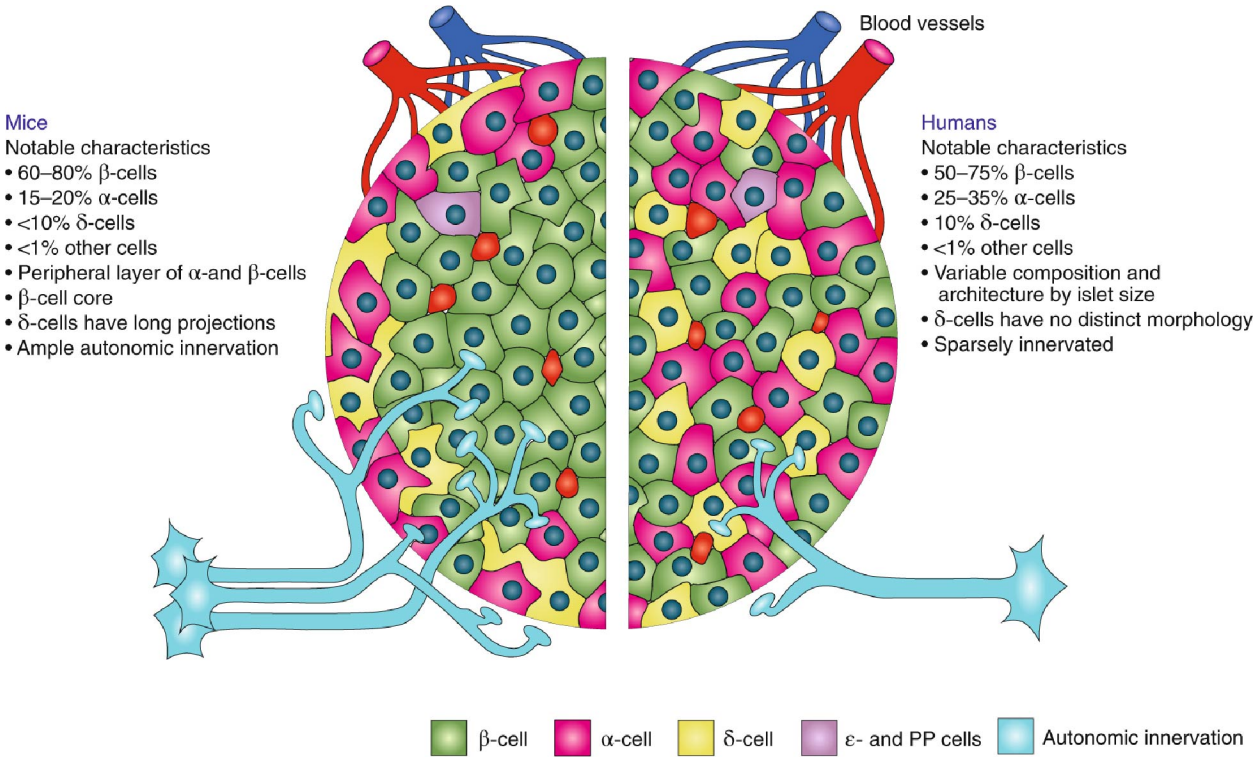
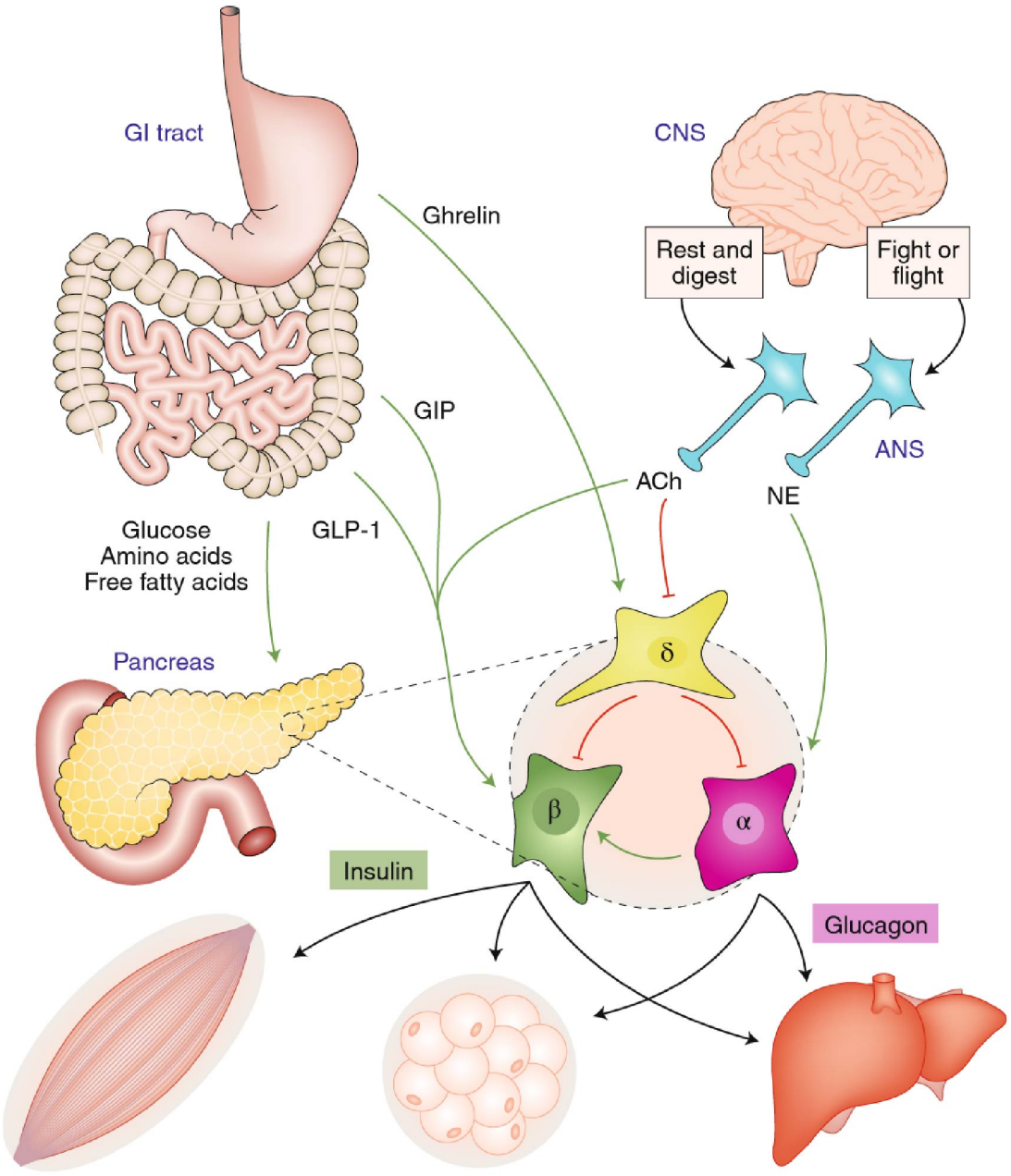


Figure 1.1 Comparative architecture of pancreatic islets of mice and humans. Pancreatic islets of mice and humans differ in important ways, but also share many features in common. These shared features make mouse islets useful experimental models to study many aspects of human islet biology. The relative proportions of endocrine cell types in mouse (left) and human islets (right) are similar with β-cells (β; green) comprising the majority of the islet cell mass followed by α-cells (α; light red) and δ-cells (δ; yellow). Other islet endocrine cells such as pancreatic polypeptide and epsilon cells (PP & ε; purple) are more sparse in number. Human islets occur in a wide variety of sizes and conformations that range from highly structured to more random distributions of cells. Mouse islets exhibit a more uniform architecture with α- and δ-cells at the islet periphery surrounding a β-cell core. Islets in both species are vascularized (dark red) and innervated (dark blue) for rapid sensing of changing energy needs, although mouse islets are more densely innervated than humans.

Collectively these insulin actions restore normoglycemia following a meal. α-cells are the second most abundant islet cell type, accounting for approximately 15-20% and of the endocrine cells in mice, and 25-35% in humans (Figure 1.1)[3-5]. α-cells secrete glucagon as a counterregulatory signal in response to hypoglycemia, and is additionally potentiated (amplified) by adrenergic stimulation and circulating amino acids. Glucagon increases

hepatic glucose production primarily via increased glycogenolysis and gluconeogenesis (Figure 1.2)[11]. δ-cells make up 5-10% of the islet[3] and release somatostatin dose-dependently in response to high glucose[12,13]. While insulin and glucagon are true hormones that are released into the circulation to elicit effects on target cells distant from site of release, somatostatin instead provides local inhibitory control over α- and β-cells[14-16]. Nevertheless, this local regulation helps determine the homeostatic set point for plasma glucose[17].

However, beyond glucose, multiple levels of paracrine, endocrine, neuronal, and nutritional inputs collectively determine islet cell activity. In this review, we focus on emerging themes with regards to control of islet endocrine function. Part of this discussion will incorporate insights gained from islet cell transcriptomes that have provided a wealth of information on the inputs that do, and do not, impinge on the islet cell types they were long thought to act upon. It is not our intent to cover all possible inputs that have been attributed to islets over many years. We refer the interested reader to comprehensive descriptions of important topics such as species differences[10,18], islet innervation[19], and islet cell receptors[20-22] that have been published elsewhere. Additionally, non-endocrine islet cells such as macrophages, endothelial, and stellate cells make important contributions to the islet as a functional unit (Table 1.1) that we will not address in detail. Our goal here is to focus on areas where recent insights challenge us to reconsider traditional views of the physiological mechanisms that control islet hormone release, and we discuss specific differences between rodent and human islets where appropriate. One major theme is the renewed appreciation for amino acids as significant contributors to nutrient-stimulated α-cell secretion. With regards to intra-islet crosstalk, increased evidence of α-cell-mediated β-cell potentiation has forced us to abandon the view of glucagon as a predominantly counterregulatory hormone in favor of a model where glucagon also makes significant physiological contributions to glucose-stimulated insulin secretion. Additionally, δ-cells have emerged as physiologically important modulators of



Skeletal muscle
↓ Glycogenolysis
↓ Gluconeogenesis
↑ Glucose uptake
↑ Glycogen synthesis
↑ Amino acid uptake
↑ Protein synthesis

Adipose tissue
↑ Glucose uptake
↑ Glycogen synthesis
↑ Triglyceride synthesis
↓ Lipolysis

↑ Lipolysis

Liver
↓ Glycogenolysis
↑ Glycogen synthesis

↑ Glycogenolysis
↑ Gluconeogenesis
↑ Amino acid uptake
↑ Fatty acid catabolism

Figure 1.2 Inter-organ signaling from nutrient sensing to islet-mediated metabolic effects. Nutrition-related signals from the gastrointestinal (GI) tract combine with neuronal input from the autonomic nervous system (ANS) to direct insulin and glucagon secretion from pancreatic islets. Changes in blood glucose levels are sensed by α, β, and δ-cells, which respond by restoring blood glucose to homeostatic levels. A-cells release glucagon at low glucose to increase hepatic glucose production. During hyperglycemia, β-cells lower blood glucose by releasing insulin to increase glucose storage in the liver, skeletal muscle, and adipose tissue. Insulin release is amplified by the incretin hormones GLP-1 and GIP from the small intestine as well as by glucagon from neighboring α-cells. δ-cells secrete somatostatin across a range of glucose levels, but most prominently in response to hyperglycemia. Amino acids and free fatty acids (FFAs) stimulate both α- and β-cells, and the peripheral effects of both glucagon and insulin result in reduced circulating amino acids and FFAs. The central nervous system (CNS) can augment islet secretion in conditions such as the "rest and digest" state where direct insulin secretion is further facilitate by a suppression of somatostatin secretion by acetylcholine (ACh) associated with parasympathetic innervation. Glucagon secretion is increased during the "fight or flight" response by norepinephrine (NE) released by sympathetic nerves.

insulin and glucagon secretion. Finally, we discuss how the inputs that coordinate insulin and glucagon release from healthy islets are affected by diabetes and how a better understanding of the physiological inputs into the healthy islets may be leveraged towards improved management in disease.

1.3.1 The complexities of studying islet endocrine cells

Multiple layers of nutrient, paracrine, endocrine, and neuronal signals modulate islet cell hormone secretion in addition to glucose levels. The mammalian islet is highly vascularized allowing for both rapid sensing of changes in nutritional status or circulating hormones, and for swift delivery of insulin or glucagon to peripheral tissues. Islets are also tightly innervated by autonomic neurons which supports sympathetic and parasympathetic modulation of insulin, glucagon, and somatostatin release (Figure 1.2). Briefly, the net effect of sympathetic stimulation is an increase in glucagon release and a decrease in insulin and somatostatin release[19,24]. Net parasympathetic signaling activates

PP/gamma cells
Pancreatic polypeptide (PP) cells are the fourth islet endocrine cell type, which comprise <5% of human and <2% of mouse islet mass[167]. PP cells are found in the islet and sparingly throughout the gastrointestinal tract, and release PP in response to meals[168]. PP regulates satiety and decreases appetite and food intake in rodents and humans with no apparent paracrine effect on insulin and glucagon levels[169].
Epsilon cells
Epsilon cells are the fifth endocrine cell type and are defined by the expression of ghrelin, classically known as the "hunger hormone". The epsilon cells increase in number throughout development reaching as high as 30% of the islet mass before decreasing to <5% in neonatal and <1% in adult islets[170]. Whether they play a role in both developing and adult islets is currently undefined.
Endothelial cells
Endothelial cells that make up the microvasculature of the islet are essential to proper endocrine function as the islet cells require high blood flow and blood volume to effectively sense nutrients and distribute their hormones. β-cells (and to some extent α-cells) produce a number of angiogenic and angiostatic factors that target endothelial cell receptors including VEGF-A and Angiopoietin 1[171]. Defects in β-cell-endothelial cell crosstalk in mice result in impaired GSIS and angiogenesis is vital for successful integration of transplanted islets[172].
Pericytes
Pericytes associate closely with islet capillaries, and dynamically regulate blood flow by constricting or dilating capillaries in response to signals from β-cells, endothelial cells and peripheral nerves[173]. The pericytes exhibit a certain amount of plasticity as vascular damage to islets during type 1 diabetes increases pericyte density as a possible healing response. Conversely, in type 2 diabetes the opposite occurs and vascular coverage decreases[174]. This likely contributes to impaired diabetic GSIS due to compromised in blood flow. Interestingly, pericytes also regulate β-cell function independent from their role in controlling vasculature. β-cell insulin content and expression are reduced and GSIS is impaired when pericytes are ablated suggesting that pericytes sustain β-cell maturity in a paracrine fashion[175].
Glial/Schwann cells
Glial cells are peripheral neuronal cells that have been shown to both penetrate the core of islets and form a peripheral sheath around the islet mantle. This sheath becomes more dense in response to injury such as stress or autoimmune attack during type 1

diabetes, reflecting a protective role of the glial cells[173]. The glial cells also serve a paracrine role: glial-derived neurotrophic factor increases β-cell mass and insulin content, which improves glucose tolerance[176].
Resident macrophages
The islet also contains resident macrophages, which, under non-inflammatory conditions, contribute to endocrine cell development in mice by supporting normal α- and β-cell expansion[177,178].
Stellate cells
Fibroblasts and myofibroblasts are uncommon in healthy islets, but contribute to fibrosis seen in pancreatic diseases like pancreatitis and pancreatic cancer. Stellate cells are quiescent myofibroblast-like cells that secrete fibrous extracellular matrix proteins upon activation. Stellate cells are primarily responsible for fibrosis that is occasionally observed in type 2 diabetes, which has been linked to reduced insulin expression and apoptosis among β-cells[179].
Acinar cells
The exocrine pancreatic acinar tissue releases digestive enzymes and is affected not just by nutrient status following food intake, but also by local signaling from the endocrine islet. Insulin potentiates amylase release while somatostatin and pancreatic polypeptide both inhibit exocrine secretion[180].

Table 1.1 The contributions of non-canonical endocrine cells and non-endocrine cells to proper islet function. What follows are brief descriptions of the various other cells within the islet and their relation to intra-islet signaling and diabetes.

both insulin and glucagon secretion while decreasing somatostatin. Interestingly, islet cells also synthesize a number of classic neurotransmitters such as GABA, acetylcholine, and serotonin for intra-islet signaling independent of innervation (detailed below)[25-27]. And while mouse islets have historically been suggested to be more highly innervated than human islets[28], there are also reports showing prominent autonomic innervation of humans islets[23].

The plethora of input signals that target the islet as a functional unit has made it a challenge to distinguish direct versus indirect mechanisms that modulate α- and β-cell activity. In recent years, islet cell type-specific reporter mice[29] and antibodies[30], supported

by advances in RNA-Seq approaches, have made it possible to disentangle how multiple layers of external and intra-islet signals affect each individual cell type. These efforts have generated comprehensive, high-quality bulk and single cell transcriptomes of mouse and human islet cells[31-34]. Advances in functional imaging and electrophysiology have similarly made characterizing islet responses much more cell-type specific: Genetically-encoded calcium indicators have improved upon traditional calcium dyes by enabling targeted functional imaging of populations of a single cell type[33]. In parallel, patch-clamp recordings aggregated across hundreds of islet cells provide us with cell type-specific electrophysiological fingerprints[35]. And Patch-Seq provides a unique approach to validate single cell transcriptomes with direct functional correlates acquired by patch-clamp measurements on the same single cell[36,37]. These technical advances now make it more feasible than ever to distinguish direct versus indirect actions on islet cells with single cell resolution.

As detailed in this review, the collective inputs that influence islet secretion are similar enough between mice and humans that mouse models, with their ease of experimental manipulation, offer unparalleled advantages in understanding islet biology. Nevertheless, species differences do exist. Mouse islets are organized as a core of β-cells surrounded by a mantle of α- and δ-cells (Figure 1.1)[3]. This same architecture is seen in islets from young humans, but adult human islets exhibit a variable assortment of islet architecture from the rodent-like mantle-core organization to a more intermingled distribution of α, β, and δ-cells – a setup well suited for paracrine signaling through the interstitial space[10,38,39]. And while many islet paracrine signals are shared between species, although some such as islet amyloidogenic polypeptide (IAPP) and peptide YY (PYY) are notably enriched in mouse over human islets[22,40]. Morphologically, human δ-cells are relatively compact while mouse δ-cells have long, neuron-like projections, which may help them overcome the distance from mantle to core when releasing somatostatin to inhibit

β-cell activity[15]. In spite of these differences, mouse and human islets share responses to many external factors and intra-islet paracrine signals that shape the final islet output.

1.4 Nutrient stimulation of α, β & δ-cells

Most textbooks offer the glucose-centric view that insulin secretion is triggered when glucose values rise over a threshold of 5 mM (7 mM in mice)[41]. Meanwhile, α-cells release most glucagon under hypoglycemic conditions, and demonstrate modest glucagon secretion under hyperglycemic conditions. However, insulin and glucagon play a large role in the metabolism of not only carbohydrates, but also of lipids (free fatty acids)[9,42] and proteins (amino acids)[9,43] as detailed in this section.

1.4.1 Glucose-stimulated insulin and somatostatin secretion

Glucose is arguably the single most important signal that controls insulin release, although glucose fluctuations in healthy subjects are relatively modest and would not by themselves elicit robust insulin secretion. Full insulin secretion *in vivo* requires glucose stimulation that is potentiated by the combined actions of other nutrients, endocrine, and paracrine factors. Glucose-stimulated insulin secretion (GSIS) is initiated when β-cells sense increases in blood glucose via glucose transporters (GLUT1 in humans and GLUT2 in mice)[44,45] (Figure 1.3). This glucose serves as a substrate for glycolysis and oxidative phosphorylation, generating ATP and leading to an accompanying drop in ADP levels. This shift in ATP/ADP ratio closes ATP-sensitive potassium channels (K_{ATP} channels), which causes membrane depolarization. This in turn opens L-type voltage-gated calcium channels (VGCCs), leading to a calcium influx and calcium-induced calcium release that triggers exocytosis of insulin secretory granules[18]. While L-type VGCCs are responsible

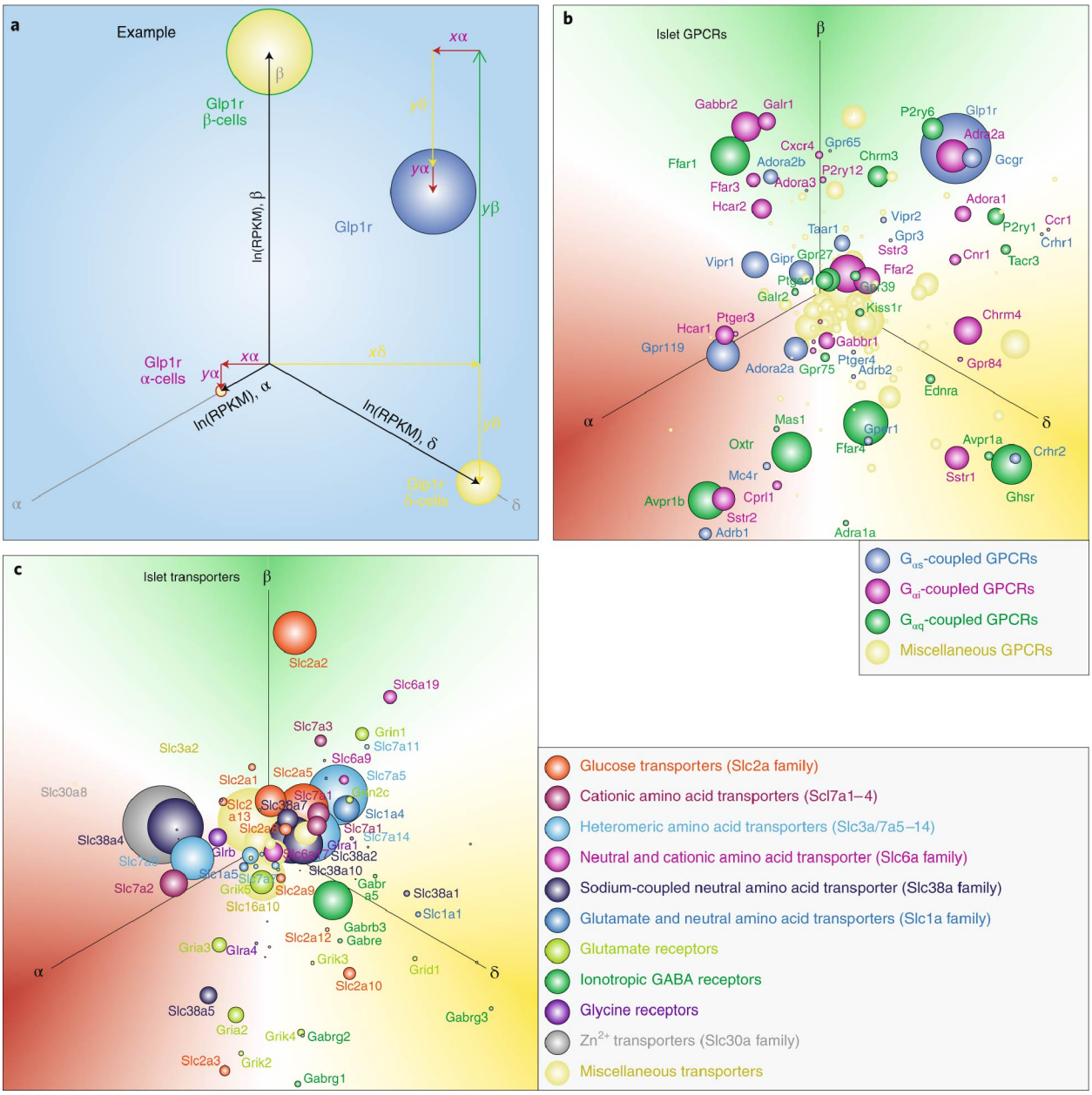


Figure 1.3 Visual representation of the abundance and cell-type selective enrichment of GPCR and transporter gene expression in α, β, and δ-cells. We used the natural log of the normalized expression values for a gene (ln[RPKM]) to plot the relative position of that gene along three axes representing α, β, and δ-cells. These expression values are derived from transcriptomes of FACS-purified mouse α, β, and δ-cells described elsewhere[33]. **a)** Each of these three individual gene expression values are converted into x and y vectors and then consolidated into a single set of x, y coordinates that represents the overall selectivity of the expression of that gene. The origin represents equal expression (*i.e.* no enrichment) in each of the three islet cell types, whereas. placement in any direction along one of the axes reflects enrichment in the...

Figure 1.3 cont. corresponding cell type. Sphere and font sizes are proportional to abundance of the gene based on the highest RPKM value for that gene in α, β, or δ-cells. **b)** The top 150 most abundant G protein-coupled receptors (GPCR) of the islet cells are color coded in accordance with the predominant signaling cascade associated with each receptor. Blue genes are $G_{\alpha s}$-coupled, green are $G_{\alpha q}$, red are $G_{\alpha i}$, and yellow is 'unknown' or ambiguous based on receptor classifications from IUPHAR (International Union of Basic and Clinical Pharmacology)[166]. **c)** Non-GPCR receptors and transporters are color-coded according to the class of signaling molecules utilized by each receptor/transporter, following IUPHAR classification for solute carriers.

for the majority of calcium currents in mouse β-cells, in humans P/Q-type VGCCs are about equally as involved as L-type channels[41].

The δ-cell signal transduction for glucose-stimulated somatostatin secretion (GSSS) shares many features with β-cells[15] albeit with a few distinctions. K_{ATP} channel closure and subsequent membrane depolarization is required, but in contrast with β-cells, calcium-induced calcium release plays a much larger role in GSSS than in GSIS[46]. This calcium-induced calcium release is mediated by both R-type VGCCs and by cAMP-driven amplification pathways[47]. Additionally, δ-cells have been suggested to be electrically coupled to β-cells[48]. The propagation of depolarization from glucose-activated β-cells to δ-cells may help potentiate somatostatin release. However, δ-cells are active at lower glucose concentrations than β-cells, possibly due to a difference in K_{ATP} activity[49]. Moreover, somatostatin secretion in response to hyperglycemia is synchronous with insulin secretion, albeit with a 30 second to 2 minute delay[50]. Such a delay is not readily reconciled with a model where δ-cells operate in lock-step with β-cells mediated solely through gap junction-mediated coordination and suggests an important paracrine component to the coordination between β- and δ-cells.

