FAILURE OF PANCREATIC ALPHA CELLS TO RESPOND TO HYPOGLYCEMIA IS LINKED TO IMPAIRED GLUTAMATE RECEPTOR SIGNALING IN DIABETES

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ABSTRACT

Glucagon secretion from the pancreatic alpha cells is crucial to prevent hypoglycemia. People with type 1 diabetes, however, lose this glucoregulatory mechanism and are susceptible to dangerous insulin treatment-induced hypoglycemia. We established that activating glutamate receptors of the AMPA/kainate type in alpha cells is needed for decreases in glucose levels to elicit full glucagon responses from mouse and human islets. We performed functional studies using living pancreas slices from donors with type 1 diabetes and found that alpha cells had normal glucagon content and responded typically to KCl depolarization, but failed to respond to decreases in glucose concentration and had severely impaired AMPA/kainate receptor signaling. Reactivating residual AMPA/kainate receptor function with the positive allosteric modulators cyclothiazide and aniracetam partially rescued glucagon secretion in response to hypoglycemia. Positive allosteric modulators of AMPA/kainate receptors already approved to treat other conditions could thus be repurposed to prevent hypoglycemia and improve management of diabetes.

INTRODUCTION

The alpha cell of the pancreatic islet secretes glucagon to promote gluconeogenesis and glycogenolysis in target organs such as the liver, thus rising blood glucose levels. Although it is now known to be involved in several homeostatic circuits, a very important and clinically relevant function of the alpha cell is to secrete glucagon in response to a drop in glycemia. Indeed, glucagon plays a preeminent role in a concerted physiological response named glucose counterregulation that efficiently prevents life-threatening hypoglycemia (Rizza et al., 1979). As a result, hypoglycemia is rare in healthy people. For reasons still unknown, in type 1 diabetes, and to some extent in type 2 diabetes, the alpha cell is not capable of regulating its secretion in response to changes in glycemia (Gerich et al., 1973). This puts people with diabetes at risk for dangerous hypoglycemia if they are treated with excessive insulin. Understanding the pathophysiological mechanisms that make alpha cells unresponsive to hypoglycemia is thus important to improve the management of diabetes.

The alpha cells are endowed with a molecular machinery that converts changes in glycemia into electrical impulses and ultimately glucagon secretion. This so-called stimulus-secretion coupling has been delineated with some detail now. It is clear that the alpha cell's intrinsic properties allow it to respond to glycemia very differently to how the insulin secreting beta cell responds (MacDonald et al., 2007). Still, the alpha cell is located in close vicinity to the beta cell in the pancreatic islet, which allows for strong paracrine regulation. The alpha cells release factors that potentiate insulin secretion, while the beta cell releases factors that inhibit glucagon secretion. As beta cells are selectively destroyed in type 1 diabetes, the inhibitory input from beta cells wanes. Not surprisingly, basal plasma glucagon levels are high in diabetes, contributing to promote hyperglycemia in diabetes (Unger and Orci, 1976). Given that they should be disinhibited, it is

puzzling that alpha cells do not react any longer to decreases in glycemia (Gerich *et al.*, 1973). Paradoxically, it appears that inhibition of alpha cells is a permissive signal for future activation by hypoglycemia. It is likely that the mechanisms that allow the alpha cell to respond to hypoglycemia require a recovery from a refractory state, a recovery that is normally provided by beta cell input but is lost in diabetes.

Indeed, the alpha cell has functional properties that display use-dependent inactivation or desensitization. A salient example is the inactivation of voltage gated Ca^{2+} and Na^{+} channels (Ramracheya et al., 2010). Alpha cells also express ionotropic glutamate receptors of the AMPA/kainate type whose activation is needed for efficient glucagon secretion (Bertrand et al., 1993; Cabrera et al., 2008; Cho et al., 2010; Hayashi et al., 2003; Weaver et al., 1996). These receptors display strong desensitization that is reversed only if glutamate levels subside (Mayer and Armstrong, 2004). Glutamate is co-secreted with glucagon (Cabrera *et al.*, 2008), and therefore prolonged exposure to glutamate may diminish glutamate receptor signaling in diabetes. We tested the hypothesis that the autocrine positive feedback glutamate provides in alpha cells is defective in type 1 diabetes, thus impairing the glucagon counterregulatory response. We used living pancreas slices from non-diabetic donors and donors with type 1 diabetes to determine alpha cell responses to (a) changes in glycemia, (b) agonists, antagonists, and positive allosteric modulators of glutamate receptors, and (c) reference stimuli such as adrenaline and KCl depolarization. We further performed *in vivo* studies using a mouse model in which beta cells are ablated to demonstrate that defective glucagon secretion can be partially restored by reactivating the glutamate feedback loop in alpha cells.

RESULTS

It is difficult to study islets in people with type 1 diabetes because there is a limited availability of tissues from donors worldwide and conventional techniques may not be adequate to study the obtained islet samples. To overcome these limitations, we used living pancreas slices, which allow functional assessments of damaged and infiltrated islets within their native environment (Cohrs et al., 2020; Panzer et al., 2020). We examined pancreas slices from 6 nondiabetic donors and 4 donors with type 1 diabetes, which we obtained from the Organ Processing and Pathology Core (OPPC) of the Network for Pancreatic Organ Donors (nPOD) program at the University of Florida, Gainesville (Table S1).

Alpha cells lose their glucose responsiveness in type 1 diabetes

We first investigated if living slices recapitulated known *in vivo* features of islet destruction and dysfunction in type 1 diabetes. Islets in slices from donors with type 1 diabetes displayed a severe loss of beta cells compared to nondiabetic donors (Figure 1A). In line with these results, secretory responses were substantially impaired (Figures 1 B-1F). In stark contrast to slices from nondiabetic donors, slices from donors with type 1 diabetes did not show increases in insulin secretion when stimulated with glucose (17 mM) and only minor responses to KCl depolarization (30 mM; Figures 1B and 1C). Although islets from donors with type 1 diabetes were composed mainly of alpha cells, their functional responses were severely altered. Baseline glucagon secretion was significantly increased (Figures 1D and 1E). When normalized to baseline secretion, the secretory profile revealed a complete loss of glucagon secretion in response to a lowering in glucose concentration (17 mM to 1mM, Figure 1F). However, the secretory capacity of alpha cells was not affected, as seen by the responses to KCl depolarization (Figures 1D and 1F). Glucagon content of lysed tissue slices was not different between the groups (Figure 1G).

To determine the response profiles of individual islet cells we performed imaging of cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). We loaded slices with the $[Ca^{2+}]_i$ indicator Fluo-4 AM and recorded $[Ca^{2+}]_i$ responses to changes in glucose concentration within the islet by confocal time-lapse imaging (Figures 1H-1I). We only included viable cells that responded to KCl depolarization in our analyses and distinguished alpha cells by their $[Ca^{2+}]_i$ responses to stimulation with adrenaline (Tuduri et al., 2019). Changes in fluorescence intensity were calculated as fold-change over baseline intensity and shown as heatmaps that included all recorded alpha cells in slices from nondiabetic donors (Figure 1H) and individuals with type 1 diabetes (Figure 1I). We found that the incidence and magnitude of $[Ca^{2+}]_i$ responses to a lowering in glucose concentration (17 mM to 1 mM) was diminished in the alpha cell population in donors with type 1 diabetes (Figures 1H-1J). The reduction in the average $[Ca^{2+}]_i$ response mirrored that of glucagon secretion (Figure 1J). These results indicate that the alpha cell response to a lowering the glucose concentrations is selectively impaired in pancreas slices, which parallels the loss of glucagon counterregulatory responses seen in patients with type 1 diabetes.

Alpha cells show heterogenous response profiles

To understand what goes awry in diabetes, we first sought to gain a deeper insight into the processes that allow alpha cells to respond to hypoglycemia under nondiabetic conditions. We prepared tissue slices from mice expressing a genetically encoded $[Ca^{2+}]_i$ indicator (GCaMP3) in alpha cells to study the responsiveness of the whole alpha cell population to changes in glucose concentration and other stimuli (Figure 2; Figures S1 and S2). Surprisingly, only a subset of alpha cells (~37%) responded to a lowering in glucose concentration to hypoglycemic conditions (7 mM to 1 mM, Figure 2A). A similar proportion of alpha cells (~31%) showed $[Ca^{2+}]_i$ responses when we lowered glucose levels from high glucose to normoglycemia (17 mM to 7 mM; Figure 2B).

The magnitude and incidence of $[Ca^{2+}]_i$ responses to drops in glucose concentration were smaller than those elicited by adrenaline (10 μM), kainate (100 μM) and glutamate (100 μM; Figures 2C-2F), all know to be selective and strong alpha cell stimuli (Cabrera *et al.*, 2008; Dai et al., 2014). The alpha cell population was very diverse in their responsiveness to changes in glucose concentrations and showed different patterns of activation (Figure S3). By contrast, the beta cell population was uniform in its response to an increase in glucose concentration, with most cells (>90%) responding with synchronicity (Figure 2G). Distinct alpha cell populations could be identified by charting responses to glucose, adrenaline, and kainate in response maps (Figure 2H) or in scatter plots of maximum peak responses to these stimuli (Figure 2I). Our results suggest that the alpha cell population is heterogeneous, with a third of alpha cells responding to drops in glucose levels, independently of whether or not they led to hypoglycemic conditions. Most of the glucose responsive population was also the one that responded the strongest to kainate, an agonist of ionotropic glutamate receptors (iGluRs) of the AMPA/kainate type.

