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Beta Cell Hubs Dictate Pancreatic Islet Responses to Glucose

Graphical Abstract

Highlights
- Optogenetic targeting reveals a pacemaker-like β cell subpopulation
- These cells, termed hubs, are required for normal insulin release
- Hubs are highly metabolic and transcriptionally immature
- Hubs are targeted by a diabetic milieu to induce islet failure

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In Brief
Combining optogenetics and photopharmacology, Johnston et al. show that a few (1%–10%) β cells exert disproportionate control over islet responses to glucose. These specialized cells, called hubs, are transcriptionally immature and highly metabolic. Their failure during type 2 diabetes mellitus may lead to reduced insulin secretion and impaired glucose homeostasis.
**Beta Cell Hubs Dictate Pancreatic Islet Responses to Glucose**

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**SUMMARY**

The arrangement of β cells within islets of Langerhans is critical for insulin release through the generation of rhythmic activity. A privileged role for individual β cells in orchestrating these responses has long been suspected, but not directly demonstrated. We show here that the β cell population in situ is operationally heterogeneous. Mapping of islet functional architecture revealed the presence of hub cells with pacemaker properties, which remain stable over recording periods of 2 to 3 hr. Using a dual optogenetic/photopharmacological strategy, silencing of hubs abolished coordinated islet responses to glucose, whereas specific stimulation restored communication patterns. Hubs were metabolically adapted and targeted by both pro-inflammatory and glucolipotoxic insults to induce widespread β cell dysfunction. Thus, the islet is wired by hubs, whose failure may contribute to type 2 diabetes mellitus.

**INTRODUCTION**

The release of insulin from pancreatic β cells is necessary for proper glucose homeostasis in mammals. β cells respond to glucose with increased oxidative metabolism, elevations in cytosolic ATP/ADP ratio, and closure of ATP-sensitive K⁺ (KATP) channels (Rutter et al., 2015). The consequent plasma membrane depolarization activates voltage-dependent Ca²⁺ channels (VDCCs), leading to Ca²⁺ influx and exocytosis of secretory granules (Rutter et al., 2015).

The 3D organization of β cells is also important for the normal regulation of insulin secretion. Thus, β cells throughout the islet microorgan display rhythmic activity patterns in the presence of high glucose (Benninger et al., 2008; Santos et al., 1991). A role for specialized β cells in orchestrating these dynamics has long been postulated, including the presence of putative “pacemakers” (Ammalà et al., 1991; Benninger et al., 2014; Squires et al., 2002). Indeed, isolated β cells possess discrete metabolic characteristics and secretory profiles (Katsuta et al., 2012; Klekens et al., 1992; Salomon and Meda, 1986), and phase lags in the onset of electrical activity can be detected between distant islet regions (Benninger et al., 2008; Meda et al., 1984; Palti et al., 1996). More recent studies have revealed functional differences between hundreds of individual β cells monitored in situ in the intact islet (Hodson et al., 2013; Li et al., 2011; Stolzer et al., 2013). Such heterogeneity may be relevant for type 2 diabetes pathogenesis, since specific insults might target single cells or defined islet regions to induce insulin secretory failure. However, whether particular subsets of cells drive the behavior of others has so far been difficult to prove empirically.

Over the past decade, optogenetics has allowed reversible control of neuronal activity with light (Zhang et al., 2007). In parallel, photopharmacology has harnessed the power of azobenzene photoreceptive units to produce exogenously applied compounds that turn ion channels and G protein-coupled receptors into endogenous photoswitches (Broichhagen et al., 2015). As both these approaches are applicable to electrically excitable endocrine tissue (Broichhagen et al., 2014; Reinothe et al., 2014), they afford the unique opportunity to precisely manipulate cell activity with high spatiotemporal fidelity. Using all-optical interrogation of individual β cells in situ, we therefore set out to probe the topology that regulates population glucose responsiveness, with the aim of identifying the islet-resident pacemaker.
RESULTS

Hubs Are a Feature of β Cell Population Dynamics

To visualize the large-scale organization of β cell activity underlying calcium (Ca²⁺)-dependent exocytosis of insulin granules, intact mouse islets were subjected to high-speed (2–8 Hz) multicellular Ca²⁺ imaging (Hodson et al., 2012). This was combined with Monte Carlo-based correlation analyses in which repeated shuffling of Ca²⁺ events (>9,999 iterations) is used to determine whether cells are coordinated due to chance or not (i.e., contributing to the same insulin release event). Together, these approaches allow online mapping of the islet functional circuitry. Initial experiments confirmed that β cells form a scale-free network (Stozer et al., 2013), which supports the synchronous propagation of glucose (11 mM)-stimulated Ca²⁺ waves by efficiently connecting distant islet regions (R² = 0.72; Figure 1A).