1.4.2 Glucose-mediated glucagon secretion

The mechanism underlying glucose-mediated α-cell activation remains incompletely understood. α-cells express analogous machinery to that used for GSIS in β-cells and

similarly rely on K_{ATP} channels and VGCCs for secretion, yet α-cells are active at low as opposed to high glucose. While there is no consensus paradigm for glucagon secretion, one current model is that increasing glucose induces a K_{ATP}-driven depolarization to inactivate voltage-gated Na^+ channels[51]. By driving Na^+ channels to a non-conducting state, α-cells are unable to reach the membrane potential necessary for VGCC-opening and cease to secrete glucagon. Conversely, under hypoglycemic conditions, α-cell K_{ATP} channels operate at a low level that holds α-cells in an electrically active state and causes small depolarizations that open P/Q-type VGCCs. While this model of α-cell activation helps explain some of the dynamics of glucagon release, a full explanation of α-cell activity in response to hypoglycemia likely involves a combination of α-cell-intrinsic, paracrine, endocrine, and neural factors. Indeed, when stripped of the paracrine influence of δ-cells, α-cell glucagon release is uniformly increased across a gradient of glucose concentrations[52]. This suggests that paracrine factors such as somatostatin have significant influence on the glucose-responsiveness of α-cells, which we will revisit in more detail.

1.4.3 Amino acids

β-cells are sensitive to circulating amino acids and insulin signaling promotes both amino acid uptake and protein synthesis in skeletal muscle[9]. β-cells express high levels of cationic amino acid transporters (CATs) and sodium-coupled neutral amino acid transporters (SNATs) (Figure 1.3c)[32,33]. The mechanisms by which amino acids stimulate insulin secretion vary[18]. Many amino acids including arginine, lysine, leucine, and glutamine depolarize β-cells upon import, either directly if they carry a positive charge[53], or due to sodium co-transport[54]. The ensuing depolarization triggers calcium influx to stimulate insulin release[55]. In parallel, amino acids such as alanine, glutamic acid and glutamine can fuel components of mitochondrial metabolism, thus increasing the ATP/ADP ratio[56]. Other amino acids such as glycine potentiate insulin secretion via its

ionotropic glycine receptor (GlyR) on the β-cell surface[57] (Figure 1.3c). Furthermore, paracrine interactions contribute to the effects of amino acids on insulin.

α-cells are highly sensitive to increases in amino acids, and are stimulated by 17 of the 20 natural amino acids[58]. This largely explains the post-prandial spike in glucagon in response to a normal mixed meal[59]. It is vital that glucagon is released along with insulin in response to amino acids because the two hormones will synergistically increase amino acid uptake in response to protein ingestion while effectively countering each other's actions on carbohydrates. Glucagon signaling in the liver increases hepatic utilization of amino acid substrates in gluconeogenesis[43], leading to a decrease in circulating amino acids. This important safety mechanism ensures normoglycemia during protein-rich, carbohydrate-low diets[59]. The liver-α-cell axis that connects α-cells, amino acids, and the liver is one of the primary mediators of amino acid homeostasis, highlighting the important role of glucagon in the post-prandial state.

The exact mechanism by which most amino acids directly stimulate glucagon secretion is less characterized than in β-cells, but likely involves similar mechanisms. Arginine is a potent stimulator of glucagon secretion that directly depolarizes the α-cell upon cellular transport into the cell[60]. Other amino acids induce glucagon secretion following import via abundantly expressed CATs and SNATs (Figure 1.3c) and subsequent use as metabolic substrates[32,33]. Glycine, signaling through ionotropic GlyR, can increase intracellular calcium and stimulate exocytosis in α-cells independent of amino acid transporters[61].

Much like peripheral insulin resistance causes hyperglycemia, which in turn contributes to β-cell hyperplasia (at least in mice), a similar relationship is emerging between hepatic glucagon signaling, amino acid levels and α-cell mass. Chronically elevated amino acids can influence the total capacity for glucagon secretion by stimulating α-cell proliferation through mTOR signaling[62]. Indeed, in glucagon receptor knockout mice or mice treated with glucagon receptor antagonists, interrupted hepatic glucagon signaling

causes a marked accumulation of serum amino acids that triggers a remarkable α-cell hyperplasia[63]. This expansion is largely driven by glutamine and alanine, which activate mTor signaling in α-cells through SNAT5 (SLC38A5) in mice and an as of yet unknown amino acid transporter in humans[62,64]. These drastic α-cell phenotypes are supported by clinical evidence. Hyperaminoacidemia is accompanied by hyperglucagonemia in patients with impaired liver function[65,66]. Interestingly, the liver-α-cell axis appears to function independently of glucose levels as a comparison between diabetic and non-diabetic patients with non-alcoholic fatty liver disease revealed no correlation of glucose levels to elevated amino acids and glucagon[65,66].

1.4.4 Lipids

Insulin regulates lipid metabolism by promoting glucose uptake for conversion into triglycerides, while simultaneously inhibiting lipolysis[9]. The net effect of this is to promote glucose storage as triglycerides in adipocytes. β-cell sensitivity to circulating fatty acids in addition to glucose is therefore important to nutrient balance. β-cell secretion is stimulated by fatty acids of varying chain length and saturation level[67,68]. These effects are mediated by fatty acid receptor signaling as well as by signaling downstream of intracellular fatty acid metabolism[69]. The primary receptor for circulating fatty acids expressed by mammalian β-cells is free fatty acid receptor 1 (FFAR1 a.k.a. GPR40), a $G_{\alpha q}$-coupled G protein-coupled receptor (GPCR)[33,70] (Figure 1.3b). FFAR1 supports medium- to long-chain saturated fatty acid as well as unsaturated fatty acid signaling. Most studies on FFAR1 have focused on the actions of palmitate – one of the most abundant circulating saturated fatty acids[70,71]. The $G_{\alpha q}$ signaling cascade activates phospholipase C (PLC) and induces 1,4,5 inositol-triphosphate (IP_3) formation. This mobilizes calcium from the ER, which in β-cells triggers insulin secretion[72]. Acute FFAR1 activation on the minute-to-hour timescale in mouse[73] and human[74] islets increases insulin

secretion at high glucose levels, and the strength of insulin secretion increases with the length of the FFA chain[75]. Conversely, chronic (multi-day) palmitate exposure induces dissociation between insulin granules and VGCCs that drive secretion, which results in insulin secretion being decreased by more than 50%[74,76]. Separate of IP_3-mediated activation, fatty acids that diffuse into β-cells can be converted into triglycerides and diacylglycerol that feed into GSIS amplification pathways or directly induce insulin exocytosis[69], independent of fatty acid receptors. Indeed, palmitate increases the calcium currents and increases the readily-releasable pool of insulin-containing granules[77].

The degree to which free fatty acids directly affect glucagon secretion has been debated for decades. Early studies suggested that α-cells were actually inhibited by free fatty acids[78], but there is growing evidence of an activating role[79,80]. Similar to β-cells, human α-cells express the activating receptor, FFAR1[32]. In both mice and humans, glucagon release is directly stimulated by long-chain fatty acids via the resulting downstream increase in cytosolic calcium[79,80]. Glucagon's role is primarily to modulate hepatic lipid catabolism. Glucagon signaling at the liver increases fatty acid β-oxidation[42] and decreases lipoprotein synthesis and secretion[81,82]. At the adipose tissue glucagon stimulates lipolysis, although this is balanced by simultaneous lipolysis inhibition by insulin[9]. α-cell sensitivity to circulating free fatty acids is thus an important contributor to whole body lipid metabolism.

In both mice and humans, δ-cells preferentially express free fatty acid receptor 4 (FFAR4 a.k.a. GPR120) (Figure 1.3b)[33], which is a $G_{\alpha i}$-coupled GPCR. $G_{\alpha i}$ signaling inhibits adenylyl cyclase, which catalyzes the formation of cyclic AMP (cAMP). As cAMP potentiates hormone secretion in islet cells, activation of Ffar4 in δ-cells inhibits somatostatin secretion by 50%[80,83]. Together, these fatty acid receptor profiles illustrate a system where, in the presence of high circulating free fatty acids, the combined activation of insulin and glucagon is augmented by disabling the inhibitory actions of δ-cells to handle this increased lipid load.

1.5 Paracrine signaling in the islet

Although nutrients are significant inputs to stimulate insulin and glucagon release, the islet itself is a rich source of signals that engage in intra-islet crosstalk. Paracrine signaling provides an additional layer of control over islet endocrine output that is essential for maintaining establishing and maintaining the homeostatic glucose setpoint. In particular, we will focus on recent developments in the paracrine actions of α-cells on β-cells – and vice versa – as well as the overarching role of intra-islet δ-cell signaling.

1.5.1 α-cell-mediated β-cell activation

It would seem intuitive for β- and α-cell activity to be mutually suppressive given that insulin and glucagon are functional antagonists, at least when it comes to maintaining euglycemia. Yet, while β-cell activity suppresses α-cells (mediated at least in part via δ-cells as will be discussed later), glucagon from α-cells has long been known to stimulate insulin secretion[84]. While an arrangement where α-cells stimulate β-cells, β-cells inhibit α-cells, but stimulate δ-cells, and δ-cells inhibit both may appear counterintuitive (Figure 4a), modeling studies validate that this is the most stable way to organize a 3-node interaction[85]. It is increasingly apparent that β-cells require α-cell inputs for full insulin secretion and establishing normoglycemia[86]. This realization warrants a paradigm shift in our thinking of α-cells as not only mediators of counterregulatory hepatic glucose production, but also as an important local source β-cell potentiation. The following section highlights local α-cell secretions that potentiate β-cell activation, namely glucagon as well as acetylcholine and corticotropin-releasing hormone.

The Traditional Incretin Effect. Incretins are classically defined as enteroendocrine hormones that potentiate GSIS. The two main incretins are glucagon-like

peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). GLP-1 is produced by L-cells in the intestinal ileum and derives from the same precursor protein as the glucagon that is released by pancreatic α-cells. In L-cells, proglucagon is cleaved by proprotein convertase 1/3 (PCSK1/3) to produce GLP-1, while α-cells express PCSK2 that produces glucagon instead. GIP is released from K cells in the duodenum and jejunum[87].

Together, incretins are believed to be responsible for as much as half of the insulin response to a carbohydrate meal[88,89]. B-cells express relatively high levels of the GLP-1 receptor (GLP1R), while the GIP receptor (GIPR) is more broadly expressed in multiple islet cells (Figure 1.3b). Both receptors are $G_{\alpha s}$-coupled GPCRs that potentiate insulin secretion primarily through adenylyl cyclase activation and the resulting cAMP-mediated signaling cascade[87,90]. In concert with this cAMP pathway, experiments using picomolar (more physiological) concentrations of GLP-1 revealed the involvement of the PLC/protein kinase C (PKC) pathway in β-cell depolarization and action potential firing[91]. PLC/PKC signaling ultimately opens non-selective TRPM4 and TRPM5 cation channels and the resulting Na+ current through these channels drives depolarization-stimulated insulin secretion[91]. This potentiation of GSIS is known as the incretin effect, whereby oral glucose consumption results in a markedly higher insulin response compared to intravenous administration in which glucose bypasses the GI tract. Incretins also stimulate an expansion of β-cell mass via proliferation, which further augments total insulin secretory capacity[92]. However, the incretin effect is observed during the cephalic phase of a meal, before nutrients enter the gastro-intestinal tract. This constitutes a disconnect with regard to the source of the GLP-1 that potentiates GSIS long before food reaches the ileum where most L-cells are located. A resolution of this conundrum may necessitate a re-evaluation of the physiological role of glucagon and incretin receptors and how they interact to potentiate GSIS.

Intra-Islet Glucagon Signaling. Glucagon has been known to be able to potentiate GSIS in a manner reminiscent of the incretin effect since the 1960s[84], but the importance of this local action of glucagon for islet function is only now coming into full view. Mammalian β-cells express relatively high levels of glucagon receptor (GCGR) (Figure 1.3b), as well as GLP1R and GIPR. All these receptors belong to the same family of GPCRs that share similar cAMP-mediated downstream signaling mechanisms. Glucagon and GLP-1 are both derived from proglucagon, and signal via receptors that share 47% sequence homology[93], which points to the potential for cross-reactivity between glucagon and GLP-1 and their receptors in the islet.

Indeed, multiple groups have now independently demonstrated that proglucagon-derived peptides produced by α-cells activate β-cells via either GCGR or GLP1R and that these actions are required for normal GSIS in humans and mice[94-99]. The majority of circulating GLP-1 is derived from the GI tract, and knocking out gut-derived GLP-1 is impairs oral glucose tolerance[100]. However, islet-specific GLP-1 signaling, without any contribution from the gut, is shown to be necessary for normal glucose handling[95]. Separate studies have demonstrated that simultaneously knocking down or blocking both GLP1R and GIPR in β-cells severely reduces glucose-stimulated insulin secretion and glucose tolerance[94,96]. Glucagon is the predominant α-cell ligand in mouse islets that engages both GCGR and GLP1R on β-cells to mediate most of this intra-islet signaling[94,96]. While α-cells can produce GLP-1 under certain circumstances[101], wild type mouse islets under normal circumstances secrete relatively little GLP-1[96].

The activation of GLP1R and other $G_{\alpha s}$-coupled GPCRs such as GCGR and CRHR1 (see below) on β-cells in response to locally released, α-cell-derived hormones represents a paradigm shift in our understanding of how insulin release can be potentiated. For instance, amino acids such as arginine and glutamine may elicit insulin secretion at least in part by stimulating glucagon release, which then indirectly promotes insulin release in a paracrine fashion, and not by direct stimulation of β-cells as was previously

thought[94]. Such a scenario likely requires β-cell triggering by glucose, which is going to be present along with amino acids in most mixed-meal settings. A number of groups are pursuing GLP1R/GCGR dual agonists that may prove to be a superior approach for amplifying insulin in treating type 2 diabetes given these mechanistic discoveries[102,103]. Together, these latest series of observations suggest that we need to reconsider our definition of GSIS: instead of reflecting the direct stimulatory effect of glucose on β-cells, GSIS from intact islets reflects the combined effects of glucose stimulation plus paracrine amplification via locally released glucagon and other α-cell-derived products [Figure 4a]. These observations also reconcile the traditional view of glucagon as a counterregulatory hormone with the long-known ability of glucagon to potentiate GSIS[84].

Corticotropin-Releasing Hormone. Corticotropin-releasing hormone (CRH), originally discovered as the principal hypothalamic factor to initiate ACTH release from the anterior pituitary, is also expressed abundantly in human and rat α-cells[32,104]. Interestingly, mouse α-cells do not express CRH peptide or mRNA[32], although β-cells of mice, rats, and human all express corticotropin releasing hormone receptor 1 (CRHR1) (Figure 1.3b)[105]. CRHR1 is a $G_{\alpha s}$-coupled GPCR that is related to the incretin receptors. Treating β-cells with CRH predictably induces a cAMP-mediated calcium influx[106], potentiates GSIS, protects against cytokine-induced β-cell apoptosis, promotes β-cell proliferation, and stimulates the expression of an immediate early gene signature[107]. While the physiological contribution of CRH derived from α-cells remains untested, it is another α-cell-derived peptide that is poised to potentiate GSIS.

Acetylcholine. In mice, acetylcholine originates from parasympathetic neurons innervating the islet[19] (Figure 1.2). In human islets, acetylcholine is released locally from α-cells[26,108] (Figure 1.4a). Irrespective of whether acetylcholine is of neural or paracrine origins, acetylcholine potentiates insulin secretion from mouse and human islets via the $G_{\alpha q}$-coupled muscarinic 3 cholinergic receptor (CHRM3) (Figure 1.3b)[26,108-110]. In contrast

to its stimulation of β-cells, acetylcholine inhibits glucose-induced somatostatin secretion from δ-cells. Parasympathetic tone is known to inhibit somatostatin release[24], and in mouse islets, cholinergic-mediated inhibition is prevented by pertussis toxin, suggesting the involvement of $G_{\alpha i}$-mediated signaling[111]. This is in line with the selective expression by mammalian δ-cells of the $G_{\alpha i}$-coupled muscarinic 4 cholinergic receptor (CHRM4) [Figure 3b]. These observations support a model where cholinergic signals (derived from parasympathetic innervations or from α-cells in humans islets) amplifies insulin release directly and indirectly by inhibiting δ-cells (Figure 1.2, 1.4a). It should be noted that, in direct contrast with acetylcholine-mediated δ-cell inhibition, other groups have reported IP_3-mediated stimulatory effects of cholinergic agonists on somatostatin secretion from mouse[46] and human[108] islets. Another way to use transcriptomes for decrypting signaling targets beyond just receptor profiles is to interrogate synthesis and degradation pathways for signaling molecules. δ-cells fit the profile of a target of cholinergic signaling given their receptor expression and the preferential expression of the enzyme acetylcholinesterase[33], which, in neurons, breaks down acetylcholine at the postsynaptic membrane[112]. The coordinated actions of acetylcholine on β- and δ-cells during normoglycemia may therefore contribute to the maintenance of basal insulin release between meals.

1.5.2 β-cell-mediated α-cell inhibition

There is general agreement in the field that β-cell-derived products contribute to α-cell silencing at high glucose. Insulin, serotonin, GABA, and zinc are some of the β-cell factors proposed to directly inhibit α-cells. β-cells also secrete the peptide hormone Urocortin3 (UCN3) at high glucose, which potentiates δ-cell glucose-stimulated somatostatin secretion. Given that somatostatin is a powerful inhibitor of glucagon secretion, UCN3-mediated stimulation of somatostatin release represents an indirect mechanism by which β-cells may suppress glucagon release during high glucose. Many

papers over the years have favored one signal over the other for reasons we will review here. It is plausible that there is redundancy or additivity among these β-cell-derived signals in their ability to suppress α-cells, or that they play similar roles in distinct physiological settings.

Insulin. While it is difficult to disentangle which of several β-cell-derived paracrine signals may be principally responsible for suppressing α-cells at high glucose, multiple groups have reported decrements in glucagon upon direct insulin administration[113-116]. Human and mouse α-cells express the insulin receptor (INSR)[32,33], which can maintain K_{ATP} channels in the open configuration via PI3K/AKT signaling when activated. Open K_{ATP} channels drive α-cells to a hyperpolarized state that prevents glucagon granule exocytosis (Figure 1.4a)[115]. Mice with an α-cell-specific deletion of *Insr* exhibit increased glucagon secretion, and as a result develop hyperglycemia and glucose intolerance[114]. However, this effect is milder than expected given the amount of insulin present locally within the islet and is by itself insufficient to explain β-cell-mediated α-cell inhibition. Indeed, knocking out the insulin receptor in δ-cells results in lower somatostatin release and α-cell insensitivity to insulin indicating 1) insulin also has a paracrine effect potentiating δ-cells, and 2) that the inhibition of α-cells by insulin is mediated at least in part indirectly via somatostatin[117].

Serotonin. Serotonin is a neurotransmitter derived from tryptophan that regulates mood and anxiety in the brain. β-cells express all of the components for serotonin production – tryptophan hydroxylase (TPH1,TPH2) and DOPA decarboxylase (DDC) – and vesicular loading – Vesicular monoamine transporter 1 and 2 (SLC18A1 & SLC18A2)[118,119]. β-cell serotonin synthesis is largely specific to females and is further enhanced during pregnancy and old age[120]. In humans, serotonin from β-cells is released at high glucose and acts in a paracrine manner, inhibiting neighboring α-cells via the $G_{\alpha i}$-coupled serotonin receptor 1F (HTR1F)[27,32]. Early clinical studies where healthy human

volunteers were administered serotonin antagonists reported increased glucagon secretion[121].

GABA. GABA is the classic inhibitory neurotransmitter of the CNS, and while it plays a role in islet signaling, neurogenic GABA's contribution appears to be minor. β-cells are able to synthesize GABA at some of the highest concentrations outside the CNS, with islet tissue content measurements in the millimolar range; comparable to local insulin concentrations at basal glucose[25,122]. Glutamic acid decarboxylase 1 (GAD1), the enzyme that synthesizes GABA, is highly enriched in β-cells and is a major Type 1 Diabetes autoantigen[32,33]. In rodents, GABA inhibits α-cells through ionotropic GABA A receptors[123-125] (Figure 1.3b), although metabotropic GABA B1 receptor expression is also detectable. GABA from β-cells has been proposed to be the reason α-cells are silenced at high glucose concentrations[124], but GABA treatment by itself does not fully suppress rodent glucagon secretion[126]. The fact that GABA A receptors are difficult to detect on human α-cells and that the application of GABA elicits little electrophysiological response[127] argue against GABA as a significant contributor to α-cell inhibition during hyperglycemia (Figure 1.4a).

Zinc. Insulin secretory granules in the β-cells are highly enriched for zinc ions (Zn^{2+}) to facilitate the formation of the insulin crystal[128]. Zn^{2+} co-released with insulin has therefore been considered as another β-cell product poised to inhibit α-cells[129,130]. Zn^{2+} is taken up by the zinc transporter Znt8 (SLC30A8), which is abundantly expressed by both α- and β-cells[32,33,131] (Figure 1.3c). However, whole body, β-specific, and α-specific Znt8 deletion does not affect glucagon secretion[131,132], indicating that Zn^{2+} is unlikely to contribute to β-cell-dependent inhibition of glucagon under high glucose.

1.5.3 δ-cell-mediated islet hormone coordination

The δ-cells have emerged as important inhibitory modulators of α- and β-cell activity and physiological metabolism. Somatostatin is an important local factor controlling and coordinating the amount and timing of insulin and glucagon release from the islet, which ultimately contributes to setting homeostatic blood glucose levels.

Somatostatin. Somatostatin has important inhibitory functions in the GI tract as well as the anterior pituitary gland. Since δ-cell-derived somatostatin accounts for only 5-10% of systemic circulating somatostatin content, its predominant function is likely as a paracrine regulator[15,16]. δ-cells are active throughout the majority of the physiological range of glucose with secretion starting as low as 3 mM glucose and increasing in a linear, dose-dependent manner towards 20 mM[12,15,133]. This large range of activity may have direct implications in the inhibition of both α- and β-cells.

There are five somatostatin receptor (SSTR) isoforms – all of which are $G_{\alpha i}$-coupled GCPRs[134]. The most abundant form expressed by mouse β-cells is SSTR3 (Figure 1.3b)[31,33,135]. The predominant SSTR(s) on human β-cells remains unclear as some combination of SSTR1, SSTR2, SSTR3 and SSTR5 are expressed[32] and impact insulin secretion to some degree[136,137]. In addition to preventing hormone secretion by decreasing adenylyl cyclase activity, somatostatin receptor signaling simultaneously activates G protein-gated inwardly-rectifying K^+ (GIRK) channels that can counteract glucose-mediated membrane depolarization[137] and inactivates VGCCs that are critical to insulin release[138]. Somatostatin released during high glucose provides inhibitory feedback that – under physiological conditions – does not fully shut down β-cells, but rather provides tonic inhibition. We have proposed that this arrangement is likely instrumental in preventing insulin release in excess of what is required to restore normoglycemia, and in doing so prevents insulin-induced hypoglycemia[17]. The local release of somatostatin during high blood glucose thus provides an additional layer of control to establish and stabilize blood glucose around its homeostatic setpoint.

α-cell activity is controlled by δ-cells as the majority of the glucagonostatic effect of high glucose requires the paracrine actions of somatostatin. Somatostatin inhibits α-cells primarily via SSTR2 in mouse and human islets with additional contribution of SSTR1 in humans[31,33,137,139]. While somatostatin-independent factors such as glucose contribute to inhibition, α-cells are under constant tonic inhibition from δ-cells. In mice, glucagon output increases across the full range of physiological blood glucose levels when either somatostatin is knocked out[140] or when islets are treated with SSTR2 antagonists or inhibitors of downstream somatostatin signaling[12,52,141].

Based on our understanding of the inhibition provided by δ-cells, it is clear that somatostatin as a paracrine regulator ultimately dictates the total α- and β-cell output. However, δ-cell activity itself is dependent on paracrine inputs as well, chief among these the β-cell hormone urocortin3.

Urocortin3. The peptide urocortin3 (UCN3) is the third most abundant hormone produced by β-cells, and is a member of the same peptide hormone family as CRH[14]. UCN3 is packaged in the same secretory granules as insulin and co-released with insulin during GSIS. δ-cell GSSS is potentiated by UCN3 via the $G_{\alpha s}$-coupled GPCR, corticotropin releasing hormone receptor 2 (CRHR2)[14], which is selectively expressed by δ-cells (Figure 1.3b. β-cell activation is required for full δ-cell activity at high glucose as demonstrated by impaired somatostatin release in *Ucn3* knockout mice and islets treated with a CRHR2 antagonist[14]. Additionally, these same conditions – *Ucn3* knockout and CRHR2 antagonism – both demonstrate markedly increased GSIS, demonstrating that UCN3 attenuates insulin release by potentiating GSSS from δ-cells in a classic negative feedback loop.

The physiological contribution of UCN3 to glucose homeostasis is best illustrated by the timing of its expression during development. *Ucn3* is one of the last β-cell genes that is turned on during β-cell maturation; full expression does not occur until around day

14 postpartum in [142] and at the end of the first trimester in human pancreas development[143]. The onset of *Ucn3* expression coincides with a general increase in plasma glucose levels that is correlated with a drop in circulating insulin levels[144]. Premature induction of *Ucn3* specifically in β-cells of transgenic mice from embryonic day 10.5 onwards results in prematurely elevated blood glucose relative to control littermates[14] demonstrating causality between the onset of UCN3 and the rise in blood glucose. UCN3 thus establishes the homeostatic glucose setpoint by activating somatostatin-mediated feedback inhibition of insulin (Figure 1.4c).

1.6 Islet signaling changes in diabetes

The balance between insulin and glucagon release that is so effectively maintained by the integration of a multitude of signals that converge on healthy islets is severely disrupted in diabetes. Autoimmune attack in type 1 diabetes, or peripheral insulin resistance in type 2 diabetes, ultimately leads to β-dysfunction and death[6]. α-cells in type 1 diabetes exhibit an impaired counterregulatory response to hypoglycemia[145], and conversely in type 2 diabetes they aggravate hyperglycemia by inappropriate post-prandial glucagon secretion (Figure 1.4d)[146].

There is evidence that these many clinical manifestations are tied to a breakdown of the paracrine crosstalk that so tightly regulates islet function in healthy islets. The impaired α-cell counterregulatory response has been attributed to autonomic failure, where adrenergic stimulation that assists in potentiating hypoglycemic glucagon secretion is lost[147,148]. A paracrine explanation (that need not be mutually-exclusive) is that somatostatin is elevated in response to hypoglycemia in STZ-induced diabetic rats[149], likely contributing to impaired counterregulatory glucagon release. Indeed, blockade of the SSTR2 that is selectively expressed by α-cells suffices to restore counterregulation in rats[150].