Alpha cell responses to lowering the glucose concentration depend on glutamate receptor activation

Alpha cells secrete glutamate together with glucagon and express iGluRs of the AMPA/kainate type, establishing an autocrine feedback loop potentiating glucagon secretion (Cabrera *et al.*, 2008). To determine how alpha cell responses to decreases in glucose concentration depend on this feedback loop, we challenged these responses with the AMPA/kainate receptor antagonist CNQX (100 μM; Figure 3). The incidence in the alpha cell population of $[Ca^{2+}]_i$ responses to lowering glucose concentration from 7 mM to 1 mM or 17 mM to 7 mM diminished in the presence of CNQX (Figures 3A-3D). The effects on glucagon secretion were even stronger; glucagon secretion in response to a drop in glucose from 17 mM to 1 mM disappeared in the presence of

CNQX (Figures 3E-3G). CNQX also diminished glucagon secretion at basal glucose concentrations (5 mM), suggesting that AMPA/kainate receptors were already activated under normoglycemic conditions (Figure 3G). The metabotropic glutamate receptor 4 (mGluR4) has also been implicated in glucagon secretion (Uehara et al., 2004). Strikingly, the inhibition CNQX produced on glucagon secretion was reversed in the presence of CPPG (100 μM), a potent and competitive antagonist of group II and III metabotropic glutamate receptors (Figures 3E-3G; Toms et al., 1996). These results suggest that glutamate receptor signaling has major effects on glucagon secretion and involves at least two different receptor types whose activation trigger different signaling pathways (membrane depolarization *versus* decreasing intracellular cAMP levels) that produce opposite effects on secretion.

In view of their therapeutic potential, we tested two positive allosteric modulators of AMPA/kainate receptors, namely cyclothiazide, a compound approved by the FDA to treat edema (Application numbers N018173 and N013157), and aniracetam, a drug available over-the-counter in the US used for its cognition-enhancing effects (Malykh and Sadaie, 2010). These compounds essentially eliminate rapid desensitization of the AMPA receptor (Ito et al., 1990; Yamada and Tang, 1993). Cyclothiazide (100 μM) potentiated glucagon secretion from isolated mouse islets when stimulated with low glucose, while aniracetam did not (100 nM; Figures 3H and 3I). Both cyclothiazide and aniracetam amplify AMPA receptor responses by inhibiting receptor desensitization, but aniracetam also potentiates metabotropic glutamate receptor activity (Pizzi et al., 1993). This effect could be observed as a drop in glucagon secretion (Figure 3J) and might have counteracted the positive effects on glucagon secretion. Nevertheless, our results suggest that potentiating the autocrine glutamate feedback loop can increase glucagon release during conditions of low glucose concentration.

Alpha cells show defective glucagon counterregulatory responses 2 weeks after beta cell ablation

A main feature of type 1 diabetes is that the autoimmune attack works with surgical precision to exclusively destroy beta cells (Stefan et al., 1982). The defect in glucagon secretion seems to track with the progressive loss of beta cell function (Fukuda et al., 1988). To investigate how alpha cell function changes when beta cells are destroyed, we injected mice with the beta cell toxin streptozotocin. At two weeks after streptozotocin injection, this mouse model mimicked the defective glucagon response typical of type 1 diabetes, both *in vivo* and *in vitro* (Figures 4A-4E). The counterregulatory response to hypoglycemia was defective, most likely because mice did not mount a glucagon response to lowering glucose levels (Figures 4A, 4B, 4D, and 4E; Figure S4). Fasting plasma glucagon concentrations were significantly increased (Figure 4C). Thus, with respect to glucagon secretion, this model reproduced most *in vivo* features of type 1 diabetes (Gerich *et al.*, 1973; Unger and Orci, 1976) and our *in vitro* results in human slices (Figure 1).

We then used $[Ca^{2+}]_i$ imaging to study how the response pattern of the population of individual alpha cells changed in the streptozotocin model. $[Ca^{2+}]_i$ responses to a lowering in glucose concentration were reduced in some mice (Figure S4), but the effect was not consistent as it was in human slices (Figure 1) and not as striking as the reduction in glucagon secretion (Figures 4D and 4E). However, we found substantial decreases in the magnitude and number of responses to glutamate and adrenaline (Figures 4F-4H). Responses to glutamate were particularly diminished, suggesting that glutamate receptor signaling was disrupted, which could potentially impact the autocrine alpha cell loop mediated by glutamate.

The autocrine feedback loop mediated by glutamate can be potentiated in human alpha cells

To translate the mouse findings into the human situation, we first determined whether the glutamate receptor mechanisms we found in mice were also working in human alpha cells. Glucagon responses from isolated human islets to a lowering in glucose concentration from 17 mM to 1 mM were potentiated by the positive allosteric modulator cyclothiazide (100 μM; Figures 5A, 5B, 5E, and 5F). These effects were prevented by CNQX (100 μM; Figure 5E). Aniracetam (10 nM) did not amplify the glucagon response, but the combination of aniracetam and CNQX greatly reduced the glucagon response to lowering the glucose concentration (Figures 5C-5F). This was most likely because CNQX blocked AMPA/kainate receptors, thus unmasking the effects aniracetam also has on the mGluR4 receptor (Figure S5; Pizzi *et al.*, 1993), whose activation diminishes glucagon secretion (Uehara *et al.*, 2004). We could confirm these data in additional living pancreas slices from a nondiabetic donor (Figures 5H and 5I). Here we used a higher concentration of aniracetam (1 mM) because at higher concentrations the effects on AMPA/kainate receptors predominated (Figure S5). As in the mouse, our results indicate that glutamate signaling impacts glucagon secretion from human alpha cells.

AMPA/kainate receptor signaling is strongly reduced in type 1 diabetes

To investigate how glutamate receptor signaling in alpha cells changes in type 1 diabetes, we used living pancreas slices from human donors. We performed $[Ca^{2+}]_i$ imaging in slices loaded with the $[Ca^{2+}]_i$ indicator Fluo-4 AM. We used responses to adrenaline to identify alpha cells in slices from nondiabetic donors. Responses to adrenaline could not be used to identify alpha cells in donors with type 1 diabetes because these responses were also affected. However, we anticipated (as shown in Figure 1A) that alpha cells in these donors represent the majority of cells exhibiting increases in $[Ca^{2+}]_i$. All cells were further preselected by their response to KCl depolarization (4 x SD of baseline activity) to ensure cell viability. Ca^{2+} responses to glutamate and kainate could be

readily elicited in islet cells in slices from nondiabetic donors but were greatly reduced in slices form diabetic donors (Figures 6A-6G). The incidence of responsive cells decreased from 41% to 18% for glutamate and from 69% to 21% for kainate stimulation (Figures 6A-6D).

There was a residual response in a subset of cells (responders in Figures 6F and 6G), suggesting that it could be potentiated in the context of type 1 diabetes to rescue the alpha cell responsiveness to hypoglycemia. Indeed, in slices we obtained from a donor with type 1 diabetes, we were able to increase glucagon secretion in response to a drop in glucose from 17 mM to 1 mM with aniracetam (1 mM; Figure 6H).

Targeting glutamate receptors *in vivo* improves glucagon secretion in response to hypoglycemia in the streptozotocin mouse model

We next determined the effects of the positive allosteric modulators cyclothiazide and aniracetam on glycemia and glucagon secretion *in vivo* using the mouse model. Cyclothiazide (30 mg/kg; i.p.) and aniracetam (30 mg/kg; i.p.) did not alter plasma glucagon levels under fed conditions in healthy mice or in streptozotocin-treated mice fasted to reach normoglycemic conditions (Figures 7A-7D). By contrast, the potent AMPA/kainate agonist kainate increased glucagon secretion in both groups (10 mg/kg, i.p.; Figures 7A-7D). Of note, when kainate activated glucagon secretion in healthy mice under normoglycemic conditions it decreased glycemia (Figure 7A), but it accelerated the return to hyperglycemia in the diabetic mice (Figure 7C). Thus, activating alpha cells had an impact on glycemia depending on whether or not the islet contained beta cells. The results further indicate that under normoglycemic conditions the positive allosteric modulators did not stimulate glucagon secretion or affect glycemia.

Our *in vitro* results suggest that loss of glucose counterregulation was associated with defective glutamate signaling in alpha cells. To test if reactivating residual glutamate signaling in

alpha cells can augment glucagon secretion, we induced hypoglycemia in streptozotocin-treated mice in the presence of the positive allosteric modulators (Figure 7E). As also shown in another set of experiments (Figure 4B), plasma glucagon levels did not increase in response to hypoglycemia in streptozotocin-treated mice (Figure 7F). However, injecting cyclothiazide or aniracetam at the time of the insulin administration increased the glucagon response to hypoglycemia. Although the treatment did not increase glucagon secretion to levels seen in control healthy mice (Figure 4; ~ 80 pM *versus* 20-30 pM), and barely reversed hypoglycemia (Figure 7E), these results indicate that glutamate receptors can be potentiated to promote glucagon secretion under hypoglycemic conditions.

DISCUSSION

Our study establishes that the alpha cell is not able to mount an efficient glucagon response to hypoglycemic conditions if glutamate receptor signaling is deficient. Inhibiting glutamate receptors of the AMPA/kainate type strongly reduced Ca^{2+} and glucagon responses to decreases in glucose concentration. In type 1 diabetes, glutamate receptor signaling in alpha cells is severely impaired, thus preventing the positive feedback glutamate provides for glucagon secretion. The main conclusion from our studies is that alpha cells in people with type 1 diabetes lose this important autocrine mechanism that guarantees a glucagon counterregulatory response to prevent hypoglycemia. Our results further demonstrate that residual glutamate signaling can be potentiated with positive allosteric modulators to partially rescue glucagon secretion.