Scale-free networks are ubiquitous throughout biology, are identified by their power law link-probability distribution (Hodson et al., 2010), and adopt a hub and spoke formation where a few cells possess the majority of connections. Accordingly, a stereotypical feature of such topology in islets was the non-random appearance of rare super-connected hubs, whose firing activity tended to repetitively precede and outlast that of the remainder of the population (i.e., was pacemaker-like) (Figures 1B and 1C) (Movie S1).

Such islet architecture was dependent on information exchanges through gap junctions, since reversible blockade of connexin channels using 18α-glycyrrhetinic acid (AGA) (Farnsworth et al., 2014) reduced the number of hubs, decreased coordinated population activity, and increased signal propagation path length (Figures 1D–1G). This may reflect the inability to identify hubs due to loss of cell-cell entrainment, as well as re-routing of information over longer distances by the remaining hubs. Notably, no differences in the amplitude of Ca²⁺ rises were seen in control and AGA-treated tissue (Figures S1A–S1C), suggesting minimal impact upon VDCC activity. In all cases, parallel experiments were performed using glycyrrhizic acid (BGA), the inactive precursor of AGA that exerts similar non-specific effects (Desarmenien et al., 2013). Results could not be attributed to the specific gap junction blocker used, and similar results were obtained with the fluorescent calcium indicator Fluo2 and the calcium sensor GCaMP6 (Figures 1H–1J, Figure S1), suggesting similar link-probability distributions in mouse and human islets (Figures 1K and 1L). Data are means ± SEM. *p < 0.05 and **p < 0.01. NS, non-significant. See also Figure S1 and Movie S1.
be replicated using mebeverine (Farnsworth et al., 2014) (Figure S1D), a gap junction inhibitor with no reported effects on VDCC or K_{ATP} channel activity, as well as Gjd2 shRNA to specifically silence connexin-36 at the islet surface (Figures S1E–S1H).

**Hubs Are Stable and Present across Species**

To assess network topology stability, islets were recorded and then left on the microscope for between 30 min and 3 hr before re-recording. Network topology was stable both over time and in response to perturbation, as statistically assessed.
versus α cells in this species (Bosco et al., 2010), β cell Ca\(^{2+}\) responses were not dependent on orientation toward the islet center or periphery (ΔY Fluo2 = 0.14 ± 0.01 versus 0.13 ± 0.004 AU, periphery versus center, respectively), and identical results were obtained using the genetical encoded indicator GCaMP6 (Figures 1K and 1L), engineered to interfere less with intracellular Ca\(^{2+}\) levels.

**A Strategy for All-Optical Interrogation of β Cell Function**

To functionally dissect the role of hubs, an optogenetic strategy was developed and validated, enabling electrical silencing following Ins1Cre-directed expression of the light-gated chloride (Cl\(^-\)) pump halorhodopsin (eNpHR3.0) (Zhang et al., 2007) in β cells (Figures 2A and 2B). This approach allowed the reversible silencing of single β cell or population Ca\(^{2+}\)-spiking activity and extracellular Ca\(^{2+}\) influx following illumination (λ = 560–590 nm) (Figures 2C–2G) (Movies S2, S3, and S4). Application of the depolarizing agent potassium chloride was able to overcome silencing by restoring VDCC activity (Figure 2H). Of note, wild-type β cells were refractory to silencing (Figures 2I and 2J), and eNpHR3.0-expressing β cells under irradiation were not further hyperpolarized using diazoxide to force open KATP channels (Figure 2K). As measured using patch-clamp electrophysiology, illumination induced photocurrents (Figure 3A), leading to membrane hyperpolarization and electrical silencing only in eNpHR3.0-expressing β cells (Figures 3B–3D). Thus, specific and powerful optogenetic silencing could be achieved.