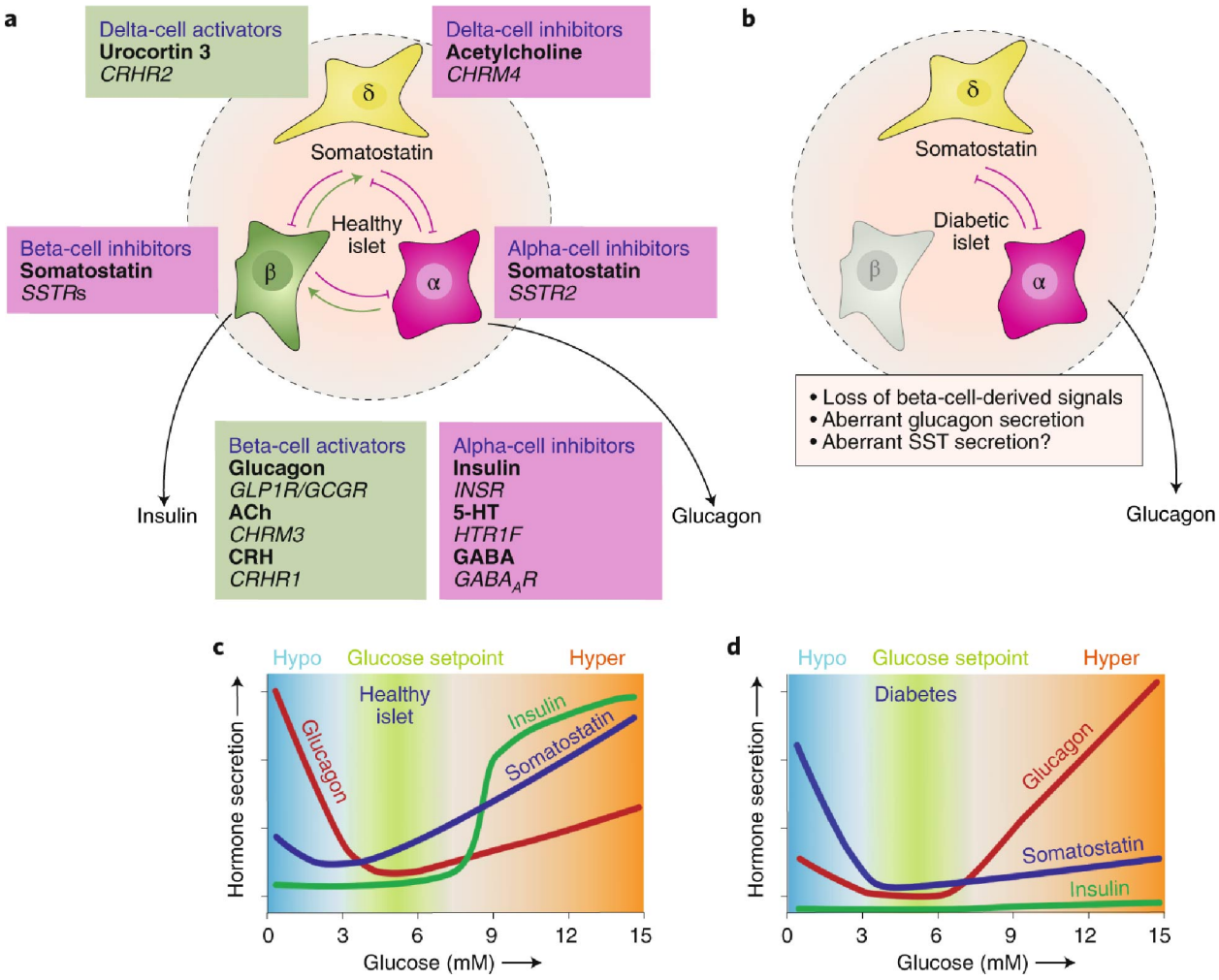


Figure 1.4 Diabetes disrupts the extensive paracrine signaling network of the islet. α, β, and δ-cells influence each other's secretion via intra-islet crosstalk. **a)** Colored text boxes (green for activating, orange for inactivating) denote the target cell type of paracrine signaling (underlined), the signal molecule involved (bold), and the target receptor gene (italicized). Each box is placed in between the target cell and the source of the signal. β-cells initiate a negative feedback loop in high glucose whereby they release UCN3 to activate δ-cells. The resulting somatostatin (SST) release feeds back...

In another example where paracrine crosstalk breaks down in diabetes, impaired UCN3 signaling likely contributes to dysregulated insulin, somatostatin, secretion in diabetes. In Type 1 Diabetes, the majority of β-cells are destroyed and no longer can serve as a source of local UCN3 (Figure 1.4b). Pre-diabetic (type 2) human, NHP, and mouse β-cells selectively down regulate *Ucn3* expression[14,151,152]. The ensuing effects of the loss of UCN3 on GSSS in human and NHP islets have not been established, but islets from

Figure 1.4 cont. to mediate insulin release, providing tonic inhibition that establishes the homeostatic glucose setpoint. β-cells also experience paracrine activation from α-cells, which release glucagon, acetylcholine (ACh), and corticotropin-releasing hormone (CRH), which all potentiate GSIS. B-cell-derived products such as insulin, serotonin (5-HT), and GABA – in addition to UCN3-induced somatostatin release – all contribute to silence α-cells during hyperglycemia. **b)** The onset of diabetes results in a loss of multiple paracrine signals. Due to autoimmune destruction, type 1 diabetic islets effectively lose all β-cell signals. In type 2 diabetes, UCN3 is severely downregulated in β-cells, blunting glucose-stimulated somatostatin secretion. **c)** The physiological impact of paracrine signaling can be visualized with glucose curves for each islet hormone. The homeostatic glucose set point is maintained by glucagon raising blood glucose during hypoglycemia and insulin lowering glucose during hyperglycemia. Somatostatin contributes as a fine-tuning mechanism via paracrine inhibition of both α- and β-cells. **d)** The absence of β-cell-derived products in diabetes results in inappropriately high glucagon secretion during high glucose, which exacerbates hyperglycemia. Glucagon counterregulation at low glucose is also impaired, possibly due to aberrant somatostatin secretion, although this is not yet fully understood. Based in part on Ref[111].

moderately diabetic leptin-deficient mice demonstrate a loss of UCN3 and consequently release little somatostatin at high glucose, despite the fact that δ-cells remain in normal, or even increased numbers in diabetes[14]. Restoring *Ucn3* expression in diabetic mice exacerbates hyperglycemia, in line with the re-activation of β-cell inhibition by somatostatin and similar to the premature embryonic *Ucn3* induction[14]. The mechanism responsible for the loss of *Ucn3* expression that precipitates the breakdown of local crosstalk early in diabetes is not known, which illustrates that a better understanding of paracrine communication between islet cells is vital to improved therapeutic options.

At the nutrient level, elevated blood glucose in diabetes provokes many functional changes in β-cells. Hyperglycemia contributes to β-cell proliferation in mice and ultimately leads to glucotoxicity and β-cell dysfunction. In the dysfunctional state, the loss of UCN3 likely removes much of the somatostatin inhibition, as stated above[14]. Unrestrained from paracrine signals including somatostatin, α-cells release glucagon that stimulates hepatic glucose production and aggravates hyperglycemia (Figure 1.4d). With hepatic insulin resistance, individuals can also develop impaired glucagon signaling, which inhibits

gluconeogenesis and leads to an accumulation of circulating amino acids. This sets off a vicious cycle of hyperaminoacidemia and hyperglucagonemia, with neither signal efficiently correcting the other[65]. Initial insulin compensation in type 2 diabetes can lead to fatty infiltration where the islet microenvironment becomes filled with adipocytes[23]. While fatty acids will contribute to the compensatory increase in insulin release via potentiating secretion and increasing β-cell mass, extended exposure to fatty acids can lead to lipotoxic stress, β-cell dysfunction and decreased insulin secretion[153]. Without intervention, these initially compensatory mechanisms to increase insulin output cause a state of partial or complete β-cell dysfunction.

1.7 The value of transcriptomics and pathways for drug discovery

Transcriptomics supported by rigorous validation experiments via imaging and hormone secretion have rapidly advanced our grasp of islet function. Such increased mechanistic understanding can inform direct translational progress. RNA-Seq performed on islet cells from diabetic donors has allowed for direct comparisons between the healthy and diseased islet [34,154]. Two examples of the insights generated by these islet cell transcriptomes are clarifications of the islet mechanisms of action of the hunger hormone, ghrelin[33], and, separately, the receptor GPR119:

1.7.1 The mechanism of ghrelin-mediated insulin suppression

Ghrelin release occurs in the fasted state and inhibits insulin release from rodent and human pancreata[155-157]. For years, these insulinostatic actions had been attributed to direct inhibition of β-cells by ghrelin[155,158], even though the growth hormone secretagogue receptor (GHSR) that mediates ghrelin's signal is a GPCR normally associated with an

activating $G_{\alpha q}$ subunit[159]. Because ghrelin's inhibitory actions were sensitive to pertussis toxin, it was proposed that GHSR couples to an inhibitory $G_{\alpha i}$ subunit in β-cells[160]. However, islet cell transcriptomes from multiple groups resolved this conundrum by demonstrating that GHSR is selectively expressed by δ-cells (Figure 1.3b), and the resulting $G_{\alpha q}$-mediated somatostatin release from δ-cells silences β-cells in a $G_{\alpha i}$-mediated fashion[31,33,161]. Coupling transcriptomics to functional assays has expanded our ability to interrogate which islet cell type expresses the requisite receptors in order to respond to each respective signal that impinges on the islet.

1.7.2 GPR119 stimulates insulin secretion indirectly via α-cells

GPR119 is a $G_{\alpha s}$-coupled GPCR that binds lipids and lipid metabolites. The receptor has been a drug target of interest for diabetes since the discovery that GPR119 activation enhances both GSIS and incretin release[162,163]. Early GPR119 research found its expression to be largely limited to the pancreatic islets and GI tract, and inaccurately built a case for GPR119 as a β-cell-specific insulinotropic receptor. Following these observation, multiple companies generated small molecule agonists for GPR119[164]. The receptor proved to be highly druggable with a number of molecules eliciting improved glucose clearance, but efforts have stalled recently in the face of common challenges related to safety and efficacy. Moreover, drug developers may have been targeting the wrong cell type – comprehensive islet transcriptomes clearly demonstrate relatively selective expression of GPR119 by α-cells (Figure 1.3b)[33,40]. This has been validated by a subsequent study demonstrating that GPR119 activation in mouse and human islets improves glucagon release during hypoglycemia[165]. The improved GSIS that is observed in response to GPR119 agonism is likely due to the actions of glucagon potentiating GSIS from β-cells that we reviewed earlier.

1.8 Conclusions

While many aspects that contribute to the regulation of pancreatic islets in health and disease remain unresolved, a brief survey of recent trends in islet research shows that in taking a small step back to appreciate the islet beyond the β-cells and glucose, the diabetes field is taking significant strides forward towards a more comprehensive understanding. Shining a light on α- and δ-cells, the intra-islet crosstalk they engage in, and non-canonical nutrient signaling may turn out to be key in tackling diabetes. Especially in the context of drug discovery, these interactions cannot be ignored. The availability of comprehensive bulk and single cell transcriptomes for each islet cell type will continue to facilitate the delineation between direct and indirect effects of hormones, nutrients, and neurotransmitters. Similarly, much of the work done to date on stem cells for a cure for Type 1 Diabetes has focused on generating insulin-secreting β-cell-like cells. However, it has become increasingly evident that β-cells require input from neighboring α- and δ-cells for mature β-cell function. Future studies should be cognizant of these interactions as Occam's Razor (the simplest explanation is likely correct) applied to the pancreatic islet must account for the islet as an interactive unit where multiple endocrine and non-endocrine cell types coordinate the overall release of insulin and glucagon from the islet.

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Chapter 2

Urocortin3 contributes to paracrine inhibition of islet α-cells in mice

2.1 Preface

This chapter has been submitted to *Journal of Endocrinology.*

2.1.1 Authorship

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The article has been modified to satisfy the formatting requirements of this dissertation.

2.2 Abstract

Pancreatic α-cell activity and glucagon secretion decrease as glucose levels increase. While part of the reduction is regulated by glucose itself, paracrine signaling by their neighboring β- and δ-cells also plays an important role. Somatostatin from δ-cells is an important local inhibitor of α-cells at high glucose. Additionally, Urocortin3 (UCN3) is a hormone that is co-released from β-cells with insulin and acts locally to potentiate

somatostatin secretion from δ-cells. UCN3 thus inhibits insulin secretion via a negative feedback loop with δ-cells, but its role with respect to α-cells and glucagon secretion is not understood. We hypothesize that the somatostatin-driven glucagon inhibition at high glucose is regulated in part by UCN3 from β-cells. Here, we use a combination of live functional calcium and cAMP imaging as well as direct glucagon secretion measurement, all from α-cells in intact mouse islets, to determine the contributions of UCN3 to α-cell behavior. Exogenous UCN3 treatment decreased α-cell calcium and cAMP levels and inhibited glucagon release. Blocking endogenous UCN3 signaling increased α-cell calcium by 26.8 ± 7.6%, but this did not result in increased glucagon release at high glucose. Furthermore, constitutive deletion of *Ucn3* did not increase calcium activity or glucagon secretion relative to controls. UCN3 is thus capable of inhibiting mouse α-cells, but, given the subtle effects of endogenous UCN3 signaling on α-cells, we propose that UCN3-driven somatostatin may serve to regulate local paracrine glucagon levels in the islet instead of inhibiting gross systemic glucagon release.

2.3 Introduction

The α-cells of the pancreatic islets of Langerhans are important physiological regulators of blood glucose that act to raise blood glucose levels via the actions of their principal hormone, glucagon. Despite being predominantly active at opposite ends of the glucose spectrum, α-cell stimulus-secretion coupling resembles its insulin-secreting β-cell counterpart in its reliance on voltage-dependent calcium influx for exocytosis and cAMP for potentiation[1–3]. α-cells exhibit calcium oscillations that are inversely correlated with glucose concentration[4] – at low glucose levels reflecting hypoglycemia, α-cells are electrically active and fire action potentials that trigger calcium currents through mostly P/Q-type and to a lesser extent L-type calcium channels[5,6]. α-cells also display high intracellular cAMP in low glucose that is linked to high glucagon secretion and is directly

modulated by the glucose concentration[3]. These measures of α-cell activity – calcium currents, cAMP, and glucagon secretion – decrease as glucose approaches hyperglycemic levels[3,7].

While intrinsic glucose-sensing machinery contributes to preventing glucagon exocytosis under high glucose conditions[8,9], increases in glucose concentration per se are not sufficient to account for the inhibition of glucagon release. Indeed, dispersed α-cells that are stripped of the influence of their neighboring islet cells are instead stimulated by glucose in a dose-dependent manner[10,11]. It has long been established that the different islet cell types dynamically regulate each other in a paracrine manner and help shape each other's true glucose response[12,13]. α-cells integrate paracrine, endocrine, and neural inputs[14] with their own intrinsic metabolic/electrophysiological response to nutrient stimulation to determine the glucagon secretion that is stimulation-appropriate[15]. These combined effects are then responsible for the glucose-dependent response of α-cells that release robust glucagon to hypoglycemia, and more measured glucagon levels in response to postprandial hyperglycemia.

Both β- and δ-cells participate in paracrine α-cell inhibition at high glucose[16,17]. The somatostatin-secreting δ-cells exert tonic inhibition on α-cells across the full range of physiological blood glucose concentrations[18] via the somatostatin receptor 2 (SSTR2), a cAMP-lowering $G_{\alpha i}$-coupled GPCR[7,19,20]. But somatostatin is particularly important to restrain glucagon secretion at glucose concentrations above 7 mmol/L[18]. The contribution of β-cells to α-cell inhibition is more complex, though the inverse relationship between insulin and glucagon has been known for decades[12,21]. β-cells have been reported to release not only insulin at high glucose, but also GABA[22] and Zn^{2+} [23]. While all three β-cell products have been proposed as the driver of α-cell inhibition[24–26], none are sufficient to fully reproduce glucose-mediated silencing seen *in vivo*[7,8,24,27].

Our lab has previously discovered that another β-cell product, the peptide hormone Urocortin3 (UCN3), is co-released with insulin at high glucose and acts as a local paracrine

signal. UCN3 is required for full glucose-stimulated somatostatin secretion from δ-cells[28]. UCN3 does so by activating corticotropin-releasing hormone receptor 2 (CRHR2), a strictly $G_{\alpha s}$-coupled GPCR[29,30] expressed specifically by δ-cells[28,31]. In line with this $G_{\alpha s}$ signaling mechanism that increases δ-cell adenylyl cyclase activity and cAMP levels, blocking cAMP in δ-cells impairs glucose-stimulated somatostatin secretion[32]. The resulting UCN3-mediated somatostatin release forms a negative feedback loop with insulin that helps establish the euglycemic setpoint during early postnatal development in mice[28]. We hypothesize that this same paracrine signaling cascade – initiated by β-cell UCN3 release under hyperglycemic conditions – also contributes to α-cell silencing at high glucose and is thus a mechanism by which β- and δ-cells cooperatively inhibit glucagon secretion. To address the role of UCN3 in glucose-induced α-cell inhibition, we performed *ex vivo* analysis of α-cell calcium activity, cAMP changes, and glucagon secretion in intact islets from both wild type and *Ucn3*-null mice.

2.4 Results

2.4.1 UCN3 inhibits a-cell activity

To observe the effects of UCN3 on α-cells, we generated α-cell-specific reporter mice by crossing the *Gcg*-CreERT2 strain that has tamoxifen-inducible Cre expressed under the *Gcg* promoter with a floxed allele of the genetically-encoded calcium indicator GCaMP6s. In islets from bitransgenic mice, this enables the imaging of α-cell calcium activity using calcium fluorescence as a proxy for active glucagon secretion. Crucially, GCaMP6s allows us to monitor α-cells in intact islets where paracrine connections are maintained – an important point given that UCN3 actions are mediated through neighboring δ-cells. Given that α-cells are less active at high glucose (16.8 mmol/L), we

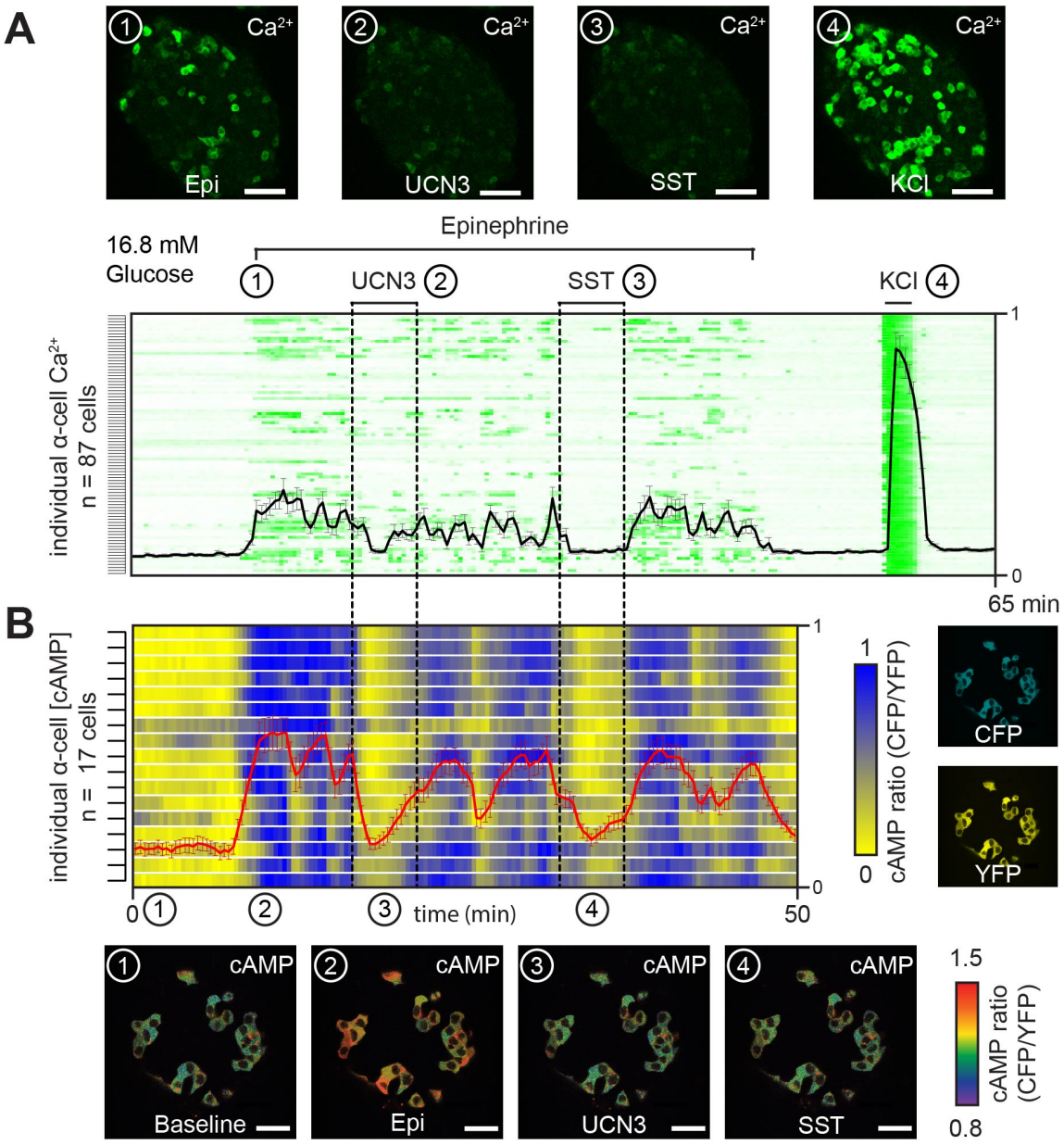

Figure 2.1: UCN3 inhibits α-cell calcium and cAMP. (A) Calcium responses of α-cells from a single intact islet from a *Gcg-CreERT2 x lsl-GCaMP6s* mouse. Islets were imaged while continuously perfused with KRB at 16.8 mmol/L glucose with epinephrine (Epi; 100 nmol/L), UCN3 (100 nmol/L), SST (100 nmol/L), and potassium chloride (KCl, 30 mmol/L) added at indicated time points. Thumbnails of imaged...

stimulated α-cells with epinephrine (100 nmol/L) to establish a baseline level of calcium fluorescence[37,38] on top of which we would be able to observe any inhibition. Epinephrine-induced calcium in α-cells was inhibited by exogenous UCN3 (100 nmol/L) (Figure 2.1A).

Figure 2.1 cont. islet correspond to each numbered treatment step. Each line along the y-axis represents a single α-cell, with level of green corresponding to GCaMP6s fluorescence intensity. The average GCaMP6s activity of all α-cells in the trace is indicated in the black line graph ± SEM. **(B)** cAMP responses of α-cells from a single islet from a *Gcg-CreERT2* x *lsl-CAMPER* mouse. Imaged following the same protocol as 2.1A without KCl viability control. Each line along the y-axis indicates the ratio of CFP:YFP for each α-cell. Yellow shows less cAMP and blue indicates higher cAMP generation. Red line indicates average CFP:YFP ratio of all cells. Thumbnails correspond to numbered treatments with colorimetric ratio scale bar for FRET ratio. Error bars = ± SEM. Scale bar = 50 µm.

Somatostatin (100 nmol/L) was applied as a positive control that is known to inhibit α-cells (Figure 2.1A)[39]. Calcium traces conclude with a brief pulse of potassium chloride (30 mmol/L), which elicits a calcium response in all GCaMP6s-expressing cells and confirms viability and responsiveness throughout the functional trace at the end of the recording.

Somatostatin inhibits α-cells by binding SSTR2, which then activates $G_{\alpha i}$ signaling. The α_i subunit of the SSTR2-coupled G proteins then decreases adenylyl cyclase activity and cAMP generation[16,19]. To confirm that the observed inhibitory effect of UCN3 is mediated by a similar cAMP decrease, we then imaged α-cell cAMP activity. To do so we crossed *Gcg*-CreERT2 mice with the floxed cAMP FRET sensor, CAMPER. Because epinephrine signals via the $G_{\alpha s}$-coupled β1-adrenergic receptor in α-cells, it elevates cAMP upstream of calcium influx and glucagon exocytosis[40–42]. Following the same protocol used in the calcium imaging, UCN3 and SST both inhibited the epinephrine-generated cAMP response (Figure 2.1B).

To assess whether the observed effects of UCN3 on α-cell calcium translated to attenuated hormone release, we measured glucagon secretion from isolated wild type mouse islets. We incubated islets with UCN3 (10 nmol/L) at both low glucose (1 mmol/L), where there would be modest glucagon secretion to be able to resolve inhibition, and at high glucose (16.8 mmol/L), where α-cells are already mostly inactive, but δ-cells demonstrate strong activity. Indeed, Ucn3 attenuated glucagon secretion by 59% at low glucose and by 66% at high glucose (Figure 2.2).

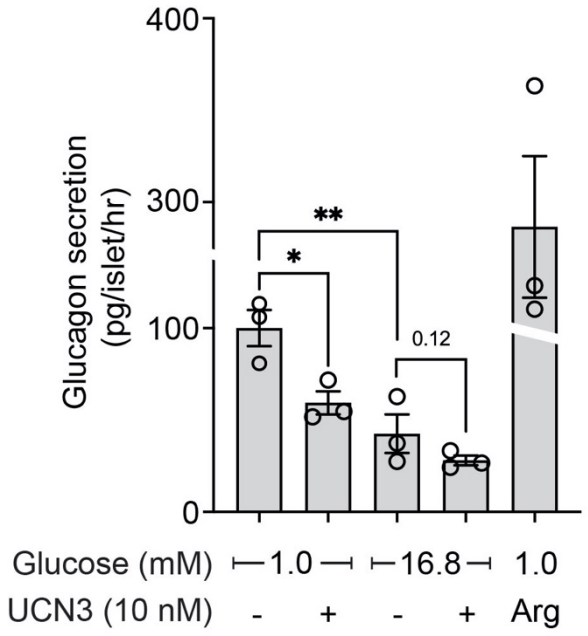

Figure 2.2: UCN3 inhibits glucagon. Glucagon secretion from intact wild type mouse islets as measured by radioimmunoassay. Islets incubated in KRB at indicated glucose levels in the presence and absence of UCN3. Arginine (Arg; 10 mmol/L) treatment used as a positive glucagon secretion control (n=3 wells per treatment). Error bars = ± SEM. *p<0.05 **p<0.01.

To further attribute UCN3-driven inhibition of glucagon release to downstream $G_{\alpha i}$ signaling, we pre-treated islets with pertussis toxin (PTx; 200 ng/mL), which prevents the activation of the α subunit of $G_{i/o}$ proteins specifically. Non-treated control islets were inhibited by UCN3 as well as SST when measuring both calcium (Figure 2.3A) and cAMP (Figure 2.3B). PTx-treated islets displayed no inhibition in response to either UCN3 or SST in calcium (Figure 2.3C) and cAMP (Figure 2.3D), and glucagon secretion (Figure 2.3E).