Thanks to the nPOD initiative to procure and characterize pancreases from cadaveric organ donors with type 1 diabetes, we were able to study the physiology of pancreatic islets in rare specimens from type 1 diabetic donors. This allowed us to determine that the deficiencies commonly reported for glucagon secretion in patients with type 1 diabetes can be translated to pancreatic slices *in vitro*, as also recently shown for isolated islets (Brissova et al., 2018). Strikingly, the main alpha cell impairment appears to be selective. Both *in vivo* and *in vitro*, full glucagon responses can be elicited by stimuli such as arginine, adrenaline, and KCl depolarization, and glucagon content was not reduced, suggesting that alpha cells retain their secretory potential (present study; (Brissova *et al.*, 2018; Gerich *et al.*, 1973). By contrast, glucagon responses to decreases in glycemia or glucose concentration are barely detectable. That this defect is present in isolated islets or pancreatic slices not only implies that the deficiency is intrinsic to the pancreatic islet, but also that pathophysiological mechanisms can be studied *in vitro*, as we did here.

To understand how alpha cells become dysfunctional, we first sought to address how they respond as a population to different stimuli, including changes in glucose concentration, under healthy conditions. A striking finding was that alpha cells responded to any drop in glucose concentration, that is, even if the drop was from high to normoglycemic conditions. That alpha cells are not linear detectors of the absolute glucose concentration (as beta cells are) was previously reported but is often overlooked (Santiago et al., 1980).The other surprising result was that only a third to half of the alpha cell population responded to decreases in glucose concentration. This was consistent in mice and human beings. These data were obtained with Ca^{2+} imaging, which does not report all the processes that contribute to glucagon secretion [e.g., rises in cAMP, (Hughes et al., 2018)]. Nevertheless, the Ca^{2+} channels that need to be activated to elicit glucagon secretion require such a large membrane depolarization that it may activate several other types of voltage-dependent Ca^{2+} channels (Ramracheya *et al.*, 2010), likely producing a Ca^{2+} influx large enough to be detectable in the cytoplasm. On the other hand, not all Ca^{2+} that is measurable in alpha cells may lead to glucagon secretion, indicating that Ca^{2+} imaging may overestimate the number of secreting cells. These caveats notwithstanding, it is important to note that this glucose responsive alpha cell population responded strongly to activation of glutamate receptors of the AMPA/kainate type.

Alpha cells are endowed with different types of glutamate receptors. There is a long history of physiological studies showing the presence of functional ionotropic as well as metabotropic glutamate receptors in alpha cells (see Introduction). Databases now show that alpha cell expression of genes encoding these receptors or their subunits (GRIA3, GRIA2, GRIK2, GRM4) is higher than in other endocrine cells of the pancreas and as high as in the brain, where glutamate signaling is predominant (Cao et al., 2017; Park et al., 2019). We previously reported that

activation of AMPA/kainate receptors increases glucagon secretion from human islets (Cabrera *et al.*, 2008), but did not study the role of metabotropic glutamate receptors in human alpha cells, as had been done in rodent alpha cells (Uehara *et al.*, 2004). We now provide evidence that both receptor types are activated when alpha cells are exposed to a lowering in glucose concentration. This finding is puzzling because activating these receptors triggers different signaling pathways (membrane depolarization *versus* a reduction in cAMP) that produce opposite effects on glucagon secretion. It is clear that the autocrine loop involving glutamate is more complex that what we originally reported (Cabrera *et al.*, 2008).

A major result of this study is that glutamate receptors of the AMPA/kainate can barely be activated in alpha cells of people with type 1 diabetes. We don't know yet for sure the reason for this deficit. There might be a general loss of alpha cell excitability (Camunas-Soler et al., 2020), although responses to KCl depolarization *in vitro* and arginine *in vivo* show otherwise (Brissova *et al.*, 2018; Gerich *et al.*, 1973). Expression of genes encoding for the glutamate receptors may be decreased (Brissova *et al.*, 2018; Camunas-Soler *et al.*, 2020). However, given the high basal glucagon secretion we measured in slices and the high plasma glucagon levels prevalent in type 1 diabetes, it is also likely that these receptors do not respond because they are chronically desensitized. AMPA/kainate receptors are known to strongly desensitize in the sustained presence of glutamate, and recovery from desensitization requires the dissociation of glutamate, that is, it needs extracellular glutamate levels to subside (Robert and Howe, 2003). But this cannot happen because glutamate is likely secreted at high rates together with glucagon. To recover from receptor desensitization, the alpha cell needs to rest and reset.

It is clear that what is missing in type 1 diabetes is the inhibitory influence from beta cells. Of note, all secretory products from beta cells have been reported to inhibit alpha cells. That we

and others were able to recapitulate alpha cell deficiencies in the streptozotocin rodent model in the absence of autoimmunity indicates that the absence of beta cells is crucial (Zhou et al., 2004). While we cannot rule out intrinsic defects in alpha cell function (e.g., responses to adrenaline were diminished), the abnormal alpha cell response to glucose levels can be attributed in part to a loss of inhibitory signaling. Indeed, it was proposed that a cessation of these inhibitory signals from beta cells in the islet during hypoglycemia is a necessary signal for the glucagon response from neighboring alpha cells [the "switch-off" hypothesis; (Hope et al., 2004; Zhou *et al.*, 2004)]. This helps explain how alpha cells are activated by hypoglycemia, but the mechanisms were not explored. Here we propose that the inhibition of glucagon and glutamate secretion by the inhibitory signals from beta cells is needed for AMPA/kainate receptors in alpha cells to recover from desensitization so that in a subsequent drop in glucose levels the glutamate autocrine feedback loop can be fully activated.

We reasoned that, if AMPA/kainate receptors are chronically desensitized but still present in alpha cells in type 1 diabetes, they could be targeted by positive allosteric modulators. Our results show that cyclothiazide, an inhibitor of AMPA/kainate receptor desensitization, potentiated glucagon secretion in response to a decrease in glucose concentration in mice and human beings *in vitro* and partially rescued glucagon secretion in response to hypoglycemia in the streptozotocin mouse model. Given its complex pharmacology, aniracetam produced less consistent results. Its use nevertheless allowed us to unmask an inhibitory contribution of metabotropic receptors to glucagon secretion. At higher concentrations, however, aniracetam effects were mostly potentiating. We were fortunate to obtain slices from a donor with type 1 diabetes late in our study to determine that aniracetam can increase glucagon secretion under hypoglycemic conditions.

However, in contrast to cyclothiazide, aniracetam was less specific in its action, and at high concentration also increased basal glucagon secretion.

Based on our results, we conclude that in type 1 diabetes alpha cells lose an important mechanism that allows efficient glucagon secretion in the context of glucose counterregulation. Our *in vitro* data demonstrate that AMPA/kainate receptor signaling has a strong impact on glucagon secretion and that this signaling is impaired in type 1 diabetes. It will be important to test the role of the glutamate autocrine feedback loop *in vivo*, in particular because several drugs modulating these receptors are already approved for use in human beings (e.g., cyclothiazide and several other nootropics and ampakines). Testing these compounds in hypoglycemic clamps could not only reveal the impact of glutamate receptor signaling on glucagon responses, but also provide evidence that these receptors can be targeted to prevent recurrent hypoglycemia in people with type 1 diabetes.

Limitations of the study

A major limitation for the research community in general is the limited availability of pancreas tissues from donors with type 1 diabetes. We received all the pancreas slices that were processed by nPOD for physiological studies during a period of two years (n = 6 non-diabetic and n = 4 donors with type 1 diabetes). Because human samples are variable in general, it is challenging to obtain consistent results. We made an effort to present data with all their dispersion and sometimes we had to normalize the recordings to internal references (e.g., basal secretion). To gain deeper mechanistic insight, we complemented the slice experiments with studies using isolated human islets and recruited the streptozotocin mouse model. We measured hormone secretion in perifusion studies and performed Ca^{2+} imaging, but could not extend the studies to record other physiological parameters such as cytoplasmic cAMP levels that are also important for alpha cell function. As

with most pharmacological compounds, cyclothiazide and aniracetam may not be completely selective. We already mentioned that aniracetam also activates metabotropic glutamate receptors. Cyclothiazide was also found to act as a $GABA_A$ receptor negative allosteric modulator, potently inhibiting $GABA_A$-mediated currents (Deng and Chen, 2003), which have been reported in alpha cells. This could explain why the AMPA/kainate receptor antagonist CNQX did not fully block the effects of cyclothiazide. These limitations notwithstanding, we consider that our studies may impact our model of how alpha cells becomes dysfunctional in type 1 diabetes.