Lastly, to confirm UCN3 is acting through δ-cells, we directly measured δ-cell activity. Using islets from *Sst*-Cre x *lsl*-CAMPER mice, in which δ-cells express the FRET sensor, we observe a potent activation of cAMP with UCN3 (100 nM & 1 μM) (Figure 2.4A). Corroborating this UCN3-driven δ-cell stimulation, when we removed paracrine signaling by FACS-purifying CAMPER-expressing α-cells, we saw no UCN3-mediated

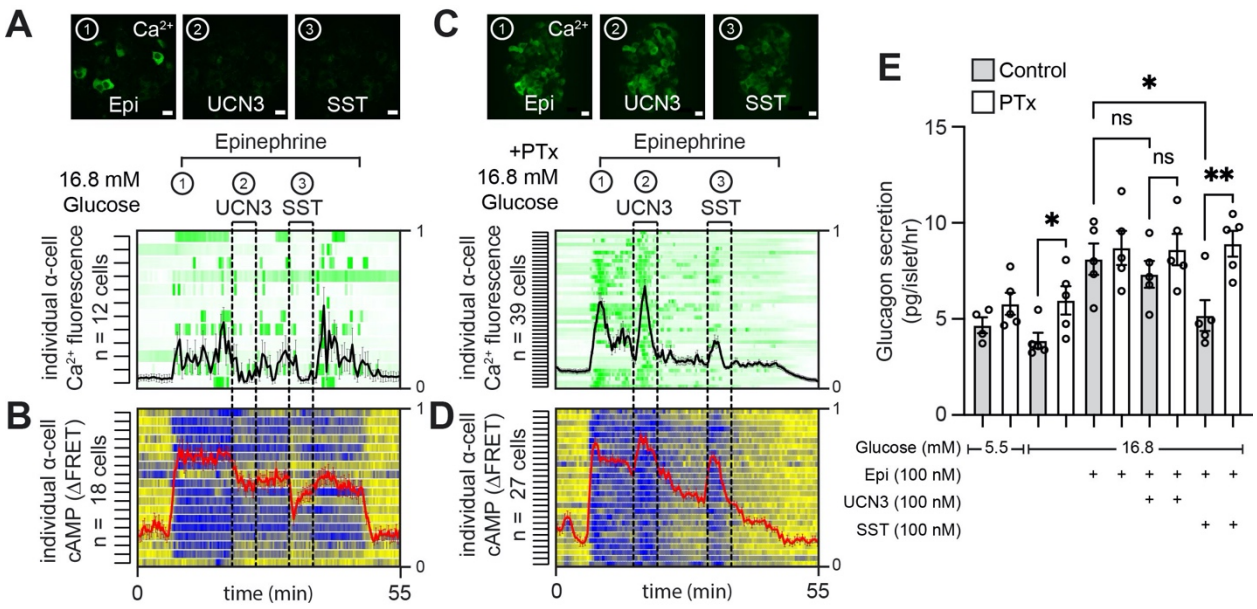

Figure 2.3: UCN3 inhibition occurs via $G_{\alpha i}$ signaling. **(A)** Islet with GCaMP6s-expressing α-cells imaged as in Figure 2.1A responding to epinephrine (100 nmol/L), UCN3 (100 nmol/L), and SST (100 nmol/L) at 16.8 mmol/L glucose. Control islet without pertussis toxin (PTx) pre-treatment. Green lines indicate calcium fluorescence of each individual α-cell. Black line graph represents average calcium fluorescence of all cells ± SEM. Scale bar = 50 µm. **(B)** Islet with CAMPER-expressing α-cells imaged as in Figure 2.1B. Blue indicates high cAMP, yellow indicates low cAMP. Red line graph tracks average cAMP ratio of α-cells ± SEM. **(C)** GCaMP6s-expressing islets incubated with PTx (200 ng/ml for 18h) prior to imaging. **(D)** α-cells from intact islet expressing CAMPER, incubated with PTx as in 3C, imaged as in 3B. Scale bar = 50 µm. **(E)** Glucagon secretion from intact islets incubated with and without PTx (200 ng/ml, 18h) and exposed to different glucose levels, epinephrine (Epi), UCN3, and SST (n=4 or 5 wells per treatment) as measured by luminescence radioimmunoassay. *p<0.05 **p<0.01.

inhibition in the single α-cells, but intact islets from the same mouse retained UCN3 sensitivity (Figure 2.4B,C). Together, these data show that UCN3 is able to inhibit α-cells and does so in a $G_{\alpha i}$-dependent manner that requires paracrine signaling, likely from δ-cells.

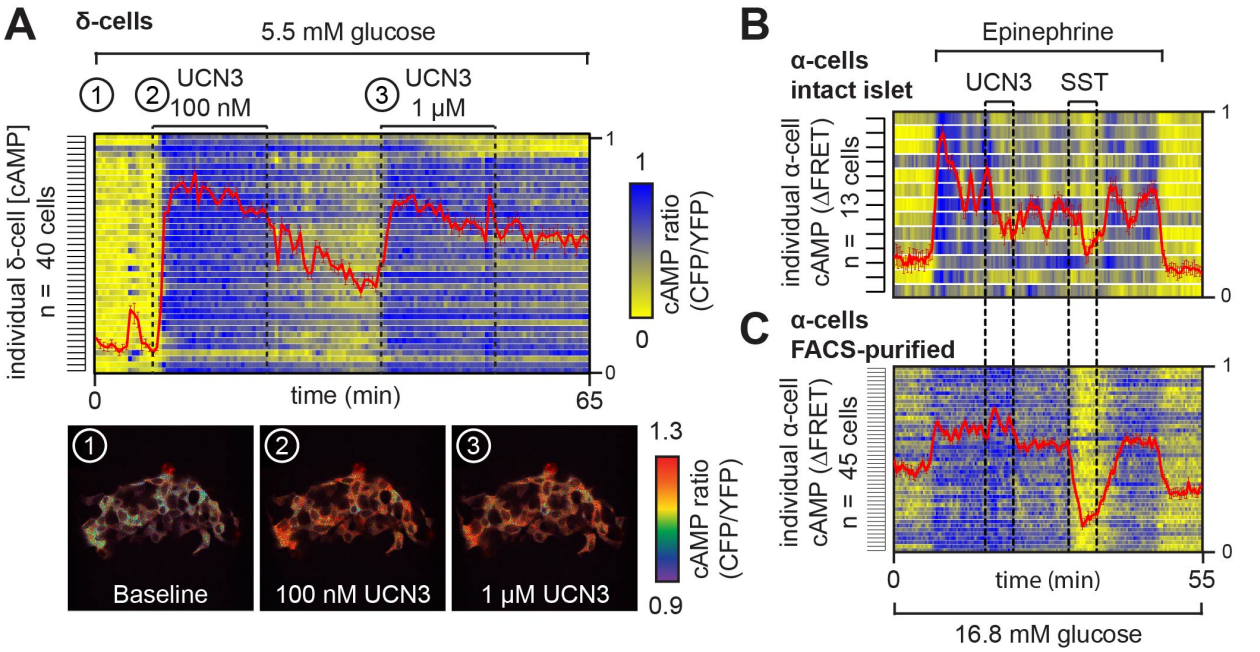

Figure 2.4: UCN3 activates δ-cells and requires paracrine signaling for α-cell inhibition. **(A)** Islet with CAMPER-expressing δ-cells treated with 100 nM and 1 μM UCN3. Thumbnails correspond to numerically-indicated timepoints. **(B)** CAMPER-expressing α-cells from intact islets and **(C)** FACS-purified single α-cells from islets from the same mouse imaged with the same protocol as in Figure 2.1B. Blue indicates high cAMP, yellow indicates low cAMP. Red line graph tracks average cAMP ratio of δ-cells ± SEM.

2.4.2 α-cell inhibition in high glucose is partially mediated by UCN3

In order to determine the role endogenous UCN3 plays in α-cell inhibition, we imaged calcium activity while blocking the receptor for UCN3, CRHR2, which is selectively expressed by δ-cells. At high glucose, treating islets with a selective CRHR2 antagonist, astressin2b (100 nmol/L)[43], induced calcium oscillations in 26.8 ± 7.6% of α-cells (Figure 2.5A). Blocking somatostatin signaling directly with a selective SSTR2 antagonist, 406-028-15 (100 nmol/L) [44], de-inhibited 56.3 ± 7.8% of α-cells. In addition to the potassium chloride viability control, we added arginine vasopressin (AVP; 10 nmol/L), which activates a robust calcium response downstream of the AVP receptor 1b selectively

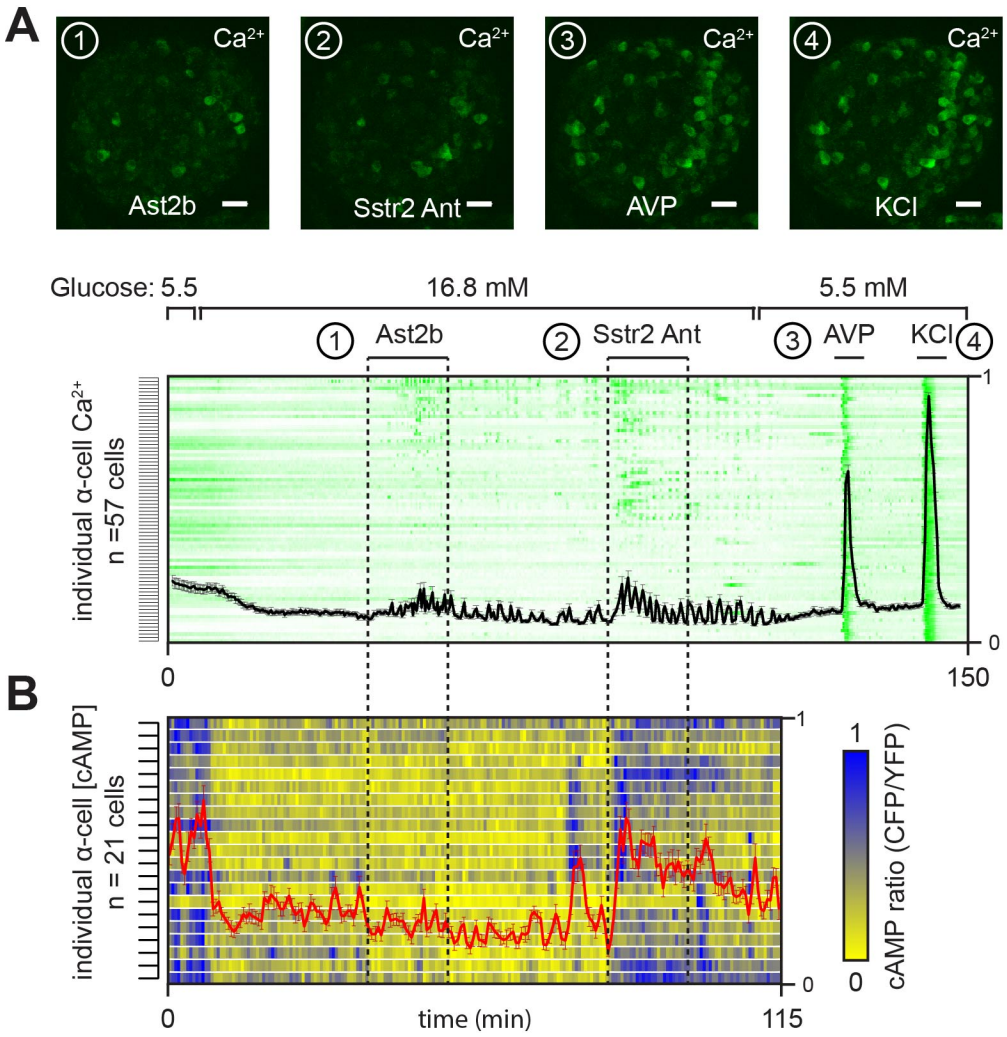

Figure 2.5: Blocking endogenous UCN3 de-inhibits α-cells. (A) Islet with GCaMP6s-expressing α-cells imaged over time with successive treatments of Astressin2b (Ast2b, 100 nmol/L), SSTR2 antagonist (SSTR2 Ant, 100 nmol/L) at high glucose, and arginine vasopressin (AVP, 10 nmol/L) and KCl (30 mmol/L) at basal glucose. Numbered thumbnails from indicated time points. Black line graph represents average calcium fluorescence of all cells ± SEM. Scale bar = 50 µm. **(B)** Islet with CAMPER-expressing α-cells imaged as in 2.4A, without AVP and KCl. Red line graph tracks average cAMP ratio of α-cells ± SEM.

expressed by α-cells, as we have previously shown[45]. AVP thus serves as a functional control to further confirm the α-cell-specific expression of the GCaMP6s sensor. CAMPER islets imaged with the same protocol revealed no discernable de-inhibition with astressin2b, but a rise in cAMP activity due to blocking SSTR2 (Figure 2.5B).

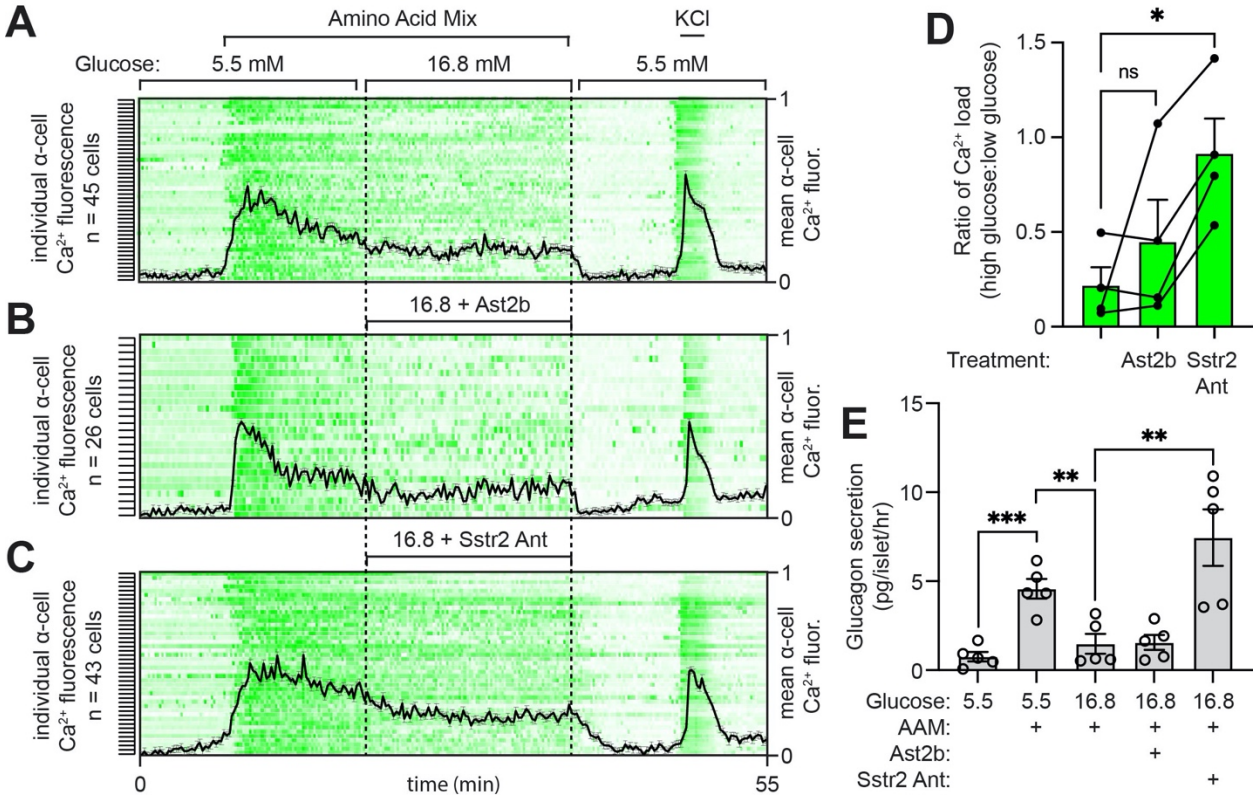

Figure 2.6: High glucose inhibition of glucagon does not require UCN3. **(A)** α-cell GCaMP6s in islets imaged with 2 mM amino acid mixture (AAM, 2 mmol/L each glutamine, alanine, arginine) at basal and high glucose. **(B)** Ast2b (100 nmol/L) or **(C)** SSTR2 antagonist (100 nmol/L) were applied at high glucose. Black line graph represents average calcium fluorescence of all cells ± SEM. **(D)** Ratio of calcium load from imaged islets such as in 2.5A-C comparing α-cell calcium fluorescence (AUC) at high glucose with AAM to basal glucose with AAM. Lines connect calcium load ratios from islets from the same animals. n = 4 animals. **(E)** Glucagon secretion from wild type mouse islets incubated in KRB with indicated treatments as measured by radioimmunoassay. AAM (2 mmol/L), Ast2b (100 nmol/L), SSTR2 Ant (100 nmol/L). n = 5 wells per treatment. *p<0.05 **p<0.01 ***p<0.005.

We then monitored α-cell glucose sensitivity with GCaMP6s in the presence of a 6 mmol/L mixture of three amino acids (alanine, arginine & glutamine at 2 mmol/L each; referred to as AAM) to better recapitulate glucagon flux in mixed meal situations. Similar to epinephrine, this amino acid mixture induces a robust calcium activation in α-cells on top of which we can quantify any inhibition by UCN3 or SST[18,46]. This approach is conceptually similar to the epinephrine stimulation we used before (Figure 2.1A), but

avoids the confounding actions of epinephrine-induced inhibition of β- and δ-cells that would prevent endogenous UCN3 and SST release, respectively[40]. In the presence of amino acids, α-cells demonstrated a reduction in calcium activity upon transitioning from basal glucose (5.5 mmol/L) to high glucose (Figure 2.6A). A reduction in activity still occurred in the presence of astressin2b (Figure 2.6B), but was largely prevented by 406-028-15 (Figure 2.6C). To tease out subtle changes, we calculated relative calcium load, as measured by the ratio of the area under the curve for 10 minutes in high glucose with amino acids to the AUC of 10 minutes in basal glucose with amino acids. The relative calcium load shows robust inhibition by high glucose in non-treated control islets (Figure 2.6D). Blocking CRHR2 with astressin2b modestly, though non-significantly, de-inhibited α-cells at high glucose. Meanwhile, calcium activity in the presence of 406-028-15 only decreased by 5% in high glucose – α-cells were almost fully de-inhibited by blocking SSTR2. Glucagon secretion from islets exposed to the same conditions reflects the calcium responses: amino acids stimulate glucagon secretion at basal glucose that is inhibited by high glucose, and while 406-028-15 prevents this inhibition astressin2b does not (Figure 2.6E).

2.4.3 UCN3 is not required for α-cell inhibition at high glucose

Our results thus far showed that exogenous UCN3 is capable of inhibiting α-cell activity and glucagon release. Conversely, blockade of *endogenous* UCN3 actions increased α-cell signaling, but this did not drive a meaningful increase in glucagon secretion. To further assess whether the inhibition of α-cells requires endogenous UCN3, we utilized a constitutive *Ucn3*-null mouse line[36]. Measuring α-cell calcium in islets from triple transgenic *Ucn3*-null x *Gcg*-CreERT2 x lsl-GCaMP6s mice and *Ucn3*+ control mice (Figure 2.7A) showed that α-cells are inhibited by high glucose despite loss of UCN3 (Figure 2.7B). To test whether this silencing at high glucose is due to residual UCN3-

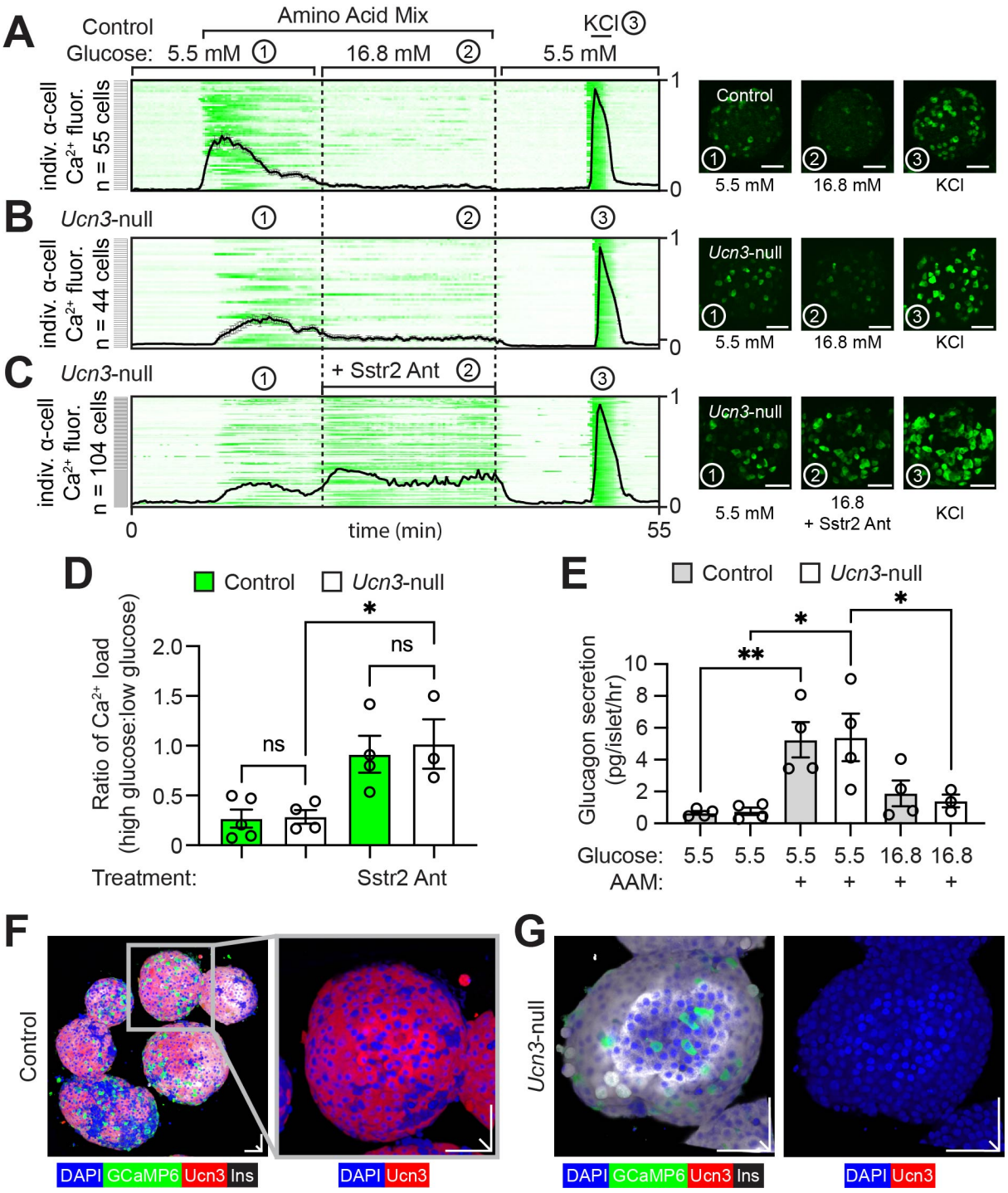

Figure 2.7: α-cell behavior at high glucose in UCN3 knockout does not change. (A) α-cell GCaMP6s in islets from *Ucn3*-expressing control mice imaged with 2 mM amino acid mixture (AAM, 2 mmol/L each glutamine, alanine, arginine) at basal and high glucose. Black line graph represents average calcium fluorescence of all cells ± SEM. Thumbnails correspond to numbered time points. Thumbnail scale bars = 50 µm...

Figure 2.7 cont. (B) α-cell GCaMP6s in islets from *Ucn3*-null mice imaged as in 2.6A. **(C)** *Ucn3*-null α-cell calcium imaged as in 6A with Sstr2 Ant (100 nmol/L) applied at high glucose. **(D)** Ratio of calcium load (AUC for 16.8 : 5.5 mmol/L glucose) for α-cell GCaMP6s in control islets (green bars) compared to *Ucn3*-null islets (white bars). Control bars contain some data points from Figure 5D. n = 5, 4, and 3 mice. **(E)** Glucagon secretion from control (grey bars) and *Ucn3*-null islets (white bars) as measured by radioimmunoassay with indicated glucose concentrations ± AAM (2 mmol/L). *p<0.05 **p<0.01. **(F)** Whole mount immunofluorescence of islet from control mouse expressing GCaMP6s in α-cells. β-cell insulin is stained in red, and β-cell UCN3 expression is confirmed by staining in far red (white). α-cell GCaMP6s expression is counterstained with GFP (green). DAPI (blue) marks nuclei. Scale bars = 50 (x) x 50 (y) x 50 (z) µm. **(G)** Whole mount immunofluorescence of islet from *Ucn3*-null mouse with α-cell GCaMP6s. *Ucn3* knockout is confirmed by lack of far red UCN3 staining. Scale bars = 50 (x) x 50 (y) x 40 (z) µm.

independent somatostatin signaling, we applied 406-028-15 at high glucose in *Ucn3*-null islets. Blocking SSTR2 with 406-028-15 in *Ucn3*-null islets was able to sustain α-cell activity at high glucose (Figure 2.7C). Quantification of the relative calcium load of the islets in high and low glucose shows no difference between the glucose sensitivity of wild type control and *Ucn3*-null islets, and confirms that SSTR2 antagonism still rescues calcium signaling In *Ucn3*-null α-cells (Figure 2.7D). These data are reflected in glucagon secretion measurements showing no difference in the glucagon output of control islets from *Ucn3*-null islets (Figure 2.7E). In both the presence and absence of UCN3, amino acids stimulated glucagon secretion at basal glucose, but lack of UCN3 did not prevent suppression of glucagon under high glucose-mediated suppression. At the conclusion of these live calcium imaging traces, we fixed and stained GCaMP6s islets to confirm UCN3 expression in control mice (Figure 2.7F) and absence in *Ucn3*-null mice (Figure 2.7G).

2.5 Discussion

The timely attenuation of glucagon secretion is critical to maintain proper glycemia as blood glucose rises[47–49]. Among the network of intercellular and extracellular signals

that determine islet cell activity, paracrine signaling plays a critical role in modulating the total endocrine output of α- and β-cells at different glucose levels. Upon high glucose stimulation, β-cells activate a paracrine negative feedback loop set by their release of UCN3 and the resultant inhibitory feedback via potentiation of δ-cell somatostatin[28]. Given the role of UCN3 in regulating proper β-cell insulin secretion, the obvious follow-up question was whether UCN3 signaling affects α-cells. We find that exogenous UCN3 inhibits α-cells, and it does so in a manner that suggests the involvement of $G_{\alpha i}$ signaling given the UCN3-driven decrease in cAMP that mirrors that of SST. This inhibition is visible across three independent measures of α-cell activity: calcium, cAMP, and glucagon secretion. Utilizing epinephrine for these experiments served a dual purpose of activating the α-cells to visualize any UCN3-mediated inhibition and simultaneously inhibiting β-cells to prevent confounding insulin, GABA or Zn^{2+} or endogenous UCN3 signaling[28,40]. Additionally, endogenous UCN3 participates in α-cell silencing at high glucose. By blocking UCN3-mediated δ-cell potentiation, α-cells are effectively de-inhibited and display calcium activity at high glucose, reflective of the type of behavior seen by others when α-cells are isolated[10,11].

Given that α-cells de-inhibited by CRHR2 antagonism represent a relative minority in the islet and that glucagon secretion remains unchanged when UCN3 signaling is blocked or knocked out, it is possible that the role of UCN3 may be to modulate paracrine (as opposed to endocrine) levels of glucagon at high glucose. Although glucagon is often described as a counterregulatory hormone, it has a well-known insulinotropic actions[50] that have recently been shown to drive proper glucose-stimulated insulin secretion (GSIS)[13,50–52]. The classic "incretin effect" describes the simultaneous activation of β-cells by high glucose and further potentiated by circulating glucagon-like peptide 1 (GLP-1) from enteroendocrine L-cells or GIP from enteroendocrine K cells in the gastrointestinal tract[53]. However, a number of studies have demonstrated that potentiation of β-cell GSIS via the GLP-1 receptor (GLP-1R) additionally relies on α-cell-derived proglucagon

peptides including glucagon[13,54]. α-cells from healthy islets produce minimal GLP-1, but glucagon shows considerable cross-reactivity between its native glucagon receptor (GCGR) and GLP-1R, both of which are expressed by β-cells[13,54,55]. In isolated islets, free from the influence of gut L-cells, blocking GCGR/GLP-1R or disrupting *Gcg* gene expression severely impairs GSIS despite the fact that relatively little glucagon is released at high glucose[13,54]. Completely shutting down α-cells at high glucose is thus counterproductive to maintaining proper glycemia. Instead, we propose a model where the contribution of UCN3-driven somatostatin is not to prevent glucagon release, but instead to temper it to allow for glucagon's local insulinotropic actions without releasing enough systemically to increase hepatic glucose output.

The importance of UCN3-mediated inhibitory feedback is particularly relevant in the context of irregular glucagon secretion in the context of diabetes. A well-documented aspect of type 2 diabetes is the inappropriate post-prandial release of glucagon, which contributes to diabetic hyperglycemia[47–49,56]. Isolated α-cells exhibit a bimodal glucose curve with the expected exocytosis at low glucose, but also increased glucagon release above a threshold of roughly 6 mmol/L glucose[11,17]. The fact that glucagon secretion *in vivo* and in intact islets is inhibited by high glucose suggests external factors are implicated in the suppression, and one of the key factors in this is somatostatin[18]. Indeed, in islets from type 2 diabetic patients, glucagon secretion is increased at both low and high glucose, and this defect in normal α-cell behavior correlates with a downregulation in the expression of the somatostatin receptor on the plasma membrane of α-cells[17]. Likewise, in type 2 diabetic human islets as well as mouse and non-human primate models of type 2 diabetes there is a dramatic decrease in the expression of UCN3 in β-cells[28]. It remains to be determined whether the loss of β-cell UCN3 in type 2 diabetes, which by extension decreases somatostatin release, contributes to the dysregulation of glucagon. At first glance, our observations in the *Ucn3*-null mice would not support such a scenario. However, the constitutive nature of the *Ucn3* knockout leaves open the possibility that

compensation could have built up in the constitutive absence of the influences of UCN3. Something very similar occurs with SST itself, where exogenous SST is capable of robust inhibition of insulin secretion in *Sst*-null islets, but even very high (1 µM) exogenous SST does not inhibit insulin secretion in response[20]. To accommodate for loss of a somatostatin potentiating signal, α-cells may have become more sensitive to the decreased somatostatin release in *Ucn3*-null mice.

In summary, we demonstrated here that UCN3 inhibits α-cells by potentiating somatostatin secretion from neighboring δ-cells. The application of exogenous UCN3 silences α-cell signaling and glucagon secretion. Blocking endogenous UCN3 signaling de-inhibits calcium signaling in a proportion of α-cells in the islet at high glucose. While endogenous UCN3 has minimal effects on the actual glucagon output, there is potential for nuanced regulation below the threshold of detection we used to measure α-cell activity in this series of experiments. Similar to α-cells displaying minimal activity at high glucose but playing an essential role in potentiating GSIS with the scant glucagon that is released, UCN3-potentiated somatostatin may fine-tune α-cells to a lower level of activity than they otherwise would be. This would be in line with β-cells from *Ucn3*-null mice displaying higher GSIS[28] – a possible combination effect of lower inhibition from somatostatin and increased glucagon potentiation.

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2.7 Materials & Methods

2.7.1 Animals

Mice were maintained in group housing on a 12 h light/dark cycle with free access to water and standard rodent chow. Mice expressing *Gcg*$^{em1(cre/ERT2)Khk}$ (Gcg-CreERT2; MMRRC stock #42277)[33] were used for α-cell-specific expression of fluorescent reporters for calcium, *Gt(ROSA)*$^{26Sortm96(CAG-GCaMP6s)Hze}$ (*lsl-GCaMP6s*; JAX stock #024106)[34], or cAMP, Gt(ROSA)26Sor$^{tm1(CAG-ECFP*/Rapgef3/Venus*)Kama}$ (*lsl-CAMPER*; JAX stock #032205)[35]. Experimental mice used for imaging were bitransgenic offspring that were hemizygous for both *Gcg-CreERT2* and *lsl-CGaMP6s* or *lsl-CAMPER*. Cre expression was induced via oral administration of tamoxifen (Sigma-Aldritch, T5648) dissolved in sunflower seed oil (Trader Joe's, Monrovia, CA, USA) given once at 125 mg/kg. Euthanasia and islet isolation were carried out after a 3-day washout period. Δ-cell-specific CAMPER expression was achieved by crossing Sst$^{tm2.1(cre)Zjh}$/J (*Sst-Cre*; JAX stock # 013044) with lsl-CAMPER mice. *Ucn3*-null mice have been described previously[36]. For all experiments, mice heterozygous for the *Ucn3*-null allele (*Ucn3*+/-) were used to produce knockout mice (*Ucn3*-/-, referred to as *Ucn3*-null) and control littermates (*Ucn3*+/+ or *Ucn3*+/-). For calcium imaging, *Ucn3*+/- mice were crossed with *Gcg-CreERT2 x lsl-GCaMP6s* hemizygous mice to produce *Ucn3*+/-x *Gcg-CreERT2 x lsl-GCaMP6s* mice. These offspring were then crossed with *Ucn3*+/- mice to produce *Ucn3*-null and control *Gcg-CreERT2 x lsl-GCaMP6s* mice. Mice used were between 4 and 6 months of age unless otherwise indicated. All mouse experiments were approved by the UC Davis Institutional Animals Care and Use Committee and were performed in

compliance with the Animal Welfare Act and the Institute for Laboratory Animal Research (ILAR) Guide to the Care and Use of Laboratory Animals.

2.7.2 Islet isolation

Islets were isolated by injecting 2 mL collagenaseP (0.8 mg/mL, Roche Life Sciences, Penzberg, Germany) dissolved in HBSS (ThermoFisher, Waltham, MA, USA) into the pancreas via the common bile duct while the ampulla of Vater was clamped. The inflated pancreas was removed and submerged in an additional 2 mL of collagenase solution, incubated at 37°C for 11 min and then gently shaken manually. The dissociated pancreas was washed three times with cold HBSS containing 5% NCS + 1 mM $CaCl_2$, then passed through a nylon mesh (pore size 425 μm, Small Parts) and isolated by density gradient centrifugation on a Histopaque gradient (1.077 g/mL, Sigma, St. Louis, MO, USA) for 25 min at 1400 g with no brake. Islets collected at the gradient interface were then washed once with the cold HBSS+NCS+$CaCl_2$ media and handpicked under a dissecting microscope before culturing in RPMI 1640 containing 5.5 mmol/L glucose, 10% FBS and penicillin/streptomycin.

2.7.3 FACS

Islets were dispersed by incubating for 2 min in 0.25% Trypsin-EDTA at 37°C and then gently pipetting. Dissociated islet cells were sorted at the UC Davis Flow Cytometry core on a MoFlo Astrios EQ (Beckman Coulter, Brea, CA, USA) using the 488 excitation for CAMPER and sorted directly into culture RPMI. Sorted cells were pelleted at 5 min x 114 g then resuspended with culture RPMI and loaded into imaging chambers.

2.7.4 Glucagon secretion

Islets cultured overnight were washed three times in Krebs Ringer Buffer (KRB) containing 5.5 mM glucose and 0.1% bovine serum albumin (MP Biomedicals, Santa Ana, CA, USA) then incubated at 37°C for 1 hour. Islets were then picked into 48-well plates, 20 islets per well, and incubated with respective treatments at 37°C for 90 minutes. The supernatant was collected and secreted glucagon was measured via radioimmunoassay (GL-32K, EMD Millipore, Hayward, CA, USA) or luminescence immunoassay (Lumit, Promega, Madison, WI, USA) as indicated in figure legends.

2.7.5 Functional imaging

Islet calcium and cAMP fluorescence were recorded in real time on a Nikon A1R+ confocal microscope using a 20X, 40X, or 60X lens. Islets were loaded in custom-made polydimethylsiloxane (PDMS) perfusion chambers (SYLGARD 184 Silicone Elastomer, Sigma; 10:1 base:curing agent ratio), cast into molds printed using a FormLabs Form 3 printer. Channels for islet loading and microfluidic perfusion were punched with biopsy needles (0.75 mm, Robbins Instruments, Chatham, NJ, USA) and the chambers were plasma bonded (BD-20A High Frequency Generator, ElectroTechnicProducts, Chicago, IL, USA) to glass-bottom culture dishes (35 mm, MatTek, Ashland, MA, USA). For GCaMP6s islets, 3-dimensional cross sections of the islets were recorded. For CAMPER islets, a single focal plane close to the cover slip was imaged. Pertussis toxin (PTx)-treated islets were cultured 18 hours in the presence of 200 ng/ml PTx (Tocris, Minneapolis, MN, USA) prior to imaging. Imaged islets were continuously perfused with KRB (and indicated added treatments) via a microfluidics system (Elveflow, Paris, France) at constant 200 µL/min flow. Islets were perfused with KRB at the indicated starting glucose concentration for 10 min to establish baseline fluorescence prior to starting each recording. Regions of interest were drawn around single cells or single islets to obtain fluorescence

intensity using Nikon Elements software. CAMPER ΔFRET ratio was calculated as CFP/YFP.

2.7.6 Immunofluorescence

Post-hoc whole-mount immunofluorescence staining of GCaMP6s islets was performed by fixing islets with 4% paraformaldehyde for 15 minutes, followed by three washes with PBS, and then incubation in PBS for 15 minutes on ice. Islets were then incubated overnight in PBS supplemented with 2% donkey serum and 0.4% Triton X-100 (donkey block) overnight at 4°C. On day 2, islets were incubated in donkey block with primary antibodies overnight at 4°C. On day 3, primary antibodies were washed off with PBS + 0.1% Tween (PBS-T) and incubated overnight at 4°C. On day 4, secondary antibodies diluted in donkey block were added to dishes and incubated overnight at 4°C. On day 5, secondary antibodies were washed out and islets were incubated in 4% PFA overnight at 4°C. On day 6, PFA was washed out with PBS-T and incubated at room temperature for 30 minutes six times. Islets were then bathed in RapiClear (SunJin Lab, Hsinchu City, Taiwan) clearing agent and imaged on a Nikon A1R+ confocal microscope. Primary antibodies used were guinea pig polyclonal anti-Insulin (1:500, #A0564, Dako, Santa Clara, CA, USA), rabbit polyclonal anti-UCN3 (1:1000, #7218, gift from Dr. Wylie Vale, La Jolla, CA, USA), rabbit polyclonal anti-GCG (1:400, #2760, Cell Signaling, Danvers, MA, USA) and goat anti-GFP (1:1000, #600-101-215, Rockland, Gilbertsville, PA, USA). Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA) and used at a 1:600 dilution.

2.7.7 Chemicals

General chemicals used were glucose (G7021, Sigma), epinephrine (E4642, Sigma), and potassium chloride (P9541, Sigma). Amino acid mixture (AAM) was made up of 2 mmol/L each of L-glutamine (G3126, Sigma), L-alanine (A7627, Sigma), and L-arginine (A6969, Sigma). Except for somatostatin (1157, Tocris), all peptides were generously synthesized and provided by Drs. Michael Beyermann and Jean Rivier: UCN3, arginine vasopressin (#138-046-15), astressin2b (#352-272-15), and SSTR2 antagonist (#406-028-15).

2.7.8 Statistical analysis

Data were analyzed by Student's t-test and represented as mean ± SEM, with n representing either number of cells, number of wells, or animals indicated in figure legends. Differences were considered significant when P value was <0.05. Statistics were computed using Prism (GraphPad Software). Mean fluorescence was calculated by normalizing GCaMP6s fluorescence or CAMPER ΔFRET ratio within each cell to its own maximum and minimum (normalized 0 to 1), then averaging and calculating the ± SEM for the normalized values of all cells. To account for natural variation in GCaMP6s brightness between islets from different animals, we calculated the ratio of calcium load within each individual imaging experiment. To do so, we calculated the area under the curve of α-cell calcium fluorescence for 10 minutes in 16.8 mmol/L glucose + AAM and divided that by the AUC for 10 minutes in 5.5 mmol/L glucose + AAM. The resulting ratio removes experiment-to-experiment variation in total GCaMP6s brightness and makes comparison and statistical analysis possible.

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Chapter 3

Functional heterogeneity among pancreatic α-cells

3.1 Preface

This chapter is being prepared for submission to *eLife*.

3.1.1 Authorship

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The article has been modified to satisfy the formatting requirements of this dissertation.

3.2 Abstract

The regulation of glucagon secretion from α-cells in the pancreatic islets is an essential component in maintaining blood glucose homeostasis, including the rapid correction of dangerous hypoglycemia. Dysregulated glucagon release is a hallmark of diabetes that is not completely understood, in part because there is a lack of consensus

on healthy α-cells function in the first place. α-cells respond to not only glucose levels, but also integrate other nutrients like amino acids, neurohypophysial hormones like arginine vasopressin (AVP), and catecholamine signals such as adrenal epinephrine into shaping the appropriate glucagon response to different physiological situations. In this study, we used genetically-encoded calcium and cAMP indicators to record the behavior of hundreds of α-cells, thus enabling a population-level view of α-cell intracellular signaling leading to glucagon secretion. We observe surprising heterogeneity within calcium responses to glucose, amino acids, epinephrine, and AVP, as well as between calcium and cAMP responses to the same stimuli. We find that previously observed cAMP elevation in α-cells at low glucose is able to significantly potentiate glucagon secretion when combined with calcium-stimulating signals like amino acids and AVP. Furthermore, we observe that α-cell responses to these signaling molecules are impaired in mouse models of type 2 diabetes, with transcriptomic analysis pointing to a shift in overall α-cell identity possibly being the reason for this.

3.3 Introduction

The α-cells of the pancreatic islets are among the chief regulators of blood glucose homeostasis in the body. They are defined by their principal hormone output, glucagon, which is classically known for stimulating hepatic glucose production in response to hypoglycemia[1]. When blood glucose drops below a euglycemic threshold, glucagon and a number of other hormones are mobilized as a unified hypoglycemic counterregulatory response (CRR)[2–4]. Conversely, when blood glucose is high, α-cell activity decreases and pancreatic β-cells release insulin to facilitate glucose clearance and storage.

While insulin impairment drives much of the characteristic elevated blood glucose in type 2 diabetes mellitus (T2DM), glucagon dysregulation is also widely recognized in the pathophysiology of the disease. In T2DM, inappropriately high postprandial glucagon

secretion is a major contributor to diabetic hyperglycemia[5,6]. On the other side of the glucose spectrum, glucagon release has been shown to be impaired in the CRR of T2DM patients[7–10]. Studies have uncovered many promising potential mechanisms underpinning dysfunctional α-cell signaling, namely disrupted paracrine signaling from neighboring β- and δ-cells[11], but α-cell-autonomous changes remain less understood.

Historically, the most common measures of individual α-cell activity have been calcium fluorescence and electrophysiology. The utility of these owes to the fact that α-cells are electrically active and glucagon secretion is tightly associated with calcium influx downstream of depolarization-mediated opening of voltage-gated calcium channels[12]. However, the inherent difficulty of both patch clamp electrophysiology and capturing a sizable portion of the α-cell population with calcium dyes have caused studies to struggle with throughput with conclusions often drawn from a few dozen down to single-digit number of cells. Additionally, while calcium is a required component for exocytosis of glucagon-containing granules, Yu et al. and others[13–16] have shown that calcium does not actually follow glucose levels well. cyclic AMP signaling, on the other hand, is a well-known amplifying mechanism for secretion[17,18] and is highly glucose-responsive, with low glucose driving marked cAMP generation in α-cells[13]. Thus, although calcium remains a permissive step in exocytosis, cAMP appears to be the key mediator of α-cell glucose sensing and amplification.

Much like calcium is not the only key second messenger intracellularly, glucose is just one of many extracellular variables modulating α-cell activity. The body does not often ingest glucose alone – a mixed meal typically includes amino acids and fatty acids, both of which increase glucagon secretion[19–21]. Glucagon signaling at the liver induces increased uptake and metabolism of amino acids as a fuel source for gluconeogenesis as well as increased fatty acid beta-oxidation and decreased lipogenesis, all resulting in increased energy production[22,23]. Likewise, the counterregulatory response is a physiological mobilization of many hormones to counteract dangerous hypoglycemia,

including epinephrine, arginine vasopressin (AVP), oxytocin, cortisol, & growth hormone[2,4]. Some of these signals, such as epinephrine and AVP, act directly on α-cells to potentiate glucagon secretion[24,25], and have been shown to be impaired in obesity and T2DM[9,26]. Thus, a glucose-centric approach may be insufficient for developing a better model of α-cell behavior.

In this study, we performed a population-level assessment of α-cell responses across multiple functional readouts. We overcame past throughput limitations by combining α-cell-specific expression of genetically-encoded calcium and cAMP indicators in mice with fast resonant scanning confocal microscopy of 3D intact islets and FACS-purified α-cell behavior in real time at single cell-resolution. With these tools we captured an average of 91.0 ± 9.9 α-cells per mouse, with a minimum of 3 mice and 215 total α-cells per experiment. Using these methods, we directly compared cAMP and calcium responses to an array of physiologically-relevant secretagogues – glucose, amino acids, epinephrine, and AVP. We observed considerable heterogeneity between calcium and cAMP activation, which correlated with glucagon secretion differences between stimuli. Furthermore, this functional heterogeneity was altered in *ob/ob* mouse models of T2DM, and correlated with obesity-induced transcriptomic changes in signaling pathways associated with sensing the stimuli. These findings corroborate previous studies showing the discordance of cAMP and calcium in α-cells, and clarify the physiological implications of the interplay between the two intracellular signaling molecules in healthy and diabetic α-cells.

3.4 Results

3.4.1 Different stimuli elicit heterogeneous calcium responses

As with many secretory cells, glucagon exocytosis requires calcium for fusion and release of the hormone-containing granules[12], thus calcium is often used as a proxy for α-

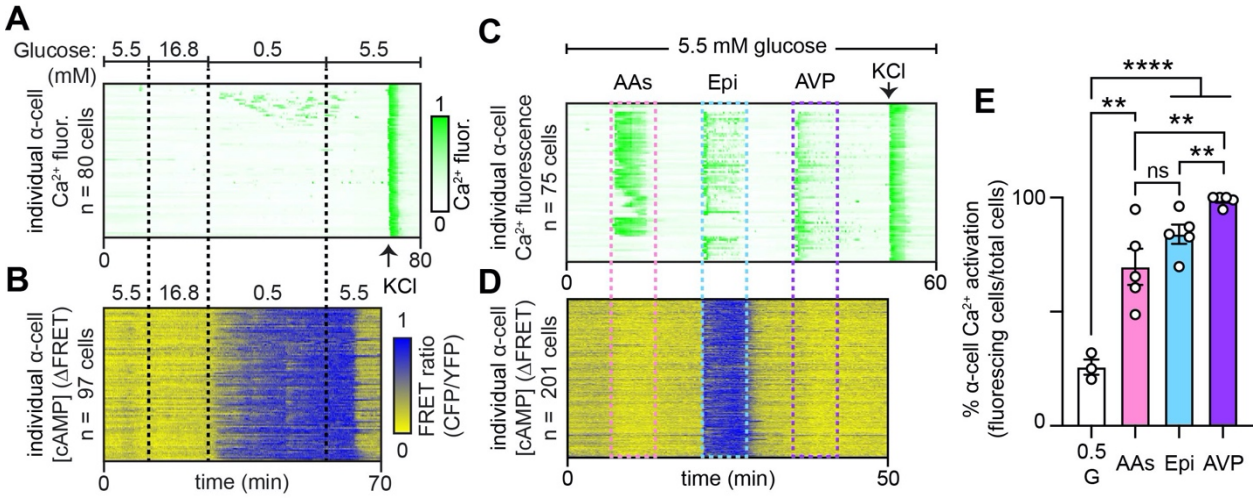

Figure 3.1: α-cells show heterogeneous calcium responses to physiological stimuli. (A) Intensity plot of α-cell calcium (GCaMP6) responses in an intact islet to basal, high, and low glucose (5.5, 16.8, and 0.5 mM, respectively). All GCaMP6 recordings end with a pulse of 30 mM KCl as a viability control. Each line on the y-axis represents the calcium response in green of a single α-cell over time (x). Representative trace of 3 recordings from 3 mice. **(B)** Intensity plot of α-cell cAMP (CAMPER) responses in an intact islet to same protocol as 1A, without KCl. Each line along the y-axis indicates the ratio of CFP:YFP for each α-cell. Yellow shows less cAMP and blue indicates higher cAMP generation. Representative trace of 5 recordings from 5 mice. **(C)** Intensity plot of calcium and **(D)** cAMP responses to a mixture of amino acids (AAs, 2 mM each glutamine, alanine, arginine), epinephrine (10 μM), and arginine vasopressin (AVP, 10 nM). All imaging experiments were performed in 5.5 mM glucose unless otherwise noted. (C) is representative of 5 traces from 5 mice. **(E)** Quantitative comparison between calcium responses to each stimulus. Bars represent mean percentage of α-cells that respond to each stimuli ± SEM. Student's t-test **$p < 0.01$, ****$p < 0.001$.

cell activation and active glucagon secretion. Yet, the connection between α-cell calcium and low glucose alone (the physiological condition in which glucagon is most often associated) is tenuous. In order to confirm previous studies showing that α-cell calcium is minimally glucose-responsive[13], we imaged islets from bitransgenic Gcg-CreERT2 x lsl-GCaMP6 mice in which the genetically-encoded calcium indicator, GCaMP6, is specifically expressed in α-cells (Supplemental Figure 3.1). We measured the calcium fluorescence of α-cells in intact islets responding to treatments in real time: first basal glucose (5.5 mM) followed by high glucose (16.8 mM), and then 30 minutes at low glucose

(0.5 mM) reflective of the length of time for CRR to take effect[3]. All GCaMP6 traces conclude with a 2 minutes pulse of potassium chloride (KCl; 30 mM) to both confirm cell viability and also as a positive control that depolarizes every cell and thus indicates the total number of activatable α-cells in the islet. We found while there is an increase in calcium influx at low glucose, this only occurs in a quarter of the α-cell population (25.8 ± 3.3%; n = 3 mice, 85.7 ± 11.6 α-cells/mouse) (Figure 3.1A). Spontaneous calcium firing at basal or high glucose occurred with negligible frequency. These data agree with studies showing that with glucose as the only variable, α-cell calcium response is infrequent and surprisingly minimal.

Unlike calcium, α-cell cAMP has been shown to faithfully and predictably reflect changes in glucose levels[13]. To confirm this, we measured cAMP changes with a modified Epac2 FRET biosensor, CAMPER, expressed specifically in α-cells. We exposed islets from Gcg-CreERT2 x lsl-CAMPER mice to the same conditions as Figure 3.1A (KCl control is unnecessary because even at low cAMP levels the CAMPER sensor emits a resting signal in all cells in which it is expressed). In response to the low glucose condition 100% of α-cells displayed an large increase in cAMP (n = 5 mice, 43.0 ± 14.6 cells/mouse) (Figure 3.1B). Thus, low glucose directly correlates with increased cAMP levels, but a corresponding calcium influx is not guaranteed in all cells.

Given the disconnect between α-cell calcium and cAMP in low glucose, we next tested other physiological stimuli often used as positive controls for α-cell activation/glucagon secretion and compared how the responses read out across hundreds of α-cells. To simulate a protein-rich meal, we used a mixture of amino acids (arginine, alanine, and glutamine; 2 mM each)[27–29]. Epinephrine (stress response/CRR; 10 μM) and AVP (CRR; 10 nM) were also used at concentrations reflective of the literature [30,31]. Each were applied successively to GCaMP6 and CAMPER α-cells, all in basal glucose – a concentration at which both α-cell-intrinsic and paracrine signaling are lowest[28], thus allowing for determination of signal-specific firing with minimal contaminating

background activity. We observed considerably heterogeneous calcium responses with 69.6 ± 7.9% of α-cells responding to amino acids, 81.5 ± 3.7% to epinephrine, and nearly all (98.9 ± 0.64%) activated by AVP (n = 5 mice, 91.2 ± 24.6 cells/mouse) (Figure 3.1C). Meanwhile, 100% of α-cells showed increased cAMP with epinephrine, and none responded to the amino acid mixture or AVP (Figure 3.1D). This pattern of cAMP responses was expected as epinephrine activates adenylyl cyclase/cAMP signaling via $G_{\alpha s}$-coupled β_1-adrenergic receptors (ADRB1) on the plasma membrane of α-cells[32,33], while amino acids and AVP affect calcium via non-cAMP-dependent mechanisms – depolarization/intracellular metabolism and PLC/IP_3 signaling, respectively[34–36]. The differential calcium and cAMP responses indicate that, much like low glucose, epinephrine-induced cAMP is upstream of calcium influx, and that the former does not guarantee the latter. Taken together, low glucose is indeed a poor modulator of α-cell calcium compared to other physiological cues (Figure 3.1E). At the same time, any signal that generates cAMP is appears to do so uniformly across all α-cells, making it an excellent readout, where appropriate.