FIGURES

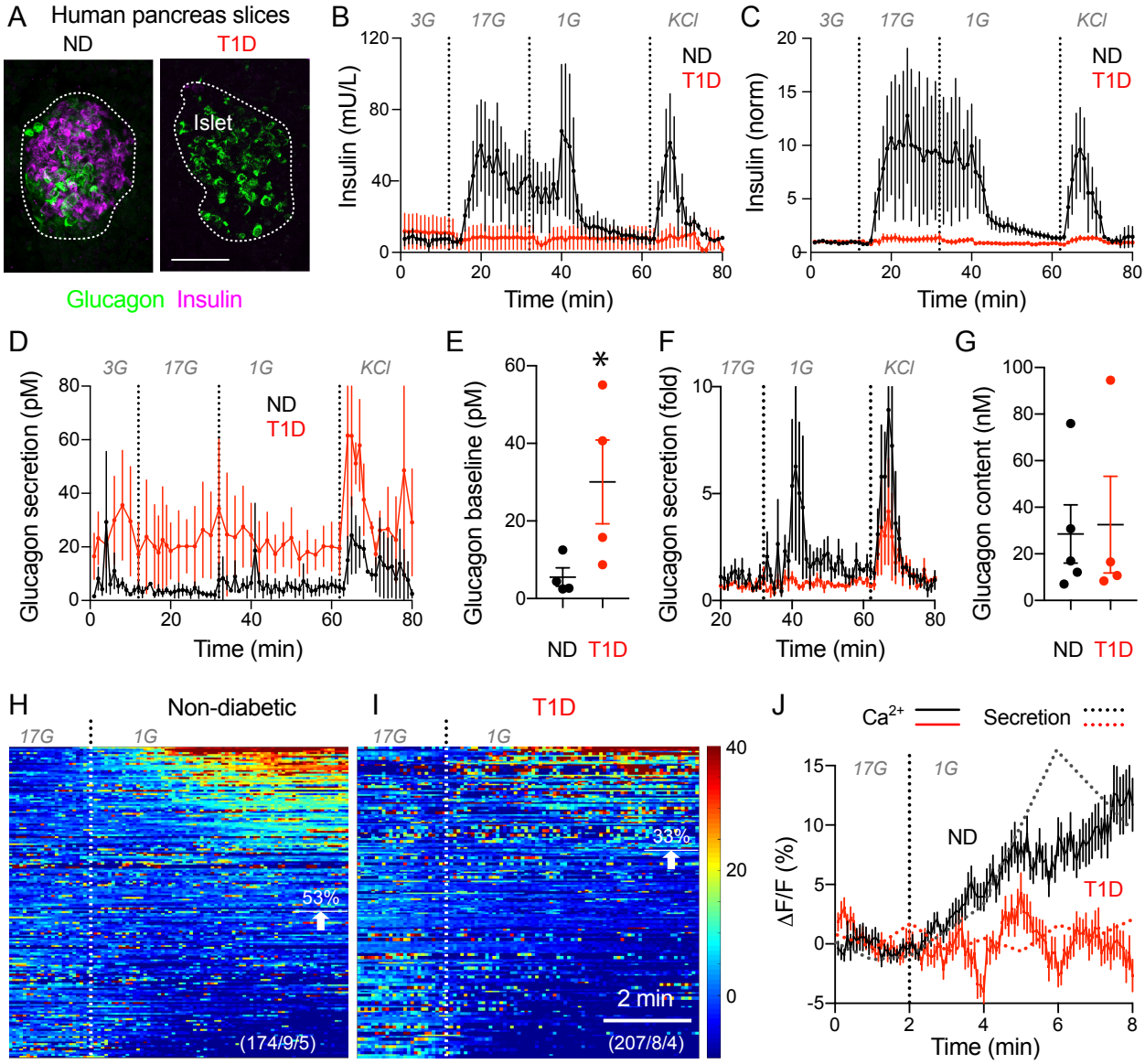

Figure 1. Alpha cells lose their glucose responsiveness in type 1 diabetes

(A) Z stack of confocal images of human tissue slices from a nondiabetic donor (ND) and an individual with type 1 diabetes (T1D) showing beta cells (insulin, magenta) and alpha cells (glucagon, green). Dotted line denotes islet border. Scale bar 50 µm.

(B) Dynamic insulin secretion of human pancreatic tissue slices from nondiabetic donors (ND, n = 5) and individuals with type 1 diabetes (T1D, n = 4) during perifusion.

(C) Data shown in (B) expressed as fold increase to the mean basal secretion at 3G (Insulin norm.).

(D) Glucagon secretion measured during the same perifusion experiment shown in (B) for nondiabetic donors (n = 4) and donors with type 1 diabetes (n = 4) expressed as absolute glucagon (pM).

(E) Average glucagon secretion during the first 10 min of baseline (3G) shown in (D).

(F) Data shown in (D) normalized to average baseline secretion (Glucagon norm.).

(G) Glucagon content of all perifused slices after secretion experiment.
(H-I) Heatmaps showing in vitro Ca^{2+} dynamics of alpha cells expressed as the fluorescent intensity of Fluo-4 normalized to the basal signal intensity at 17 mM glucose (17G) at stimulation with low glucose (1G) for (H) 5 nondiabetic donors and (I) 4 individuals with type 1 diabetes. Each row represents a single cell followed over time in the x-axis and their response in magnitude change (%) of the fluorescent intensity over baseline ($\Delta F/F$) shown in the color scale from blue (low intensity) to red (high intensity).
(J) Average trace of Ca^{2+} dynamics shown in (G and H) compared to average glucagon secretion trace shown in (F) for stimulation with low glucose (1G).
Data in (B-D), (F) and (J) are shown as average traces (± SEM). Data in (E) and (G) are shown as dot plots and analyzed with Student's t test. Asterisks denote significance (*$p < 0.05$)

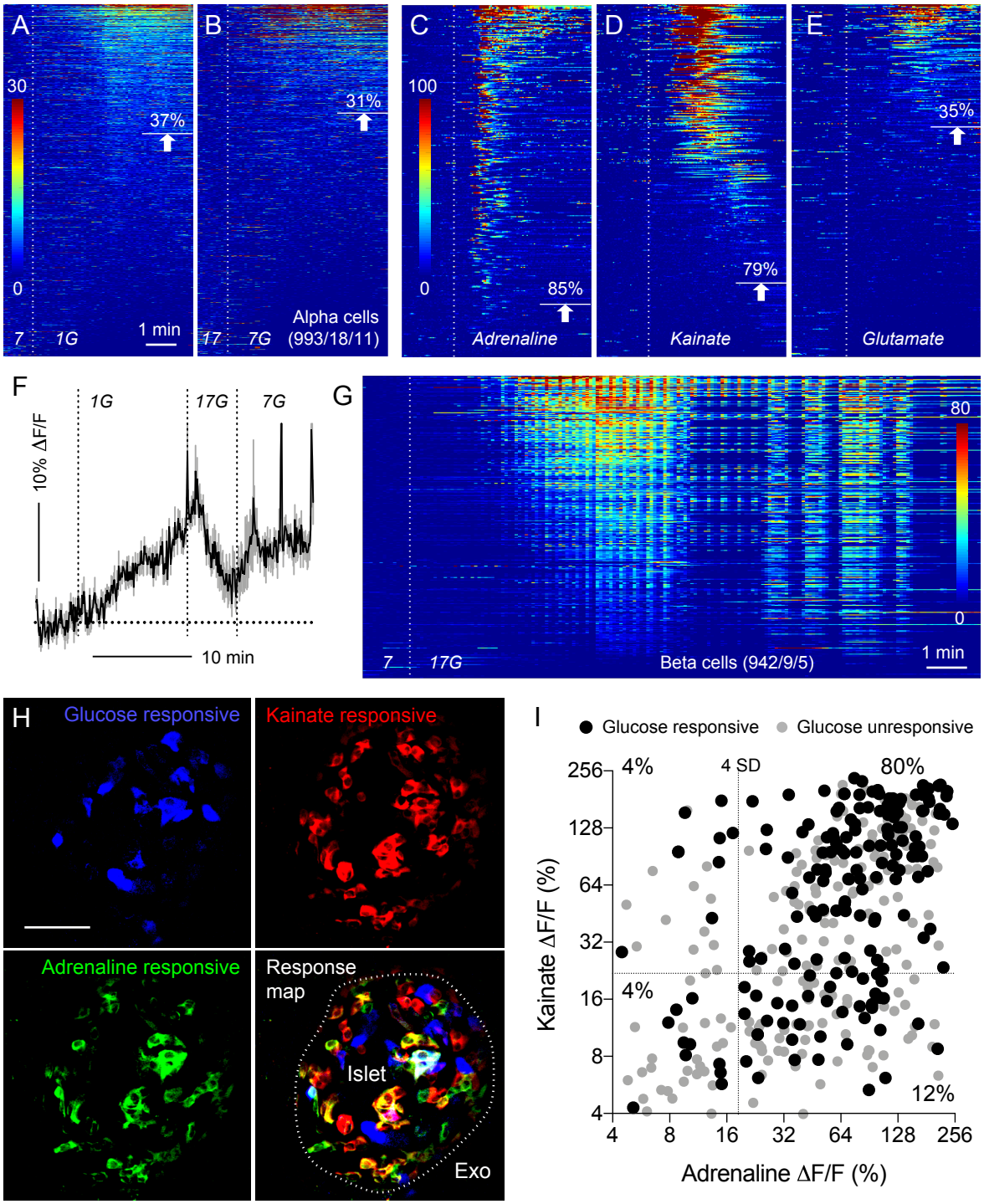

Figure 2. Alpha cells show heterogenous response profiles

(A-E) Heatmaps showing in vitro Ca^{2+} responses of alpha cells from Gcg-Cre-GCaMP3 mice within tissue slices for lowering in glucose concentration (A) from 7 mM to 1 mM and (B) from 17 mM to 7 mM. Alpha cell responses to stimulation with (C) adrenaline (10 µM), (D) kainate (100 µM), and (E) glutamate (100 µM). Responses are expressed as the fluorescent intensity over baseline of GCaMP3 signal. Each row shows a single cell followed over time in the color scale from low intensity (blue) to high intensity (red) expressed as change in magnitude (%).

(F) Average trace (± SEM) of alpha cells responding to changes in glucose concentration shown in (A) and (B) expressed as fold change to the average baseline intensity (%).
(G) Heatmap of beta cells within slices of Ins-Cre-GaMP3 mice showing responses to elevation in glucose from 7 mM to 17 mM.
(H) Z stack of confocal images of an islet within a tissue slice from a Gcg-Cre-GCaMP3 mice. Images shown as specific response to each stimulus subtracted from baseline activity during time of stimulation.
(I) Scattered dot plot showing maximum amplitudes (%) as fluorescent intensity over baseline ($\Delta F/F$) of individual glucose responsive cells (black dots) and glucose unresponsive cells (grey dots) to stimulation with kainate and adrenalin. Percentages represent fraction of glucose responsive alpha cells for each stimulus response (threshold of 4xSD over mean baseline).
Numbers of cell/islets/animals are indicated (B) for alpha cells and (G) for beta cells.

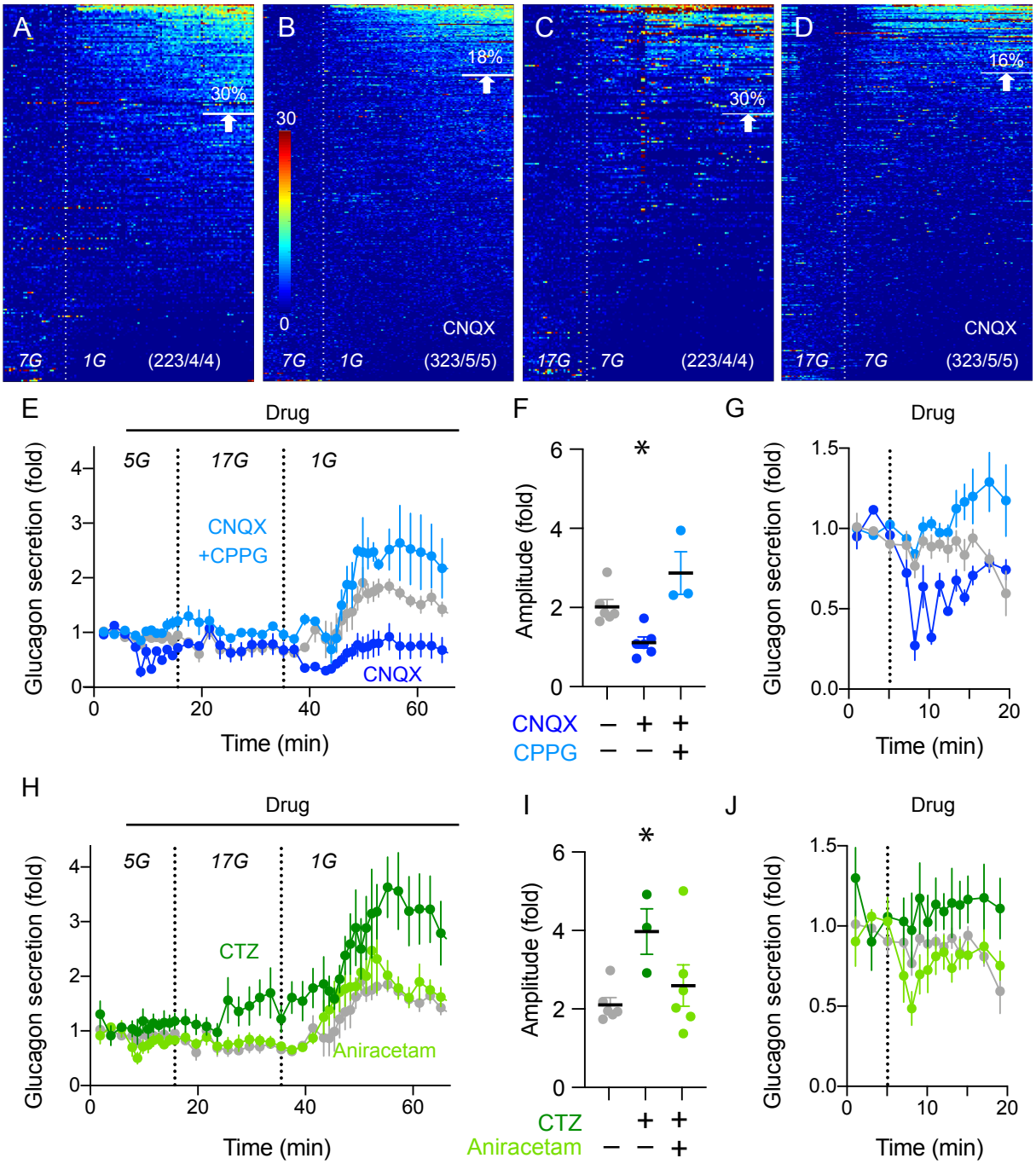

Figure 3. Alpha cell responses to lowering the glucose concentration depend on glutamate receptor activation

(A-D) Heatmaps showing in vitro Ca^{2+} dynamics of alpha cells within slices from Gcg-Cre-GCaMP3 mice. Responses shown for lowering in glucose concentration (A and B) from 7 mM to 1 mM and (C and D) from 17 mM to 7 mM (A and C) in the absence or (B and D) presence of CNQX (100 µM). Each row shows a single cell followed over time in the color scale from low intensity (blue) to high intensity (red) expressed as change in magnitude (%) of the fluorescent intensity over baseline (ΔF/F) activity.

(E) Glucagon secretion from isolated mouse islets during dynamic perifusion in response to changing glucose concentrations and in the presence of CNQX (100 μM) or combination of CNQX (100 μM) and CPPG (100 μM).
(F) Quantification of glucagon amplitudes during 1 mM stimulation.
(G) Magnification of the first 20 min shown in (E).
(H) Glucagon secretion from isolated mouse islets during dynamic perifusion in response to changing glucose concentrations and in the presence of CTZ (100 μM) or aniracetam (100 nM).
(I) Quantification of glucagon amplitudes during 1 mM stimulation.
(J) Magnification of the first 20 min shown in (H).
Numbers of cell/islets/animals are indicated in each heatmap. Data in (E), (G), (H) and (J) are shown as average trace (± SEM) normalized to baseline secretion at 7 mM glucose of 150 islets each (control n = 6, CNQX n = 6, CNQX+CPPG n = 3, CTZ n = 3, aniracetam n = 6). Data in (F) and (I) are shown as dot plot (± SEM) and analyzed by one-way ANOVA followed by multiple comparisons. Asterisks denote significance (*$p < 0.05$).

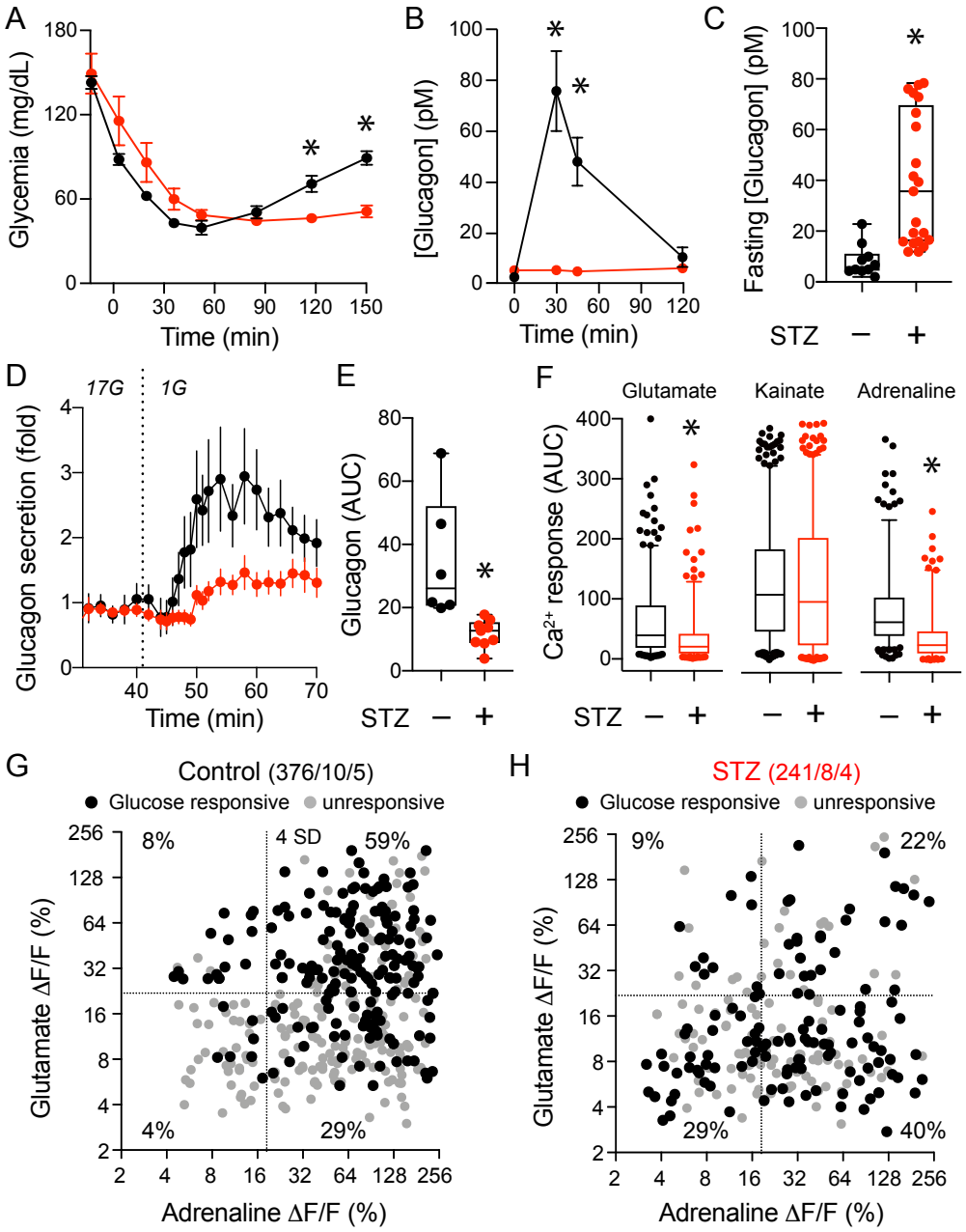

Figure 4. Alpha cells show defective glucose counterregulation 2 weeks after beta cell ablation