3.4.2 Glucagon secretion is not directly proportional to calcium response in a-cells

To test the consistency of the heterogeneous pattern of calcium activation observed in Figure 1C, we performed back-to-back imaging on the same set of α-cells. We recorded calcium responses among the cells in response to amino acids, epinephrine, and AVP, let the cells recover for 3 hours, then repeated the same experiment (Figure 3.2A, 3.2B). We used FACS-sorted α-cells, which maintain their position on the dish better than cells in intact islets and thus make it easier for downstream comparisons and analysis (Figure 3.2C). When the calcium traces of each individual α-cell are compared (Figure 3.2D), we

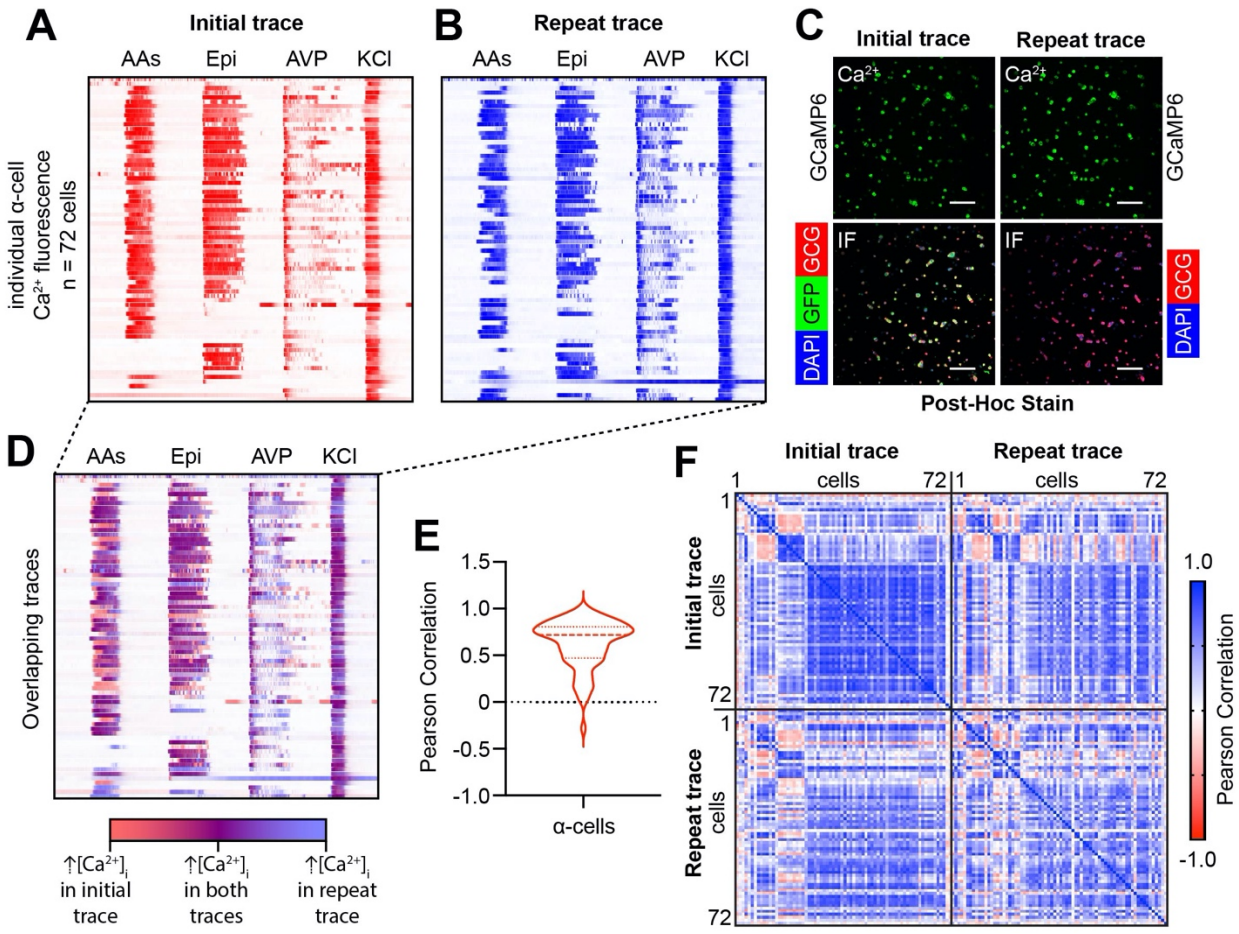

Figure 3.2: Heterogeneous calcium responses are stable over time. **(A)** Intensity plot of calcium from dispersed, FACS-purified α-cells responding to AAs (2 mM), epinephrine (10 μM), and AVP (10 nM). **(B)** Intensity plot of same α-cells as 2A, imaged 3 hours later with identical protocol. **(C)** Side-by-side snapshots of imaged α-cells from the initial and repeat calcium traces, and post-hoc immunofluorescence (IF) staining of DAPI (blue), GFP (green), and GCG (red) to confirm α-cell identity. **(D)** Overlay of initial (2A) and repeat trace (2B) with violet regions indicating similar responses. **(E)** Violin plot of Pearson correlation of each individual α-cell's initial response to its repeat response. Dotted lines indicate quartiles and median. **(F)** Heatmap of the correlation matrix for α-cell initial and repeat responses. High correlation (closer to 1) is more blue, low correlation is red (closer to -1). Correlation of each cell's initial to repeat response is in northeast or southwest quartile along the northwest to southeast diagonal (quantified in 2E).

observe strong correlation in how cells respond the first time vs. the second (Figure 3.2E, 3.2F). This indicates that heterogeneous calcium responses are a stable trait of the α-cells.

To further characterize this functional heterogeneity, we imaged dose responses for different stimuli. By increasing doses to supraphysiological levels we sought to answer whether the α-cell calcium response to any given cue reaches a maximum and if the responses are random or ordered. We imaged GCaMP6 α-cells treated with successive increasing doses of epinephrine (0.1, 1, 10, and 50 μM), followed by KCl. The proportion of α-cells that increase calcium in response to epinephrine plateaus at 88.2 ± 5.5% (n = 6 mice, 81.7 ± 16.8 cells/mouse) (Figure 3.3A). However, α-cell cAMP responses again do not match calcium – epinephrine elicits cAMP changes in 100% of α-cells starting from the lowest dose, 0.1 μM (compared to 41.5 ± 9.8% calcium-responsive α-cells at the same dose) (Figure 3.3B). These data show that calcium non-responding cells are not a byproduct of stochastic variation because every cell that responds to a low dose of epinephrine responds to each successive higher dose. Additionally, 100% cAMP activation indicates ADRB1 is distributed among all α-cells and the minimum epinephrine concentration required for receptor activation is below the concentration needed for calcium influx.

Although cAMP comparison traces are not possible with non-adrenergic compounds, calcium traces for AVP (Figure 3.3C) and 17 individual amino acids (Figure 3.3D, 3.3E, Supplemental Figure 3.2) further highlight the dose-dependent increases in responding number of α-cells. From the various compounds tested, AVP, arginine, serine, and tryptophan are the only compounds that activate nearly all α-cells, though the amino acids require an extremely high 25 mM dose to achieve this. Only histidine showed no response at any concentration.

To observe whether calcium heterogeneity translates proportionally to glucagon secretion, we compared glucagon output between signals using a six-point dose response for each of the amino acid mixture, epinephrine, and AVP at basal glucose. Glucagon secreted dose-dependently with each compound, but epinephrine-stimulated secretion substantially exceeded amino acids and AVP (Figure 3.3F). Thus, while 10 nM AVP

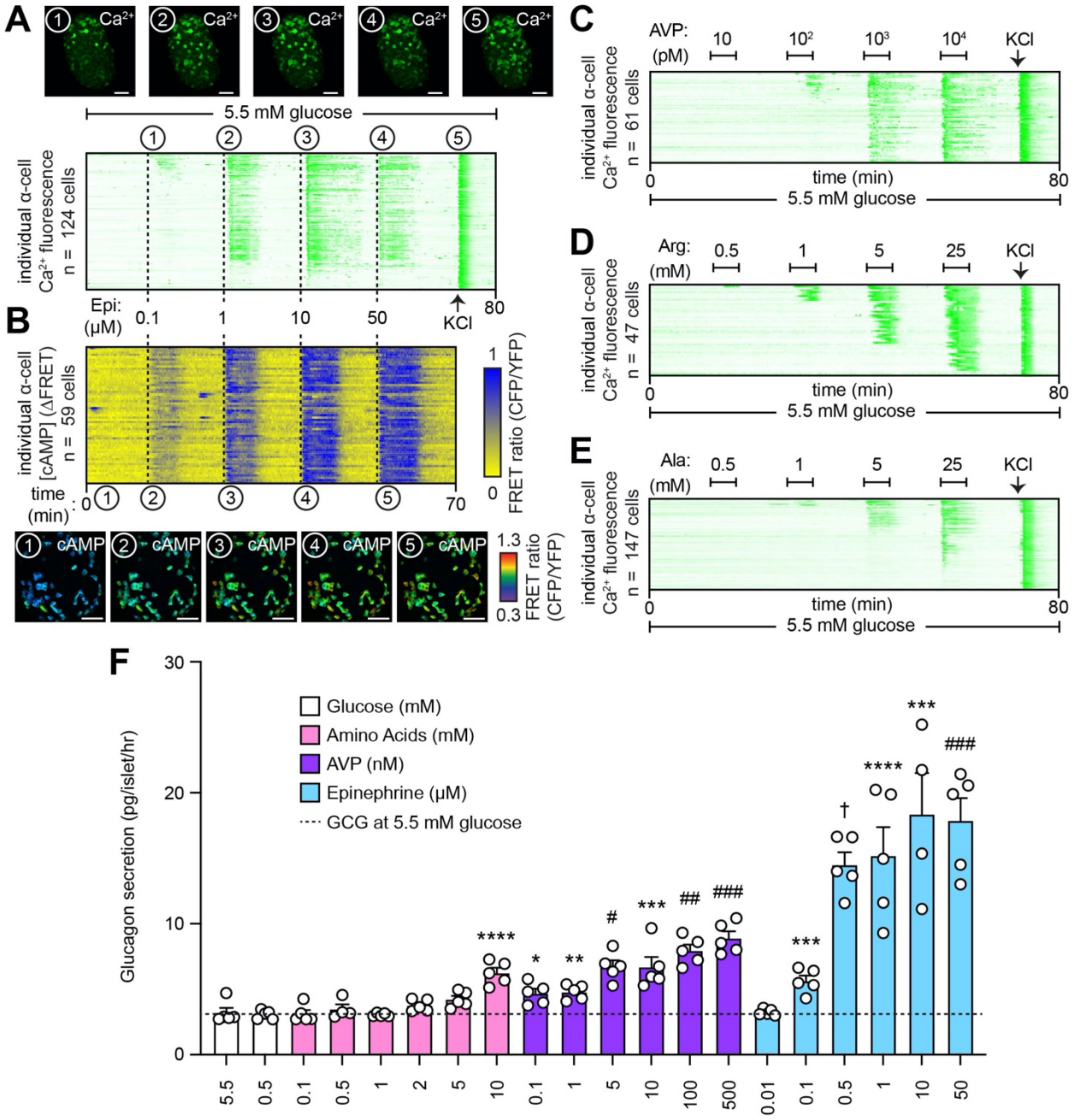

Figure 3.3: α-cell calcium does not directly correlate with glucagon secretion. **(A)** Intensity plot of calcium and **(B)** cAMP dose responses to epinephrine. Thumbnails correspond to numbered treatments with colorimetric ratio scale bar for FRET ratio. Scale bar indicates 50 μm. **(C)** AVP, **(D)** arginine, and **(E)** alanine dose responses in calcium. **(F)** Glucagon secretion from mouse islets in response to increasing doses of AAs, AVP, and epinephrine. n = 4 or 5 samples per treatment. All statistics are relative to glucagon secretion at 5.5 mM glucose alone. Student's t-test *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$, $^{\#}p < 5\text{x}10^{-4}$, $^{\#\#}p < 1\text{x}10^{-4}$, $^{\#\#\#}p < 5\text{x}10^{-5}$, †$p < 5\text{x}10^{-6}$.

activates 17% more α-cells than 10 μM epinephrine, as measured by calcium, it leads to 2.7-fold less glucagon secretion. Calcium responsiveness to each compound is not predictive of glucagon secretion beyond a dose-dependent increase within each treatment group. Given how epinephrine performs compared to the other stimuli, we began considering a model integrating cAMP and calcium to predict glucagon output.

3.4.3 Low glucose-activated cAMP increases glucagon response to stimulators of a-cell calcium

In seeking to understand the significance of calcium heterogeneity and its discrepancy with cAMP and glucagon secretion, we found prior studies showing that amino acid- and, separately, AVP-stimulated glucagon is inversely and dose-dependently proportional to glucose levels[27,37,38]. We hypothesized that this amplification is due to amino acid and AVP calcium effects synergizing with low glucose-stimulated cAMP. We first measured glucagon secretion from islets exposed to each of the three compounds at low, basal, and high glucose. We observed glucose-dependency as others have published, except with epinephrine, which maintained high glucagon secretion across the range of glucose concentrations (Figure 3.4A). At low glucose, AVP elicited 3.3-fold higher glucagon secretion than amino acids and 2.3-fold higher than epinephrine. Thus, glucagon responses to the three compounds at low glucose correlate much better with observed calcium percentages than at basal glucose.

We hypothesized that epinephrine escapes glucose-dependency because it generates cAMP independently, so we next compared the cAMP and calcium responses of low glucose and epinephrine in the same experiment. We imaged islets with α-cells expressing GCaMP6 side-by-side with islets with CAMPER, and sequentially exposed them to low glucose and epinephrine. Having islets with both reporters in the same dish ensured that any observed non-responding calcium reporter cells would not be a byproduct of

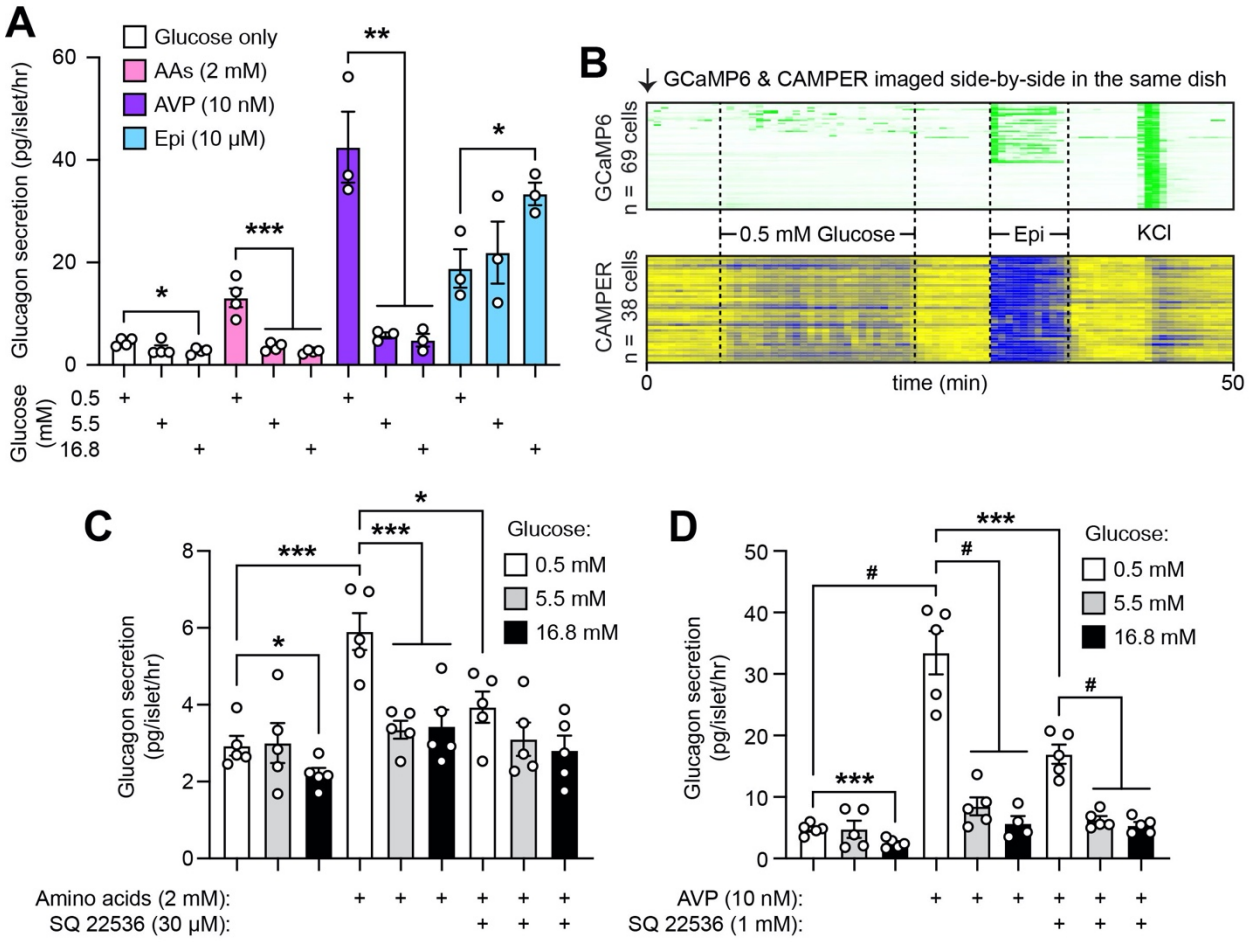

Figure 3.4: α-cell stimuli show glucose cooperativity that is driven by cAMP. (A) Glucagon secretion from mouse islets exposed to AAs, epinephrine, and AVP at low, basal, and high glucose. n = 3 or 4 samples per treatment. Student's t-test *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$. **(B)** Intensity plots of calcium and cAMP recorded simultaneously from GCaMP6 and CAMPER islets in the same dish responding to low glucose and epinephrine (10 μM). **(C)** Glucagon secretion at low, basal, and high glucose with and without AAs (2 mM) and adenylyl cyclase inhibitor (SQ 22536, 30 μM). n = 5 samples/treatment. Student's t-test *$p < 0.05$, ***$p < 0.005$. **(D)** Glucagon secretion at low, basal, and high glucose with and without AVP (10 nM) and adenylyl cyclase inhibitor (SQ 22536, 1 mM). n = 5 samples/treatment. Student's t-test ***$p < 0.005$, $^{\#}p < 5x10^{-4}$.

differences in treatment preparations. We saw partial calcium responses of the α-cell populations in line with prior traces where low glucose and epinephrine responses were measured separately (Figure 3.4B). Meanwhile, cAMP increase was reliably recorded in all cells for both conditions, and low glucose elicited effectively identical cAMP as 10 μM

epinephrine (100.0 ± 3.3%) as measured by area under the curve. Thus, epinephrine stimulating similar levels of cAMP on its own can possibly account for lack of glucose dose-dependence.

In order to test whether signals other than epinephrine exhibit glucose dependency as a function of cAMP amplification at low glucose, we measured glucagon output in response to amino acids at each glucose level while in the presence of the adenylyl cyclase inhibitor, SQ 22536 (30 μM). In line with the potentiating role of cAMP at low glucose, SQ 22536 blocked amino acid-stimulated glucagon secretion at low glucose (Figure 3.4C). Adenylyl cyclase inhibition also blocked the low glucose-mediated potentiation of AVP-stimulated glucagon secretion (Figure 3.4D), though not to the level of AVP-stimulated glucagon at basal glucose. These data show that glucose sensitivity of amino acid- and AVP-stimulated glucagon secretion is regulated by the increase in adenylyl cyclase activity and resultant cAMP production in α-cells in low glucose.

3.4.4 There is a shift in expression of receptors and transporters mediating activation in ob/ob a-cells

To discern whether α-cell functional heterogeneity is a stable state or is plastic and responsive to physiological demands, we explored α-cell identity and behavior in *ob/ob* mice. Glucagon impairment is a well-documented feature of type 2 diabetes, thus we first sought to ascertain whether changes in α-cell gene expression is underlying some of the observed dysfunction. We performed RNA-Seq on FACS-purified α-and β-cells from lean and *ob/ob* mice. Quad-transgenic lean and obese offspring of *ob/ob* x mIP-H2b-mCherry x Gcg-CreERT2 x lsl-YFP mouse crosses contain α-cells that express YFP and β-cells with nuclear mCherry, thus permitting straightforward identification during FACS. As shown in the heat map in Figure 3.4A, the identity of sorted α- and β-cells was confirmed by expression of *Gcg* and *Ins1/2*, respectively. Pulling out 19 representative genes related

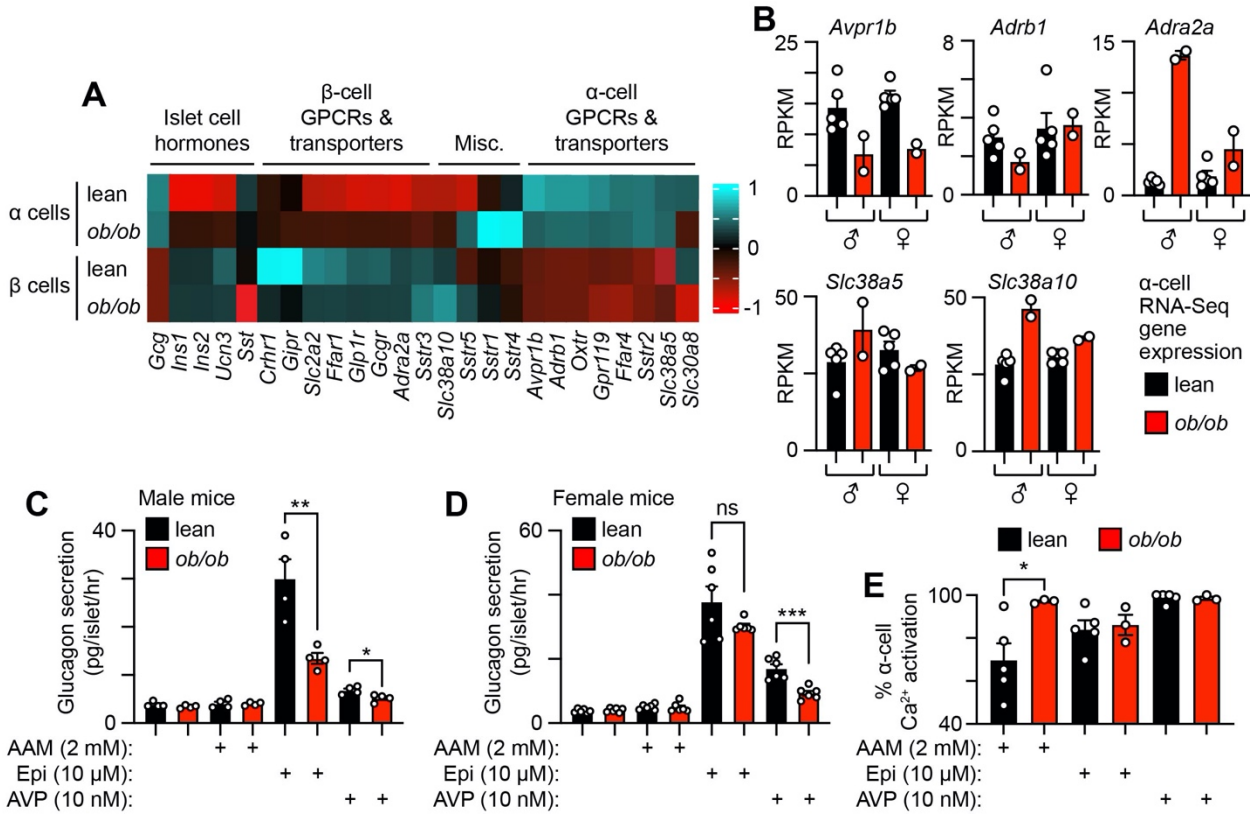

Figure 3.5: *ob/ob* α-cells show decreased sensitivity to epinephrine and AVP. (A) Heatmap of differential expression of key hormones and select GPCRs and transporters in lean and *ob/ob* α-and β-cells, measured by RNA-Seq. **(B)** RNA-Seq expression (RPKM) of receptors and transporters for AVP, epinephrine and amino acids broken down by sex and mouse metabolic status. n = 5 lean mice and 2 *ob/ob* mice per group. **(C)** Glucagon secretion from isolated islets from male and **(D)** female lean and ob/ob mice. n = 4 male samples 6 female samples/treatment. Student's t-test *p $<$ 0.05, **p $<$ 0.01, ***p $<$ 0.005. **(E)** Percentage of GCaMP6-expressing α-cells activated by AAs, epinephrine, and AVP between lean and *ob/ob* males. n = 5 lean mice and 3 *ob/ob* mice/treatment. Student's t-test *p $<$ 0.05. Bar graph includes data from Figure 1E.

to endocrine, paracrine, neuronal, and nutritional signaling, we observe transcriptomic changes suggesting obesity-induced alterations in sensitivity (Figure 3.5A). The *ob/ob* α-cells upregulate a number of receptors and transporters typically associated with β-cells, including inhibitory $G_{\alpha i}$-coupled α_{2A}-adrenergic receptor (*Adra2a*), and downregulate important signaling genes such as stimulatory *Adrb1* and the $G_{\alpha q}$-coupled AVP receptor 1b (*Avpr1b*). Interestingly, *ob/ob* α-cells also increased granularity (Supplemental Figure

3.3), and upregulated a number of β-cell identity genes and transcription factors (Supplemental Figure 3.4).

Given that there are known sex differences in susceptibility to diabetes [39], we also split out the RNA-Seq data into male and female groups to uncover potential transcriptomic differences underlying this phenomenon. We observe that *Avpr1b* expression is decreased in α-cells from *ob/ob* mice, relative to lean, across sexes (2.1-fold decrease in male, 2.0-fold female), but other genes show a sex difference (Figure 3.5B). *Adrb1* is decreased 1.7-fold in α-cells from male *ob/ob* mice, but not in females (1.1-fold higher). Meanwhile, *Adra2a*, and amino acid transporters *Slc38a5* and *Slc38a10* are upregulated in males (10.1-, 1.4-, and 1.6-fold), but show only modest differences in females (2.4-fold higher, 1.2-fold lower, 1.2-fold higher). Based on these transcriptomics data, one would predict obesity-induced changes as well as sex differences in glucagon responsiveness to amino acids, epinephrine, and AVP.