(A and B) Intraperitoneal insulin tolerance test (0.75 U/kg) performed in fasted control (n = 30) and rendered diabetic C57Bl6J mice (n = 21) 2 weeks after intravenous streptozotocin (STZ, 200 mg/kg) injection. (A) Blood glucose excursions and (B) plasma glucagon levels after injection.

(C) Quantification of fasted blood glucagon prior to ITT shown in (B) for control (n = 10) and STZ (n = 21) mice.

(D-E) In vitro glucagon secretion from isolated islets of control (n = 6) and STZ treated animals (n = 9). (D) Average trace of secreted glucagon after lowering glucose from 17 mM to 1 mM normalized to baseline activity and (E) quantification as area under the curve (AUC).

(F) Quantification of Ca^{2+} responses to glutamate (100 μM), kainate (100 μM), and adrenaline (10 μM) from individual alpha cells within slices from Gcg-Cre-GCaMP3 of control and STZ treated mice (n= 376 and n = 241, respectively).
(G-H) Scattered blots showing maximum amplitudes (%) as fluorescent intensity over baseline (ΔF/F) of individual glucose responsive cells (black dots) and glucose unresponsive cells (grey dots) to stimulation with glutamate and adrenaline for (G) control animals (376 cells, 10 islets, n = 5) and STZ treated mice (241 cells, 8 islets, n = 4). Percentages represent fraction of glucose responsive alpha cells for each stimulus response.
Data in (A), (B) and (C) are shown as average trace (± SEM). Data in (C) and (E) is shown as box-and-whisker plots and compared with one-way ANOVA followed by Tukey's multiple comparison tests. Data in (F) is shown as box-and-whisker plots of 90 percentile and analyzed with Student's t test. Asterisks denote significance (*$p < 0.05$)

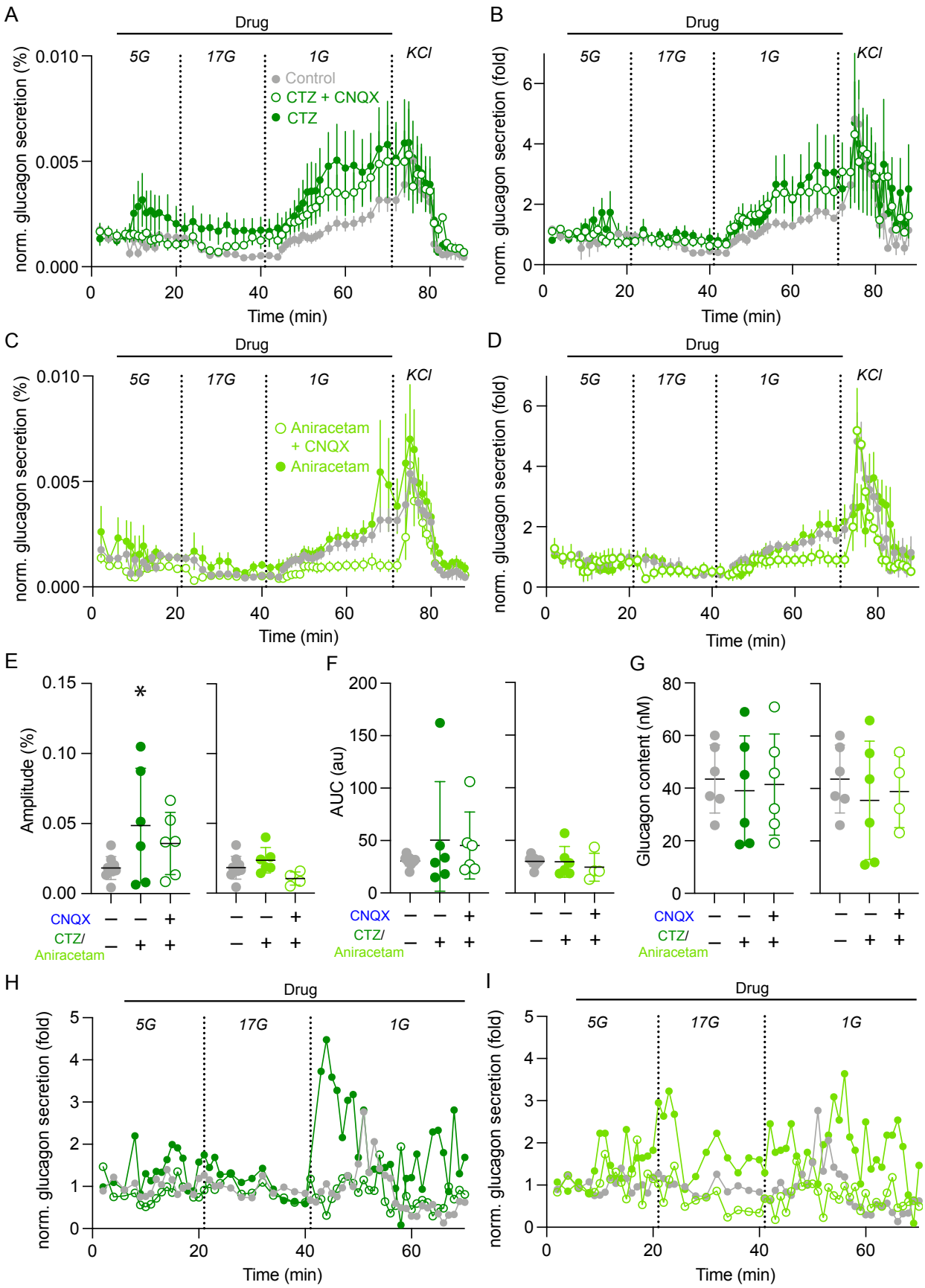
A
Drug
5G
17G
1G
KCl
Control
CTZ + CNQX
CTZ
norm. glucagon secretion (%)
0.010
0.005
0.000
0
20
40
60
80
Time (min)
B
norm. glucagon secretion (fold)
6
4
2
0
C
Aniracetam
+ CNQX
Aniracetam
D
E
Amplitude (%)
0.15
0.10
0.05
0.00
*
CNQX
CTZ/
Aniracetam
F
AUC (au)
200
150
100
50
G
Glucagon content (nM)
80
60
40
20
H
I

Figure 5. The autocrine feedback loop mediated by glutamate can be potentiated in human alpha cells.

(A-D) Glucagon secretion of isolated human islets in response to changes in glucose concentration. Perifusion experiments performed in the presence of glucose only (n = 6), cyclothiazide (CTZ, 100 μM; n = 6), a combination of CTZ and CNQX (100 μM, n = 6), aniracetam (10 nM, n = 6), or a combination of aniracetam and CNQX (n = 4). (A and C) Average traces shown as percent of glucagon content from perifused islets. (B and D) Average traces normalized to the mean baseline within the first 6 minutes.

(E-F) Quantification of glucagon amplitudes during 1 mM stimulation (E) as percent of glucagon content and (F) as area under the curve during 1 mM stimulation (AUC). au = arbitrary untis.

(G) Total glucagon content of perifused human islets.

(H-I) Glucagon secretion normalized to baseline values of human tissue slices from a single nondiabetic human donor in response to changes in glucose concentration in the presence of (H) CTZ or a combination of CTZ and CNQX and (I) in the presence of aniracetam (1 mM) or a combination of aniracetam and CNQX (100 μM).

Glucose concentrations are indicated above each trace. Average traces are shown as mean (± SEM). Data were analyzed by one-way ANOVA followed by multiple comparison. Asterisks denote significance (*$p < 0.05$).

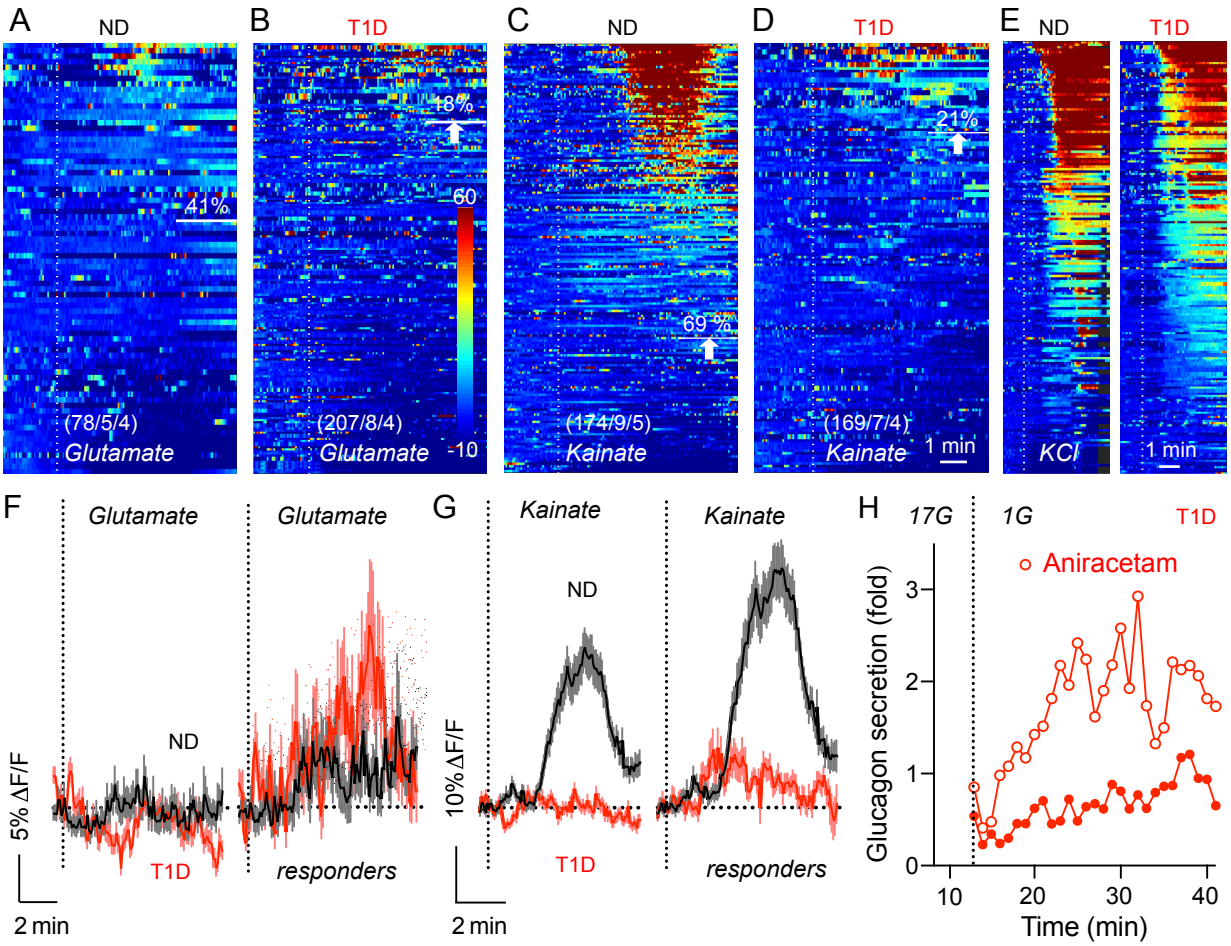

Figure 6. AMPA/kainate receptor signaling is strongly reduced in type 1 diabetes.
(A-D) Heatmaps showing in vitro Ca^{2+} dynamics of alpha cells expressed as the fluorescent intensity of Fluo4 for (A and C) nondiabetic donors and (B and D) individuals with type 1 diabetes. Each row represents a single cell followed over time in the x-axis and their response in magnitude change (%) of the fluorescent intensity over baseline (ΔF/F) activity. Alpha cell responses to glutamate (100 µM, A and B) and kainate (100 µM, C and D). Numbers of cell/islets/donors are indicated in each heatmap. For nondiabetic donors, cells were sorted by adrenaline response (10 µM).
(E) Heatmaps showing Ca^{2+} responses to membrane depolarization with KCl (30 mM) to ensure cell viability of slices from nondiabetic donors (ND) and individuals with type 1 diabetes (T1D).
(F-G) Average traces for Ca^{2+} responses of individual alpha cells from nondiabetic donors (black line) and donors with type 1 diabetes (red line). (F) Average trace of all alpha cells and responders sorted by threshold response to glutamate (100 µM). (G) Average trace of all alpha cells and responders only sorted by threshold response to kainate (100 µM). Traces are shown as mean (± SEM).
(H) Trace showing normalized glucagon secretion of slices from one single human donor with type 1 diabetes in response to lowering in glucose concentration from 17 mM to 1 mM and in the presence of aniracetam (1 mM).

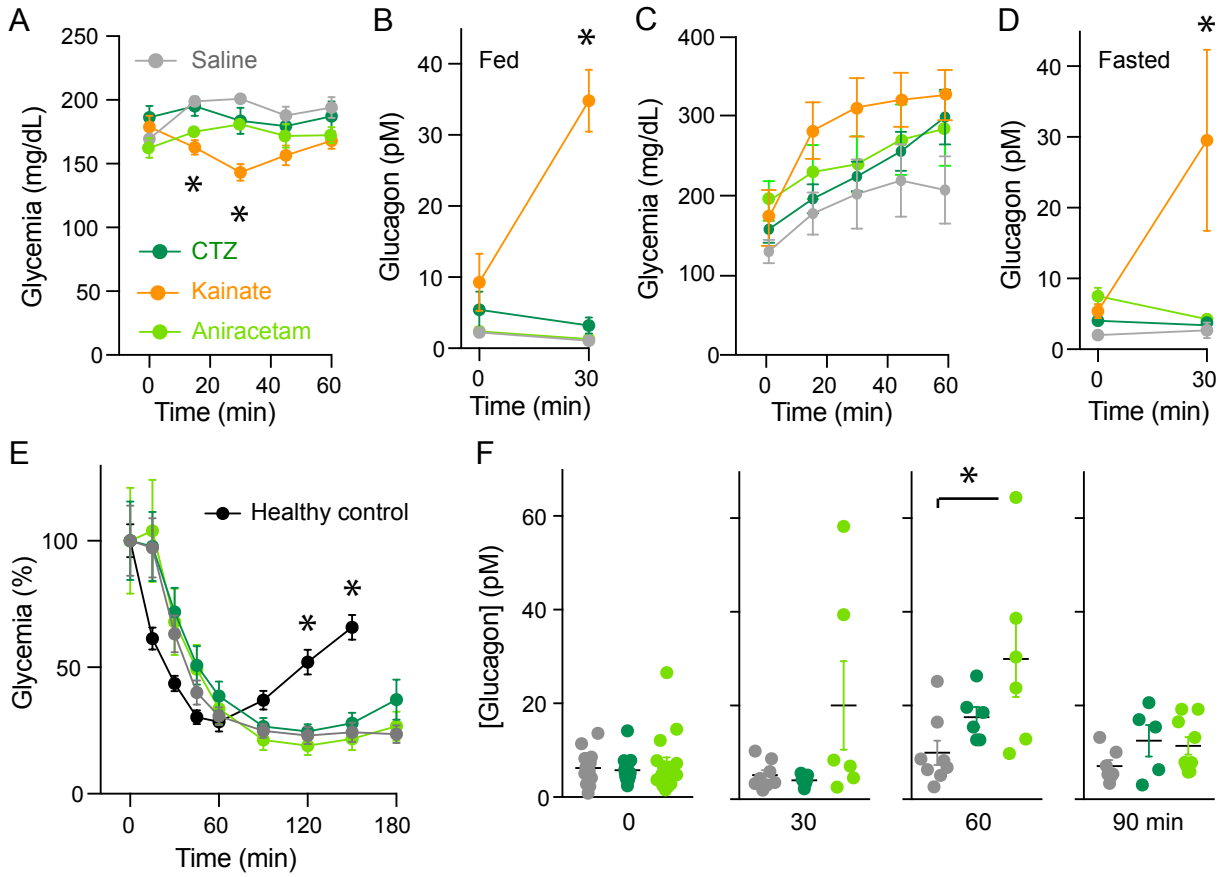

Figure 7. Targeting glutamate receptors *in vivo* improves glucagon secretion in response to hypoglycemia in the streptozotocin mouse model

(A) Non fasting glycemic levels of C57Bl6J mice after injection with saline (n = 8), cyclothiazide (CTZ, 30 mg/kg, i.p.; n = 8), kainate (10 mg/kg, i.p., n = 8) or aniracetam (30 mg/kg, i.p., n = 5).
(B) Serum glucagon levels before and 30 min after injection.
(C) Overnight fasted glycemia of rendered diabetic animals with STZ after injection with saline (n = 4), CTZ (n = 9), kainate (n = 4) or aniracetam (n = 9).
(D) Serum glucagon levels before and 30 min after injection.
(E) Blood glucose excursions after 0.75 U/kg intraperitoneal insulin injection in 6 h fasted diabetic animals compared to healthy animals shown in Fig. 4 (A). Insulin was administered at 0 min directly followed by injection with saline (n=15), CTZ (30 mg/kg, n=17) or aniracetam (30 mg/kg, n = 17).
(F) Plasma glucagon levels of diabetic animals before injection 0 min (saline n = 15, CTZ n = 17, aniracetam n = 17), 30 min (saline n = 8, CTZ n = 6, aniracetam n = 6), 60 min (saline n = 8, CTZ n = 6, aniracetam n = 16) and 90 min later (saline n = 6, CTZ n = 6, aniracetam n = 9).
Data are shown as average (± SEM) and compared with one-way ANOVA followed by Tukey's multiple comparison tests. Asterisks denote significance (*p < 0.05).