To ascertain whether the differential expression of the various receptors and transporters correlated with changes in α-cell responsiveness, we measured amino acid-, epinephrine-, and AVP-stimulated glucagon secretion from lean and *ob/ob* male (Figure 3.5C) and female (Figure 3.5D) mice. In line with sex-specific receptor expression changes, islets from male *ob/ob* mice show a 2.2-fold decrease in epinephrine-stimulated glucagon secretion, but both sexes experience decreased AVP responsiveness and no change in amino acid effects. Similarly, to determine if gene expression correlates with calcium responsiveness, we exposed islets from *ob/ob* x Gcg-CreERT2 x lsl-GCaMP6 mice to the trio of stimuli. We observed that while epinephrine and AVP-mediated glucagon secretion decreases in disease, calcium responsiveness remains constant at the concentrations used (Figure 3.5E). Corroborating increased transporter expression, 27.4% more α-cells are activated by amino acids in *ob/ob* islets.

3.4.5 Epinephrine response in ob/ob α-cells is impaired, partially due to changing receptor expression

α-cell transcriptomes and glucagon secretion show major changes in epinephrine sensitivity in *ob/ob* mice. To further characterize the epinephrine response in diabetes, we performed dose response calcium imaging experiments. In agreement with Figure 3.5E, there was no statistical difference between the number of calcium responsive α-cells at the 10 μM dose (Figure 3.6A). However, there was a 19.9% decrease at 1 μM, which may suggest a right shift in the response curve in diabetes.

To parse out the effects of adrenergic receptor expression changes, we first measured epinephrine-stimulated (200 nM) glucagon secretion from lean and *ob/ob* male islets in the presence of the β_1-adrenergic receptor antagonist CGP 20712 (200 nM) and the α_{2A}-adrenergic receptor BRL 44408 (200 nM). As in Figure 3.5C, epinephrine-potentiated glucagon secretion was markedly decreased in diabetic islets (Figure 3.6B). CGP 20712 blocked the effects of epinephrine in lean islets, but not diabetic. Interestingly, BRL 44408 also inhibited the ability of epinephrine to increase glucagon in lean islets.

To better observe the potential functional consequences of altered adrenergic receptor expression in diabetes, we imaged α-cell calcium in islets from lean (Figure 3.6C) and *ob/ob* male mice (Figure 3.6D). In order to observe possible inhibition mediated by the $G_{\alpha i}$-coupled α_{2A}-adrenergic receptor, we established basal calcium activation with amino acids before adding epinephrine (200 nM). In the middle of 30 minutes in epinephrine, we treated with BRL 44408 (200 nM), which would de-inhibit any α-cells shut off by epinephrine. In islets from lean mice, α-cells are activated normally by epinephrine (Figure 3.6C). Among *ob/ob* α-cells we observe a subset of cells comprising 2.5 ± 0.2% of the total α-cell population (n = 3 mice, 102.7 ± 29.2 cells per mouse) that are paradoxically inhibited by epinephrine and activated with BRL 44408 (Figure 3.6D). Thus, impaired glucagon secretion in response to epinephrine may be due to *Adrb1*

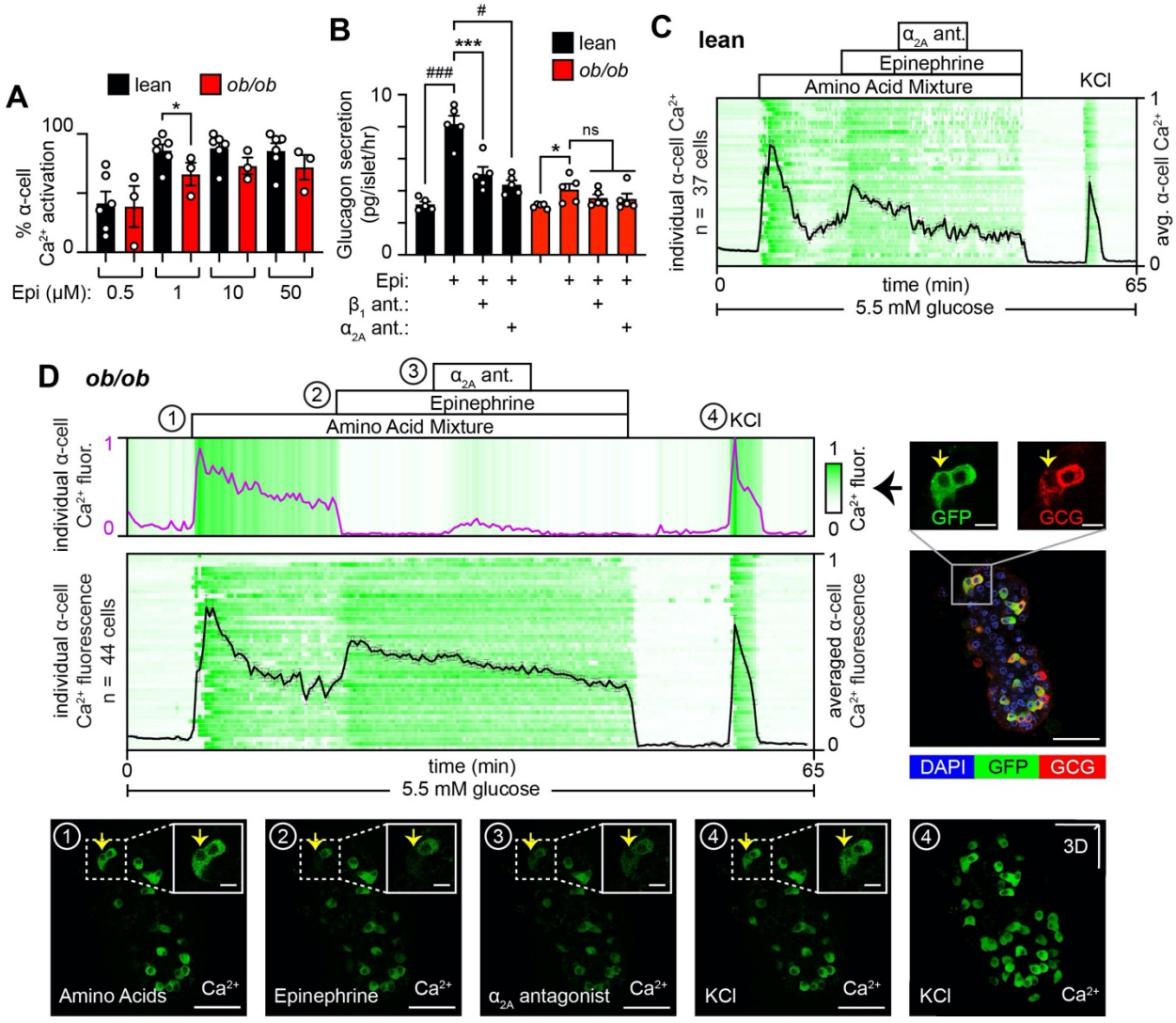

Figure 3.6: Diabetes drives changes in α-cell adrenergic signaling. **(A)** Percent calcium responses of α-cells from lean and *ob/ob* mice to increasing doses of epinephrine. n = 6 lean mice and 3 *ob/ob* mice/treatment. Student's t-test *p < 0.05. Includes quantified data from Figure 3A. **(B)** Glucagon secretion from isolated islets from lean and *ob/ob* mice in the presence of epinephrine (200 nM), β_1-adrenergic receptor antagonist (CGP 20712, 200 nM), and α_{2A}-adrenergic receptor antagonist (BRL 44408 maleate, 200 nM). n = 5 samples/treatment. Student's t-test *p < 0.05, ***p < 0.005, $^{\#}$p < $5\text{x}10^{-4}$, $^{\#\#\#}$p < $5\text{x}10^{-5}$. **(C)** Intensity plot of calcium responses of α-cells from an intact islet from a lean mouse to epinephrine (200 nM) and BRL 44408 maleate (α_{2A}-ant, 200 nM) while...

downregulation and *Adra2a* upregulation resulting in two functionally opposed α-cell subpopulations. In total, the data reveal fascinating calcium heterogeneity in healthy α-cell responses to various physiological stimuli and extending to T2DM.

Figure 3.6 cont. stimulated by AAs (2 mM). **(D)** Intensity plot of calcium from *ob/ob* α-cells using same protocol as 6C. Upper panel shows single α-cell with heterogeneous adrenergic response (purple line graph is analogous to green fluorescence intensity). Lower panel shows all other α-cells in the islet. The average GCaMP6 activity of all α-cells in the trace is indicated in the black line graph ± SEM. Thumbnails correspond to numbered treatments and immunofluorescent post-hoc confirmation of α-cell identity. Yellow arrow and inset magnification indicates heterogeneous cell from upper intensity plot. Scale bars indicate 50 μm for single plane thumbnails, 10 μm for magnified insets, 50 x 50 x 22.5 μm for 3D projection.

3.5 Discussion

In this study, we present previously uncharacterized functional heterogeneity among α-cell calcium responses to different physiological activation signals. In describing these observations, we additionally attempt to place this heterogeneity in context with how it relates to α-cell transcriptomes, cAMP levels, and glucagon release, and affects hormone output in healthy and diabetic islets. We measured hundreds of α-cell calcium and cAMP responses per experiment in order to build a population-level understanding of α-cell behavior in a variety of physiological contexts. What became immediately apparent upon comparing calcium responses to low glucose, amino acids, epinephrine, and AVP is that the α-cells are not a monolith: they are not a homogenous population that reacts *en masse* to all stimuli with every cell fine-tuning glucagon release based on the strength of signal. Rather, different compounds activate a fixed percentage of α-cells.

Past studies have reported a certain degree of α-cell calcium heterogeneity before, but have lacked the throughput seen here[14,30]. And those that managed to capture sufficient numbers have largely ignored the implications of partial activation of the islet α-cell mass[15]. Additionally, heterogeneity has never been compared between signals within the same trace. The four main stimuli used in this study were chosen on the basis of their usage in the literature as positive controls for either α-cell activation or glucagon secretion[13,27–31,33]. We confirm work from other groups showing a minority of α-cells

actually independently oscillate calcium with low glucose alone[13–16]. We observed 88% of α-cells respond to epinephrine (in line with earlier reported numbers by Liu et al., 2004) and roughly 70% to an amino acid mixture, meaning at least 1 in 10 α-cells would be missed using these as positive controls for α-cell calcium. The only signals with near-100% efficacy with calcium as the readout were AVP and oxytocin (Supplemental Figure 3.5). We did not expand on oxytocin because it appears to act through both its native receptor, OXTR, and cross-reactivity with AVPR1B[40]. These findings indicate that prior work in which α-cells were identified via activity at low glucose and/or responsiveness to epinephrine[16,33] may have, through no fault of their own, missed a large chunk of the α-cell population when drawing conclusions ostensibly describing total α-cell behavior.

When compared in the same trace, the α-cells broke out into what appear to be different subgroups of responders with the majority responding to all three stimuli, some responding to only amino acids and AVP and others responding to only epinephrine and AVP. These subdivisions were largely stable with cells maintaining the same response pattern across back-to-back imaging sessions. Breaking down the responses even further reveals heterogeneity within responses to each stimulus – amino acids elicited early and delayed responses, and epinephrine and AVP activation were either extended or showed oscillations diminishing over the course of exposure. The intrigue of these functional subgroups is further strengthened by the fact that epinephrine activates cAMP production in 100% of α-cells, thus the heterogeneous calcium is unlikely to be due to heterogeneous adrenergic receptor expression. Although we did not explore the mechanism driving the discrepancy between epinephrine calcium and cAMP response in this study, we continued pursuing the combined role of calcium and cAMP in glucagon release.

Although this heterogeneity is not stochastic, demonstrated by the dose response experiments, and calcium is required for hormone granule exocytosis, glucagon secretion responses to the secretagogues at basal glucose did not correlate with the percent responses of GCaMP6 α-cells. Interestingly, multiple groups have shown for decades that glucose

dose-dependently modulates amino acid- and AVP-stimulated glucagon secretion in an inverse fashion[27,37]. Given that work from Yu et al. and our own experiments demonstrated unambiguous glucose-dependent cAMP generation in α-cells, and evidence that cAMP amplifies secretion by increasing the number of hormone-containing granules that are primed for release (the readily releasable pool)[24] and/or lowering the threshold for calcium-mediated exocytosis[18], we postulated that the glucose effect on amino acid and AVP stimulation was due to increased intracellular cAMP. Combining amino acids, epinephrine, and AVP with low glucose led to glucagon release more closely emulating calcium heterogeneity with ranked secretion from 1) AVP, 2) epinephrine, 3) amino acid-stimulation following the percentage of activated α-cells. This low glucose amplification is indeed because of cAMP as adenylyl cyclase inhibition blocked the increase in response to amino acids and AVP. These observations follow physiology when considering that α-cell activation during the counterregulatory response involves simultaneous sensing of hypoglycemia, AVP, and epinephrine[41,42]. Similarly, a high protein diet could effectively mimic low glucose situations while increasing circulating amino acids.

As type 2 diabetes develops it is accompanied by a host of physiological changes in both the release of glucagon secretagogues in the body and the responsiveness of α-cells to the signals. In hypoglycemic clamp and acute insulin injections, T2DM subjects consistently exhibit decreased glucagon release while epinephrine and norepinephrine levels show either no change or are increased[8,10,43,44]. Increased peripheral adrenergic signaling would allow for compensation for the lost glucagon CRR as epinephrine can stimulate glucose production on its own, though this would also suggest a loss of epinephrine sensitivity among α-cells. Repeated exposure to hypoglycemic episodes results in long term T2DM patients developing hypoglycemia-associated autonomic failure (HAAF), wherein both epinephrine and glucagon are impaired thus compromising the CRR as a whole[9,45]. Amino acid alterations include significantly decreased circulating glutamine and arginine in T2DM rats and human subjects compared to controls[46,47]. The

increase in transporter expression and calcium activation we observe is potentially a compensatory response to increase uptake given the amino acid scarcity. In support of this are data showing that utilization of alanine and glutamine as substrates for gluconeogenesis is increased in T2DM[48], and glutamine-stimulated glucagon secretion from T2DM human islets is increased 2.7-fold relative to non-diabetic donors[49]. While T2DM-associated changes in AVP have not been studied, obese men have shown impaired AVP and oxytocin-induced hypoglycemic responses[26].

We have combined our calcium imaging and glucagon measurements side-by-side with bulk transcriptomic analysis of α-cells from *ob/ob* and lean mice to reveal potential mechanisms by which some of these physiological changes may arise. Decreased AVP receptor 1b expression in *ob/ob* α-cells correlated with impaired glucagon release in response to AVP stimulation. Additionally, male-specific downregulation of β_1-adrenergic receptor and upregulation of α_{2A}-adrenergic receptor carried over to lower epinephrine-stimulated glucagon secretion and heterogeneous calcium response profiles. The possible changes in cAMP in *ob/ob* α-cells resulting from competing adrenergic receptors remains to be seen. While we are unable to carry out specialized Patch-Seq experiments directly correlating α-cell electrophysiological heterogeneity with single cell RNA-Seq, as showcased by Camunes-Soler et al.[50], our transcriptomic, calcium, and glucagon data largely agree. Whether altered α-cell receptor expression is caused by T2DM changes in circulating levels of the respective ligand, or ligand levels change due to altered α-cell sensitivity is inconsequential. The two situations in which glucagon dysfunction arises do not have to be mutually exclusive as diabetes typically impairs multiple systems at once[51,52].

The increased expression of α_{2A}-adrenergic receptor on *ob/ob* α-cells is notable because it is expressed by β-cells in healthy islets, and the α-cells exhibited other signs of β-cell-like characteristics appearing. These observations are reflective of studies showing that under intense physiological stress α-cells show signs of plasticity – in models of β-cell

loss they preferentially shift to a β-cell-like entity[53,54] and in T2DM islets α-cells also alter processing of the proglucagon peptide and increase the production of glucagon-like peptide 1[55,56]. α-cells have been shown to be uniquely resistant to apoptosis, and thus seem more likely to adapt to different functional state instead[57]. Even in non-stress situations, α-cells have been shown to transdifferentiate to immature "virgin" β-cells[31]. Thus, our findings that *ob/ob* α-cells are moving both transcriptionally and functionally towards β-cells bears further exploration as we seek to better understand T2DM progression.

In summary, we find that α-cells respond to various physiological stimuli with markedly heterogeneous calcium responses. Amino acid, epinephrine, and AVP-stimulated calcium profiles are not reflected in glucagon secretion on their own, but rather need the assistance of cAMP-driven amplification achieved at low glucose. Meanwhile α-cells from *ob/ob* mice undergo transcriptomic changes in the expression of receptors and transporters that mediate the signaling for these stimuli. These expression changes correlate with altered calcium and glucagon secretion responses, and reflect clinically observed impairment of glucagon responses in type 2 diabetic individuals. This population-scale survey of multiple levels of α-cell behavior sets the stage for a deeper understanding of other α-cell secretagogues, and further exploration into α-cells in other disease states and human islets as well.

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3.8 Data availability statement

Data will be publicly available on GEO upon publication.

3.9 Methods

3.9.1 Animals

Mice were maintained in group housing on a 12 h light/dark cycle with free access to water and standard rodent chow. Mice expressing *Gcg*$^{em1(cre/ERT2)Khk}$ (Gcg-CreERT2; MMRRC stock #42277) (Amanda M Ackermann 2017) were used for α-cell-specific expression of fluorescent reporters for calcium, *Gt(ROSA)*$^{26Sortm96(CAG-GCaMP6s)Hze}$ (lsl-GCaMP6s; JAX stock #024106) (Madisen et al. 2015), or cAMP, Gt(ROSA)26Sor$^{tm1(CAG-ECFP*/Rapgef3/Venus*)Kama}$ (lsl-CAMPER; JAX stock #032205) (Muntean BS 2018). Experimental mice used for imaging were bitransgenic offspring that were hemizygous for both Gcg-CreERT2 and lsl-CGaMP6s or lsl-CAMPER. When possible, mice also expressed mIns1-H2b-mCherry (Chy; Jax stock #28589), a β-cell-specific nuclear red marker, for an additional expression control. Cre expression was induced via oral administration of tamoxifen (Sigma-Aldritch, T5648) dissolved in sunflower seed oil (Trader Joe's, Monrovia, CA, USA) given once at 125 mg/kg. Euthanasia and islet isolation were carried out after a 3-day washout period. For all *ob/ob* experiments, mice heterozygous for the *ob/ob* allele (+/*ob*) were used to produce obese (*ob/ob*) and lean control littermates (+/+ or +/*ob*). For calcium imaging, +/*ob* mice were crossed with Gcg-CreERT2 x lsl-GCaMP6s hemizygous mice to produce +/*ob* x Gcg-CreERT2 x lsl-GCaMP6s mice. These offspring were then crossed with +/*ob* mice to produce *ob/ob* and lean control Gcg-CreERT2 x lsl-GCaMP6s mice. Mouse blood glucose and body weight were measured weekly, and were considered hyperglycemic upon three consecutive glucose measurements >200 mg/dL. Male *ob/ob* mice and lean controls were used for experiments unless otherwise noted.

Both male and female mice were used for the RNA-seq experiment. Lean (+/+ or +/*ob*) and *ob/ob* mice were crossed with Chy, Gcg-Cre and lsl-eYFP to enable the purification of α-and β-cells. Mice used were between 3 and 6 months of age unless otherwise indicated. All mouse experiments were approved by the UC Davis Institutional Animals Care and Use Committee and were performed in compliance with the Animal

Welfare Act and the Institute for Laboratory Animal Research (ILAR) Guide to the Care and Use of Laboratory Animals.

3.9.2 Islet isolation

Islets were isolated as previously described [58]. The ampulla of Vater was clamped and 2 mL collagenase P (0.8 mg/mL, Roche Life Sciences, Penzberg, Germany) dissolved in HBSS (ThermoFisher, Waltham, MA, USA) was injected into the pancreas via the common bile duct. The inflated pancreas was then removed, submerged in an additional 2 mL of collagenase solution, incubated at 37°C for 11 min, and manually shaken. Islets were isolated by washing three times with cold HBSS supplemented with 5% NCS + 1 mM $CaCl_2$, then passing through a nylon mesh (pore size 425 μm, Small Parts), and centrifuging on a Histopaque gradient (1.077 g/mL, Sigma, St. Louis, MO, USA) for 25 min at 2100 g with no brake. Islets at the interface were then washed with the supplemented HBSS and handpicked under a dissecting microscope before culturing in RPMI 1640 containing 5.5 mmol/L glucose, 10% FBS and penicillin/streptomycin. For intact islet- and single cell-imaging, islets were loaded into imaging dishes the same day as isolation to adhere overnight. For RNA-Seq and glucagon secretion, islets were incubated in 10 cm petri dishes overnight and used the following day. Islets from *ob/ob* mice were cultured overnight in RPMI with glucose reflective of the average blood glucose over their final three measurements.

3.9.3 FACS

Isolated islets were washed with PBS and dissociated in 0.25% Trypsin-EDTA solution (Invitrogen, Waltham, MA, USA) for 2 min at 37 °C with gentle mechanical dissociation by pipetting. Islets from each animal were kept separate. Dissociated islet

cells were sorted at the UC Davis Flow Cytometry core on a MoFlo Astrios EQ (Beckman Coulter, Brea, CA, USA) using the 561 excitation laser for Chy, the 488 excitation for YFP/GCaMP6, with 405 excitation of DAPI to exclude dead cells. For RNA-Seq, the sorted cells were collected directly into Trizol to ensure immediate cell lysis and preservation of RNA integrity. For purifying α-cells intended for imaging, GCaMP6 in dissociated cells was stimulated with 30 mM KCl before being loaded into the machine and sorted into culture RPMI. Sorted cells were pelleted at 5 min x 114 g then resuspended with culture RPMI and loaded into imaging chambers.

3.9.4 Functional imaging

α-cell calcium and cAMP fluorescence were recorded on a Nikon A1R+ confocal microscope using a 20X, 40X, or 60X lens. Islets and sorted α-cells were loaded into custom-made polydimethylsiloxane (PDMS) perfusion chambers. Chamber molds were printed using a FormLabs Form 3 printer. PDMS was mixed at 10:1 base:curing agent ratio (SYLGARD 184 Silicone Elastomer, Sigma), poured into molds, and bubbles were popped by placing under vacuum in a glass desiccator before baking in a dry oven at 80°C. Channels for islet loading and microfluidic perfusion were punched with biopsy needles (0.75 mm, Robbins Instruments, Chatham, NJ, USA) and the chambers were bonded via plasma (BD-20A High Frequency Generator, ElectroTechnicProducts, Chicago, IL, USA) to glass-bottom culture dishes (35 mm, MatTek, Ashland, MA, USA). Imaged islets and cells were continuously perfused with KRB (and indicated treatments) via a microfluidics system (Elveflow, Paris, France). An Elveflow OB1 MK3+ pressure controller drove KRB flow through a Mux Distribution Valve into the imaging dish at 200 µL/min, all controlled via the Elveflow Smart Interface. Islets were perfused with KRB at the 5.5 mM glucose for 10 min to establish baseline fluorescence prior to starting each recording. Regions of interest were drawn around single cells or single islets to obtain fluorescence intensity

using NIS-Elements software. CAMPER ΔFRET ratio was calculated as CFP/YFP. Intensity plots were generated using in-house R script, and cells were arranged manually according to response pattern.

3.9.5 Immunofluorescence

Post-hoc whole-mount immunofluorescence staining of GCaMP6s islets was performed by fixing islets with 4% paraformaldehyde for 15 minutes, followed by three washes with PBS, and then incubation in PBS for 15 minutes on ice. Islets were then incubated overnight in PBS supplemented with 2% donkey serum and 0.4% Triton X-100 (donkey block) overnight at 4°C. On day 2, islets were incubated in donkey block with primary antibodies overnight at 4°C. On day 3, primary antibodies were washed off with PBS + 0.1% Tween (PBS-T) and incubated overnight at 4°C. On day 4, secondary antibodies diluted in donkey block were added to dishes and incubated overnight at 4°C. On day 5, secondary antibodies were washed out and islets were incubated in 4% PFA overnight at 4°C. On day 6, PFA was washed out with PBS-T and incubated at room temperature for 30 minutes six times. Islets were then bathed in RapiClear (SunJin Lab, Hsinchu City, Taiwan) clearing agent and imaged on a Nikon A1R+ confocal microscope. Primary antibodies used were guinea pig polyclonal anti-Insulin (1:500, #A0564, Dako, Santa Clara, CA, USA), rabbit polyclonal anti-UCN3 (1:1000, #7218, gift from Dr. Wylie Vale, La Jolla, CA, USA), rabbit polyclonal anti-GCG (1:400, #2760, Cell Signaling, Danvers, MA, USA) and goat anti-GFP (1:1000, #600-101-215, Rockland, Gilbertsville, PA, USA). Secondary antibodies were obtained from Jackson ImmunoResearch (West Grove, PA) and used at a 1:600 dilution.

3.9.6 Glucagon secretion

Islets cultured overnight were washed three times in Krebs Ringer Buffer (KRB) containing 5.5 mM glucose and 0.1% bovine serum albumin (MP Biomedicals, Santa Ana, CA, USA) then incubated at 37°C for 1 hour. Islets were then picked into 48-well plates, 20 islets per well, and incubated with respective treatments at 37°C for 90 minutes. The supernatant was collected and secreted glucagon was measured via luminescence immunoassay (Lumit, Promega, Madison, WI, USA).

3.9.7 Nucleic acid isolation & library prep

RNA was isolated from Trizol-preserved samples by chloroform extraction, assisted by phase lock tubes, and precipitated with isopropanol. Quality of RNA was assessed using the 2100 Bioanalyzer (Agilent, Santa Clara, CA, USA). Only samples with a RIN value of 8 or higher were used to prepare RNA-seq libraries, with the exception of one sample with a RIN value of 7.4. Indexed sequencing libraries were prepared using the TruSeq RNA sample Prep Kit v2 (Illumina Inc., San Diego, CA, USA) and sequenced at 75 cycles, single read on an Illumina MiSeq platform.

3.9.8 RNA-Seq alignment, quantification, differential expression analysis & visualization

Read quality control was performed on raw and filtered fastq files using FastQC[59]. Adapter trimming and read filtering were performed using fastp[60]. Filtered fastq files were then aligned using STAR[61] against Gencode's GRCm38.96 M19 (mm10)[62] mouse genome build. Gene-level quantification was performed on sorted BAM files using featureCounts[63]. Differential expression analysis results and RPKM values were generated using the R BioConductor package edgeR[64]. Clustering analysis was performed using Gene Cluster

3.0[65] on RPKM values, and the R BioConductor package ComplexHeatmap [66] was used to generate heatmap visuals.

3.9.9 Statistical analysis

Data were analyzed by two-tailed student's t-test and represented as mean ± SEM, with n representing either number of cells, number of wells, or animals indicated in figure legends. Differences were considered significant when P value was <0.05. Statistics were computed using Prism (GraphPad Software). Mean fluorescence was calculated by normalizing GCaMP6s fluorescence within each cell to its own maximum and minimum (normalized 0 to 1), then averaging and calculating the ± SEM for the normalized values of all cells.