METHODS

Mouse models

For in vivo and ex vivo measurements of hormone secretion we used male C57Bl6J (JAX stock #000664) mice, age 10-14 weeks, purchased from The Jackson Laboratory (JAX). After in vivo measurements, the pancreases of these mice were processed for islet isolation as described below. For ex vivo measurements of cytosolic Ca^{2+} in alpha cells, we generated mice that express GCaMP3 selectively in alpha using the Cre-Lox system. Briefly, we crossed female Gcg/tm 1.1(icre)Gkg/J mice (JAX stock #030663), with male mice that express GCaMP3 downstream of a loxP-flanked STOP cassette (JAX stock #029043). Only F1 male mice were used, 8-14 weeks old. The pancreases of these animals were used to generate pancreatic tissue slices as described below. All experiments were conducted according to protocols and guidelines approved by the University of Miami Institutional Animal Care and Use Committee.

The University of Miami complies with the Animal Welfare Act of 1966 (PL89-544) as amended by the Welfare Act of 1970 (PL91-279), adheres to the principals stated in the guide for the care and use of laboratory animals – NIH publication #85-23 (revised) and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Human organ donors

Pancreas tissues from human donors with or without T1D were procured from the nPOD program at the University of Florida in Gainesville (https://www.jdrfnpod.org). All procedures were performed according to the established SOPs by the nPOD/OPPC and approved by the University of Florida Institutional Review Board (IRB201600029) and the United Network for Organ Sharing (UNOS) according to federal guidelines, with informed consent obtained from each donor's legal representative. Demographic data, hospitalization duration, and organ transport time were obtained

from hospital records. Donor pancreata were recovered, placed in transport media on ice, and shipped via organ courier to the University of Florida. Detailed donor information is listed in Table S1.

Human Islet Preparations

Human pancreatic islets were obtained through Prodo Laboratories. The experiments were conducted with islets preparations that were obtained from non-diabetic, cadaveric donors. Detailed donor information is listed in Table S2.

Intraperitoneal Insulin-Tolerance Tests

Intraperitoneal insulin-tolerance tests (IPITTs) were performed after 6 h fasting. To induce hypoglycemia, mice were injected with insulin (intraperitoneal 0.75 U/kg) and blood glucose was monitored at predetermined time points after the injection. STZ treated animals were injected with insulin (intraperitoneal 0.75 U/kg) 3 h prior to the ITT in order to decrease glycemia to comparable levels with control animals and ensure lowering to hypoglycaemic setpoints during the experiment.

Islet isolation procedure

Pancreatic islets were isolated by enzymatic digestion using collagenase type V from *Clostridium histolyticum* (Sigma), dissolved in Hanks' Solution (Sigma) to a final concentration of 0.4 mg/mL. Briefly, mice were anesthetized with isofluorane and euthanized by cervical dislocation. 3 mL of cold collagenase solution was injected into the common bile duct. The injected pancreas was transferred in a 50 mL Falcon tube containing 2 mL of collagenase solution stored on ice. Digestion was performed in a water bath at 37°C for 20 min followed by gentle shaking by hand. The reaction was stopped by adding 25 mL ice-cold Hank's solution. After 1 min centrifugation at 1400 rpm at 8°C, supernatant was discarded, the pellet was resolved in 25 mL Hanks' solution and passed through a small sterile metal strainer. The solution was centrifuged for 1 min at 1400 rpm at 8°C,

resuspended in 25 mL Hanks' solution and centrifuged as before. The pellet was resuspended in 12.5 mL Histopaque® 1077(Sigma) and 10 mL of Hanks' solution was added carefully. After 24 min centrifugation at 3510 rpm with minimum acceleration and breaking, supernatant was collected and washed with 45 mL Hanks' solution. After 1 min centrifugation at 1500 rpm, islet pellet is resuspended in 10 mL CMRL media (Corning). Islets were handpicked and rested overnight in an incubator at 37°C and 5 % CO_2.

Preparation of living tissue slicing

Tissue slices were prepared from Gcg-Cre-GCaMP3 mice (8-14 weeks old). Briefly, mice were anesthetized with isofluorane and euthanized by cervical dislocation. Low gelling temperature agarose (1.2%, Sigma, Cat# A9414, dissolved in HEPES-buffered solution without BSA) was injected in the common bile duct using a 30-gauge needle and 5 mL syringe. After injection, small tissue pieces were cut and embedded further in agarose and placed at 4C for 10 min. Pancreatic slices were then cut (150 mm thickness) on a vibrating blade microtome (VT1000S, Leica) and incubated in HEPES-buffered solution (125 mM NaCl, 5.9 mM KCl, 2.56 mM CaCl2, 1 mM MgCl2, 25 mM HEPES, 0.1% BSA, pH 7.4) containing 5.5 or 7 mM glucose and supplemented with aprotinin (25KIU/mL, Sigma, Cat# A1153).

Dynamic Measurements of hormone secretion

To assess glucose-stimulated hormone release of human pancreatic tissue slices, 3 viable slices were placed into a closed perifusion chamber (Warner Instruments, cat. no. 64-0223 and cat. no. 64-0281 (P-5)) and connected to a perifusion system with automated tray handling (Biorep Technologies, cat. no. PERI4-02-230-FA). For isolated islets (human/mouse), ~100-150 islets were placed in perifusion columns and connected to the perifusion system. Slices/islets were perfused at a flow rate of 100 µL/minute and samples were collected in 96-well plates with a 60

sec interval. Initially tissue samples were flushed for 90 minutes with baseline Hepes buffer to wash out accumulated hormones and enzymes, followed by stimulation with high glucose, low glucose and KCl (with and without the addition of iGluR antagonists/agonists as indicated). Perfusates were stored at -20°C until insulin/glucagon content was measured using commercially available ELISA kits.

Ca^{2+} imaging

Viable human slices were incubated with Fluo4-AM (6 µM, Invitrogen cat. No. F1221) for 1h in 3 mM HEPES buffer (125 mmol/l NaCl, 5.9 mmol/l KCl, 2.56 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 25 mmol/l HEPES, 0.1% BSA [wt/vol.], pH 7.4) supplemented with aprotinin (25KIU, Sigma, cat# A1153) at room temperature in the dark. Human and mouse slices were then placed on a coverslip in an imaging chamber (Warner instruments, Hamden, CT, USA) for imaging on a Leica TCS SP5 upright laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Slices were continuously perfused with HEPES-buffer solution with basal glucose (5 mM and 7 mM for human and mouse slices, respectively), 17 mM glucose or basal glucose with 25 mM KCl. Confocal images were acquired with LAS AF software (Leica Microsystems) using a 40X water immersion objective (NA 0.8). A resonance scanner was used for fast image acquisition producing time-lapse recordings spanning 50-100 µm of the slice (z-step: 5 µm, stack of ten confocal images with a size of 512 × 512 pixels) at 5 sec resolution (XYZT imaging). Backscatter light was collected upon excitation with the 633 nm laser and Fluo4-AM fluorescence was excited at 488 nm and emission detected at 510–550 nm.

Immunofluorescent staining

Slices are washed for 10 min in PBS prior to incubation in 1x blocking solution (BioGeneX, cat No. HK085-5K, diluted in PBS 0.3% Triton X-100) for 2 hours at room temperature. Primary

antibodies are diluted in 1x blocking solution and incubated with antibodies against insulin (1:2000, guineapig, Dako, cat. no. A-0546), glucagon (1:500, mouse, Sigma, cat. no. G2654) and somatostatin (1:500, rabbit, Millipore, cat. No. MAB354) overnight at 4°C while shaking. Slices were washed three times for minimum 30 minutes with PBS and then incubated with secondary antibodies diluted in PBS overnight at 4°C while shaking. Slices were washed three times for 10 minutes with PBS and kept at 4°C in the dark until imaging.

Quantification of Cytosolic Ca^{2+}Levels

To quantify changes in intracellular Ca^{2+}levels, we drew regions of interest around individual islet cells and measured changes in mean GCaMP3/Fluo4-AM fluorescence intensity using ImageJ. Regions of interest were drawn using single planes. A detailed description of how Ca^{2+}imaging data were analyzed and quantified is provided as supplementary material (Figure S2). Briefly, changes in fluorescence intensity were expressed as percentage changes over baseline ($\Delta F/F$). The baseline was defined as the mean intensity of 3 min baseline prior to each stimulus either as heatmaps using MATLAB R2019a (MathWorks software, Natick, MA) or as average traces using Prism 9 (GraphPad software, La Jolla, CA).

Quantification of hormone measurements

Quantification of hormone release was measured using commercially available ELISA kits. Concentrations of insulin/glucagon were obtained by plotting the absorbance of calibrators versus their concentration using sigmoidal four parameter logistic with Prism 9 software (GraphPad software, La Jolla, CA). Data is presented either normalized to average baseline secretion (fold) or as percent of total hormone content (%). Hormone content was measured from lysates obtained from all islets/slices used after the experiment.

Statistical Analyses

For statistical comparisons we used Prism 9 (GraphPad software, La Jolla, CA) and performed Student's t tests (unpaired) or one-way analysis of variance (ANOVA) corrected for multiple comparisons (using Tukey's Multiple Comparison Test; each row represented matched data). P-values < 0.05 were considered statistically significant. Throughout the manuscript we present data as mean ± SEM.

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AUTHOR CONTRIBUTIONS

J.K.P. designed and performed physiological experiments with slices/islets and performed immunohistochemistry. A.T. conducted vivo experiments, maintained animal colonies, performed islet isolations and contributed to physiological experiments. J.K.P. and A.C. designed the study, analyzed and interpreted data and wrote the paper. All authors discussed the results and critically edited the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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