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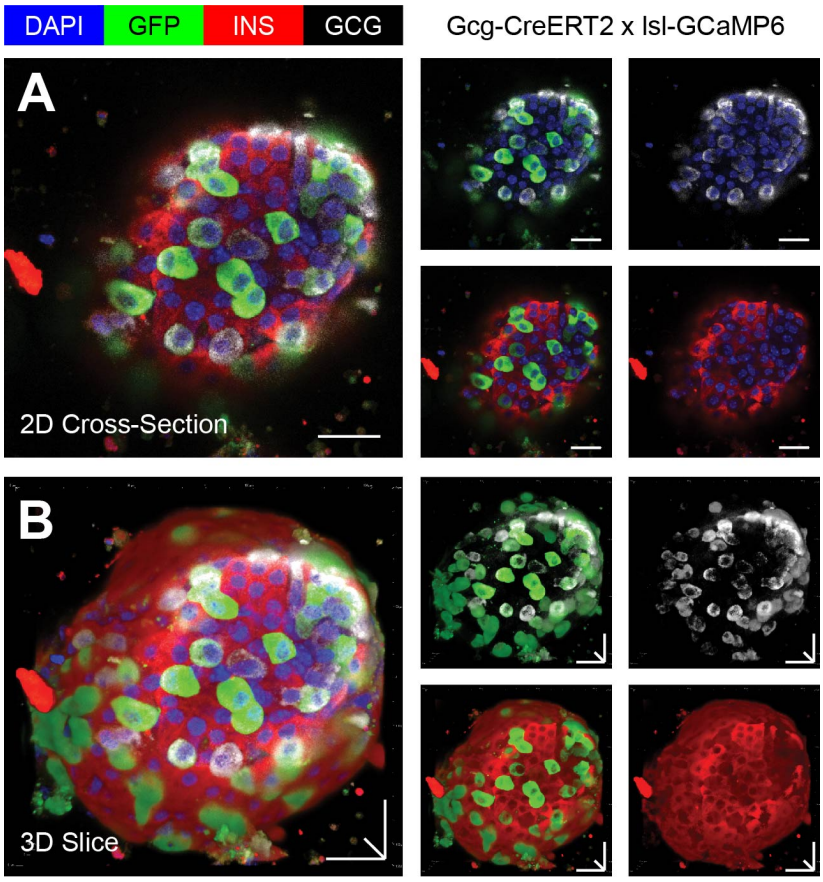
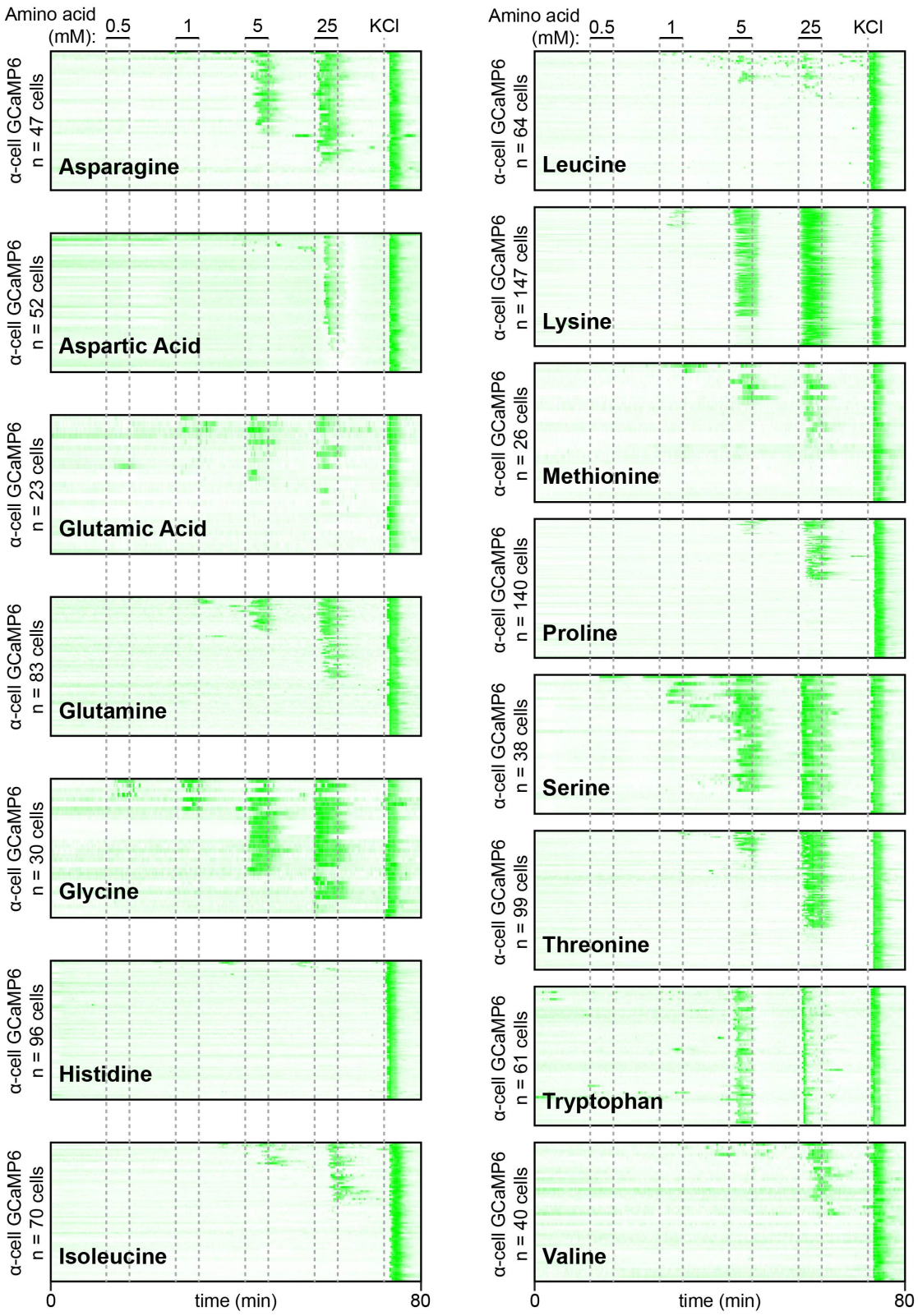
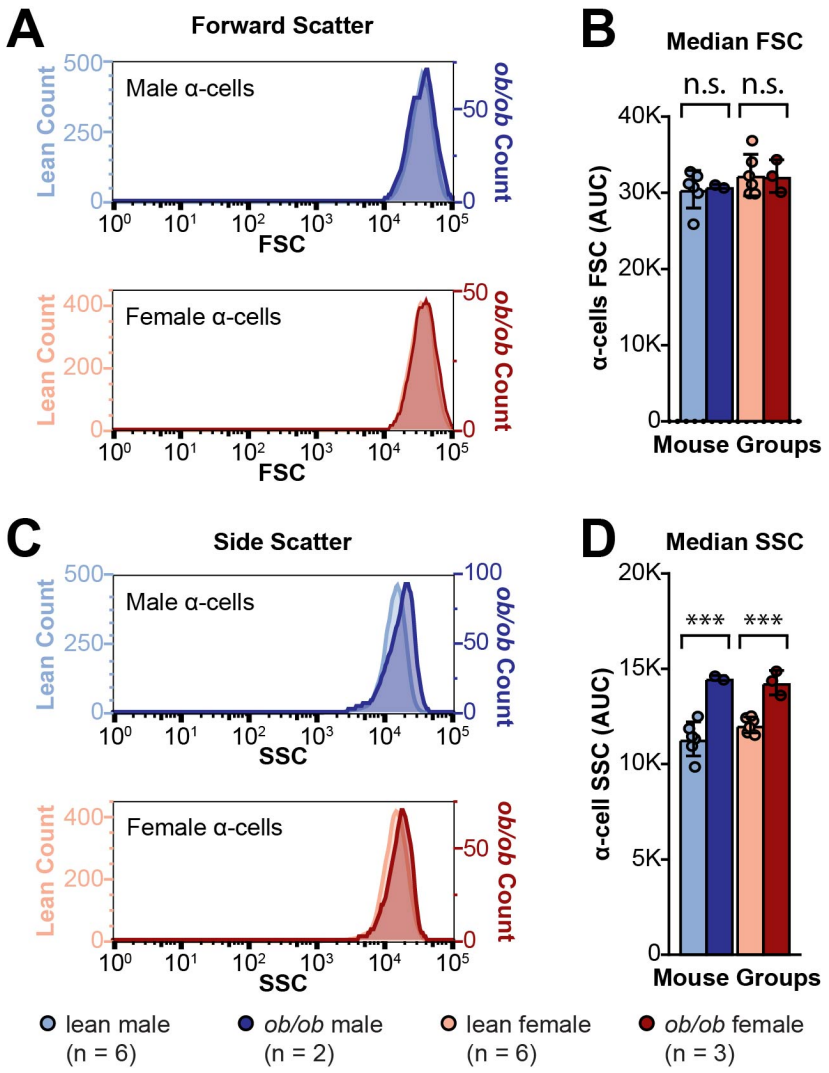


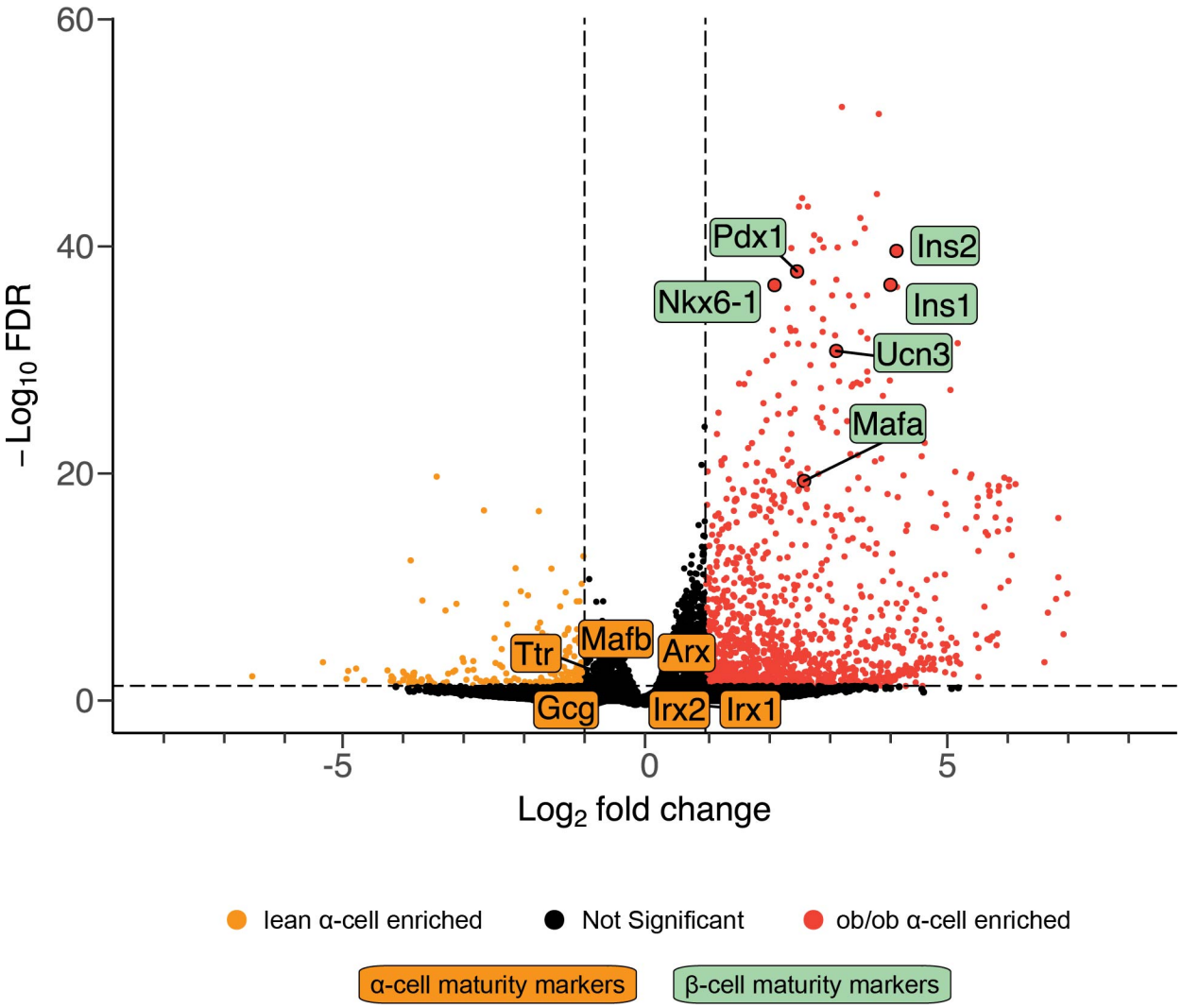
Supplementary Figure 3.1: α-cell-specific calcium sensor expression. **(A)** Post-hoc immunofluorescence stain of Gcg-CreERT2 x lsl-GCaMP6 islet demonstrating selective and efficient induction of GCaMP6 expression. Single Z plane and **(B)** 3D projection of the islet. Scale bar indicates 25 μm for single plane, 25 x 25 x 40 μm for 3D projection.



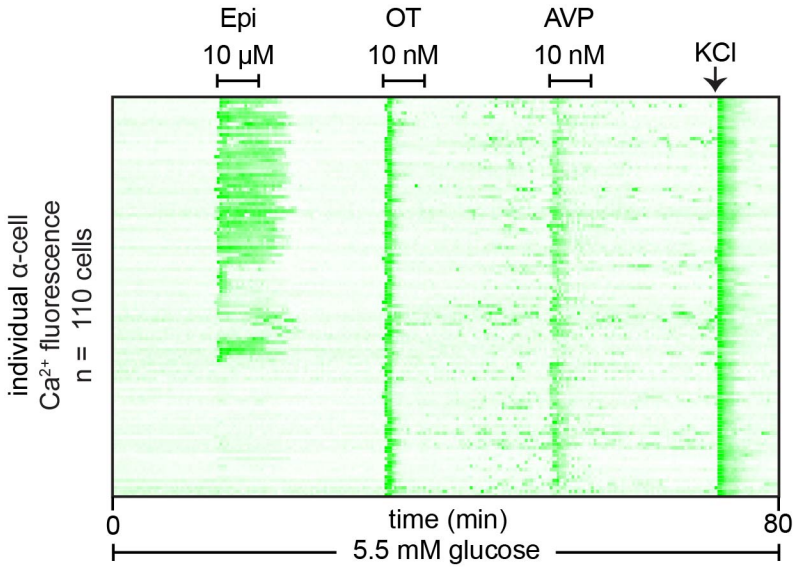
Supplementary Figure 3.2: Amino acid dose response calcium traces. α-cell GCaMP6 intensity plots of dose response experiments for 15 essential amino acids.



Supplementary Figure 3.3: Representative histograms of forward scatter and side scatter characteristics, separated by sex, with lean overlaid with its respective *ob/ob* counterpart. **(A)** Average median forward scatter (FSC) of YFP+ cells in male and female lean and *ob/ob* mice. Forward scatter in α-cells is not significantly different between lean and *ob/ob* mice. **(B)** Average median side scatter (SSC) of YFP+ cells in male and female lean and *ob/ob* mice. Side scatter is significantly higher in α-cells from *ob/ob* mice of both sexes. Error bars represent SEM. Student's t-test ***p < 0.001.



Supplementary Figure 3.4: RNA-Seq of *ob/ob* α-cells shows upregulated β-cell markers. Volcano plot showing differentially regulated genes between α-cells from lean mice and α-cells from *ob/ob* mice. Select α-cell markers are highlighted in orange and β-cell markers are highlighted in green. While α-cell markers are not differentially regulated between lean and *ob/ob* mice, there is a significant enrichment of several β-cell markers in α-cells from *ob/ob* mice.



Supplementary Figure 3.5: Oxytocin is a strong activator of α-cell calcium. Intensity plot of α-cell calcium responses to epinephrine (Epi, 10 µM), oxytocin (OT, 10 nM), and AVP (10 nM).

Chapter 4

Conclusions & Future Work

4.1 Abstract

In this dissertation I address the physiological regulation of glucagon secretion from pancreatic α-cells, with particular emphasis on the interplay between cAMP and calcium. The goal was to form a more complete picture of α-cell signal transduction, from receptor expression to receptor activation, to intracellular signaling and finally how all this contributes to shape the ultimate endocrine glucagon output, both inhibitory and stimulatory. The majority of the work performed here involved 3D live imaging of fluorescent reporters in α-cells in intact islets in real time, but these experiments were informed and supported by transcriptomic analysis of the islet cells, hormone secretion assays, as well as a thorough literature review. The following chapter summarizes the key findings and implications of the research comprising the chapters above.

4.2 Summary of Work

Chapter 1 is a comprehensive literature review of the islet and the multitude of signals that it must parse through to generate a net endocrine response. It provides an overview of the standard glucose-stimulated secretion pathways of the α, β, and δ-cells,

and delves into the contributions of amino acids and lipids to secretion as well. Islets are both innervated and highly vascularized, so we also detail the role of neuronal and endocrine signaling in affecting hormone release. We then center on the intra-islet signaling and how each cell is able to affect its neighbors, with an emphasis on the often underappreciated δ-cells and how they contribute to insulin and glucagon release. Finally, we summarize how this signaling breaks down in diabetes and how understanding the complexity of intra-islet relationships should be used to inform on future studies and therapeutic interventions.

Chapter 2 characterizes a novel mechanism of α-cell inhibition via UCN3-activated intra-islet signaling. Our lab previously observed that UCN3 from β-cells is co-secreted with insulin upon high glucose stimulation, and that the receptor for UCN3, CRHR2, is selectively expressed by δ-cells[1]. The release of UCN3 thus potentiates δ-cell somatostatin secretion and creates a negative feedback loop where insulin is then tonally inhibited. But β- and δ-cells do not exist in a vacuum – they are intermixed with α-cells as well, and δ-cell somatostatin is known to be the key source of α-cell inhibition at high glucose[2]. The outstanding question was whether UCN3 is able to inhibit α-cells via indirect δ-cell activation, and, if so, whether it is a required factor in somatostatin-mediated silencing. Thus, in many ways the work in Chapter 2 is the companion piece to our lab's 2015 Nature Medicine publication, filling in the α-cell branch of the tripartite islet paracrine network.

We found that UCN3 is indeed a capable inhibitor of α-cell activity, but is not required for somatostatin-mediated inhibition at high glucose. To observe α-cell behavior at the single cell level, we utilized a combination of calcium and cAMP fluorescent sensors. Both second messengers are key contributors to glucagon exocytosis, and thus are proxies for α-cell activity and glucagon secretion. We observed potent UCN3-mediated α-cell inhibition, as measured by calcium, cAMP, and direct glucagon secretion. We also confirmed that this inhibition was PTx-sensitive and thus driven by $G_{\alpha i}$ signaling,

reminiscent of somatostatin receptor activation, and required paracrine connections, as isolating the α-cells prevented inhibition. In measuring δ-cell cAMP, we observe a marked increase in cAMP production with the addition of UCN3. We next sought to determine the relative importance of endogenous UCN3 on α-cells by pharmacological intervention and genetic knockout. We were able to see measurable calcium de-inhibition in α-cells when antagonizing δ-cell CRHR2. However, blocking endogenous UCN3 as well as a constitutive knockout was insufficient to affect glucagon secretion. Altogether, these data point to UCN3 inhibiting α-cells by activation the release of somatostatin by δ-cells. However, the relative importance of UCN3 as a necessary component of high glucose inhibition of glucagon release is not clear.

In **Chapter 3**, I addressed α-cell activation mechanisms in both healthy and type 2 diabetic mice, and characterized a novel level of heterogeneity within α-cell responses to common physiological stimuli. The goal of this study was to expand our understanding of α-cell behavior by taking an unbiased population-level approach and observing the calcium and cAMP responses of hundreds of α-cells to different stimuli. The three major findings were that 1) α-cell heterogeneous calcium responses revealed functionally distinct subgroups, 2) glucose-dependence of the glucagon response to certain stimuli is a function of glucose-regulated cAMP levels, and 3) diabetes induces a change in the expression of adrenergic receptors among α-cells that fundamentally changes the response to epinephrine from α-cell-like (stimulated by epi) to β-cell-like (suppressed by epinephrine).

α-cells are commonly thought to be activated (and thus intracellular calcium is increased) by low glucose, but this is in fact a relatively poor stimulus on its own[3–5]. We directly compared low glucose responses to amino acids, epinephrine, and AVP, and measured calcium, cAMP and glucagon secretion. We found that only a quarter of the α-cells actually experience calcium influx at low glucose. This showcases the value of our high throughput approach because past studies have often excluded low glucose non-responders from their analysis and characterization. This heterogeneous α-cell behavior of

some cells responding and others remaining silent carried over to amino acids, epinephrine, and AVP. Once again, only a fraction of α-cells fluoresced with amino acids and epinephrine, and the cells appeared to subdivide into functional groups that respond to all stimuli or only two of the three. Interestingly, calcium heterogeneity was not mirrored in cAMP – where all cells were activated – or in glucagon secretion, in which epinephrine significantly exceeded the other stimuli. In fact, what brings glucagon release closer to reflecting the calcium response is combining amino acid, epinephrine or AVP stimulation with low glucose. We attributed this glucose sensitivity to the known low glucose activation of cAMP[3], and the ability of cAMP to amplify hormone secretion via increased vesicular recruitment[6] and/or increased calcium sensitivity[7]. Thus, amino acids and AVP – which stimulate calcium, but not cAMP – require low glucose – which stimulates cAMP but minimal calcium – for secretion commensurate with their respective calcium activation. Epinephrine is the exception to this glucose reliance because it is able to directly increase cAMP via β_1-adrenergic signaling[8].

Lastly, we aimed to understand why glucagon becomes dysfunctional during diabetes, and if/how this is due to α-cell-autonomous changes. Autonomic impairment is a feature of diabetes that involves loss of glucagon responsiveness downstream of repeated activation from hypoglycemic episodes, which can occur when the disease is poorly managed[9,10]. We performed RNA-Seq on FACS-purified α-cells from lean and *ob/ob* mice, and observed that α-cells undergo downregulation of the receptors *Adrb1* and *Avpr1b* that mediate epinephrine and AVP sensing. We correlated this with decreased glucagon secretion in response to epinephrine and AVP, and additionally showed that a subset of α-cells exhibit functionally opposing calcium responses. In diabetic animals, some α-cells are instead silenced by epinephrine, likely mediated through an upregulation of the inhibitory α_{2A}-adrenergic receptor (*Adra2a*). Together, Chapter 3 captures a large degree of heterogeneity among functional calcium responses in α-cells that adds a layer of complexity to how we both study and understand their physiological interactions.

4.3 Conclusions

The findings detailed in this dissertation add to our collective understanding of how α-cells are inhibited and activated and highlight the continuing intrigue of α-cell biology. Beyond moving the field forward in a basic biology sense, characterizing these mechanisms holds value from a disease standpoint as both the release and suppression of glucagon in diabetes become impaired. At postprandial high glucose, diabetic α-cells are unrestrained and thus contribute to diabetic hyperglycemia[11]. At hypoglycemic glucose levels, α-cells are no longer activated as part of the counterregulatory response and are unable to help rescue normoglycemia[9]. We confirm that inhibition of α-cells at high glucose is dependent on somatostatin signaling, and that exogenous UCN3 is capable of restraining α-cell activity (Chapter 2). Thus, a novel therapeutic approach could be to target δ-cells for more physiologically-relevant glucagon inhibition. We further observe that different physiological stimuli that mobilize glucagon secretion do so in a heterogeneous manner, and responsiveness to these signals is disturbed by diabetes at the level of gene expression (Chapter 3). Desensitization of α-cells thus becomes a critical point to address in diabetes treatment moving forward. Underpinning all these observations, we present the recordings of thousands of individual α-cells, which offers insight into canonical response patterns and direction for how best to approach their study in the future.

4.4 Future Work

4.4.1 Relative important of somatostatin for α- and β-cells

The findings in Chapter 2 illustrate the extent to which endogenous δ-cell somatostatin provides strong inhibition of α-cells at high glucose. However, there is a dichotomy in the way that α-cells respond to UCN3-potentiated somatostatin, and how

β-cells respond: we see negligible increases in α-cell activity when UCN3 is removed whereas β-cells experience increased in insulin secretion in the same conditions[1]. One possible explanation is the relative somatostatin receptor expression and downstream signaling mechanisms in each cell type. α-cells relay somatostatin signaling via the somatostatin receptor 2 (SSTR2) isoform[12], while mouse β-cells express SSTR3 and particularly have it localized on the primary cilia[13,14]. The strong somatostatin inhibition of α-cells, but relative lack of change when UCN3 is removed points to the possibility that α-cell SSTR2 has a low IC_{50}. Thus only a small amount of somatostatin would be sufficient to shut them down and fine-tuning with UCN3 would not be effective. Meanwhile, insulin is less sensitive to somatostatin, perhaps due to SSTR3 localization, so slight adjustments in somatostatin secretion have more visible effects. Our imaging approaches allow us to interrogate the comparative effects of somatostatin on both β-and α-cells, and would thus help to answer such questions.

4.4.2 Expanding a-cell imaging to new contexts

The value of our work in developing a protocol for imaging a large number of α-cells at once is that we can apply it to a number of other situations. We are currently expanding our ability to transduce human islets with calcium and cAMP sensors that would remain stably expressed so we can identify the imaged cells with post-hoc immunostaining. Furthermore, in this dissertation we mainly focused on the effects of type 2 diabetes on α-cell function, but defective glucagon in type 1 diabetes is equally worthy of study. Impaired hypoglycemic counterregulation in type 1 diabetes appears more frequently because of the inherent dangers of injecting glucose-lowering insulin into the body – insulin-induced hypoglycemia is what generally-unmasks counterregulatory defects[15,16]. Thus, applying the methods from this dissertation to studying calcium and cAMP behavior among α-cells from mouse models of type 1 diabetes would be a logical

next step. Finally, the methods used to stimulate α-cells in Chapter 3 include a variety of signals present in different physiological situations. They were compared because α-cells are activated in a variety of contexts beyond hypoglycemia, and often are targeted by multiple signals at once. But these experiments were still conducted in a dish with isolated islets, thus excluding other potentially relevant systems and using concentrations different from what may be seen *in vivo*. Recent developments in the field of microscopy have allowed for imaging islets with GCaMP6 with single cell resolution in the pancreata of live mice without having to surgically implant them in non-native contexts (such as the eye or in a kidney capsule)[17], though the consequence would likely be decreased throughput. Moving forward, understanding how α-cells *in vivo* integrate the multitude of physiological signals into a single net glucagon response is a potentially valuable tool to add to the islet biology researcher repertoire.

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