Animals harboring a single eNpHR3.0 allele unexpectedly demonstrated improved glucose tolerance compared to wild-type littermates, despite normal insulin sensitivity (Figures 4A–4F) and body weight/growth curves (Figures 4G and 4H). This was probably due to enhanced in vivo insulin secretion (Figure 4I), as β cell mass was apparently normal (Figure 4J). Activation of eNpHR3.0 on an Ins1Cre background also led to similar results, suggesting that alternation in insulin gene dosage in the context of the transgene was unlikely to be a contributing mechanism (Figures 4K and 4L). Pertinent to the in vitro studies here, however, isolated islets responded normally to glucose in terms of ionic fluxes and insulin release (Figures S2A–S2I), and eNpHR3.0 does not possess basal activity in the absence of light (Zhang et al., 2007) (also shown in Figure 3C).

**Hubs Orchestrate β Cell Population Responses to Glucose**

By performing analysis in real-time using islets maintained on the microscope stage, hubs could be identified and subsequently manipulated (Figures 5A–5C). Silencing of individual hubs using a pinpointing laser had catastrophic consequences for coordinated islet responses to high glucose (Figures 5D and 5E) (Movies S5 and S6), an effect reversed simply by ceasing illumination (Figures 5F and 5G). The strength of inhibition following targeting of individual hubs tended to be inversely associated with the number of these cells per islet before silencing (Figure S2J), suggesting that some redundancy is present in the system, most likely due to follower cells being controlled by more than one hub. By contrast, silencing of individual non-hub or follower cells did not significantly perturb islet dynamics (Figure 5H), demonstrating the specificity of the approach.
Figure 4. Glucose Homeostasis in eNpHR3.0 Mice

(A and B) Glucose tolerance is improved in male 8 week \textit{Ins1Cre}^{+/+}; \textit{eNpHR3.0-EYFP}^{+/+} (NpHR) animals (n = 7) compared to \textit{Ins1Cre}^{++/++}; \textit{eNpHR3.0-EYFP}^{++/++} (wild-type, WT) littermates (n = 7) (i.e., activation of \textit{Ins1Cre} on an eNpHR3.0-EYFP background), as assessed using IPGTT.

(C) Insulin sensitivity is similar in male NpHR mice animals and WT littermates (n = 6–11), as determined using ITT.

(D and E) As for (A) and (B), but female 8 week mice (n = 7–9).

(F) As for (C), but female 8 week (n = 4).

(G and H) Fasting body weight and growth curves (non-fasted) are similar in WT and NpHR animals (n = 9–13).

(I) In vivo insulin release tended to be increased in NpHR compared to WT animals at 15 min post-IP glucose injection (n = 4).

(J) \(\beta\) cell mass, \(\alpha\) cell mass, and \(\alpha:\beta\) cell ratio are similar in WT and NpHR animals (n = 3).

(K and L) As for (A) and (B), but glucose tolerance in 6 and 8 week \textit{Ins1Cre}^{+/+}; \textit{eNpHR3.0-EYFP}^{+/+} (NpHR) (n = 3–4) compared to \textit{Ins1Cre}^{++/++}; \textit{eNpHR3.0-EYFP}^{++/++} (wild-type, WT) animals (n = 8) (i.e., activation of eNpHR3.0-EYFP on an \textit{Ins1Cre} background).

Data are means ± SEM. **\(p < 0.01\). NS, non-significant. See also Figure S2.
Using a similar technique, hubs were first identified at high glucose, before inactivation using low glucose and stimulation with JB253, an exogenously applied KATP channel photoswitch based on glimepiride (Broichhagen et al., 2014). Following targeted illumination of JB253-treated islets, hub connectivity could be mimicked without activation of intervening cells, as determined by the presence of glucose- and gap-junction-dependent entrainment patterns in follower cells (conduction velocity = 47.0 ± 8.9 μm/s) (Figures 5I–5K). Such effects were unlikely to stem from diffusion of active JB253, since this molecule turns off within milliseconds in the dark (Broichhagen et al., 2014), and proximate cells remained unaffected by hub stimulation (Figures S3A–S3D).

**Hubs Are Required for Insulin Secretion**

We were unable to measure insulin secretion accurately from a single islet over the 5 min experimental period used here, since levels were below the detection sensitivity of current assays. Therefore, to link hub activity with hormone release, the cell-surface-attached fluorescent Zn²⁺ probe JP-107 (Pancholi et al., 2014) was instead employed as a surrogate to dynamically report Zn²⁺ co-released with insulin from cells at the islet surface, as previously reported with ZIMIR (Li et al., 2011). Using this

![Figure 5. Real-Time Analysis and Targeting of β Cell Hubs](image-url)
approach, silencing of follower cells or wild-type islets was without effect, as evidenced by a linear increase in fluorescence due to Zn²⁺ accumulation at the probe. By contrast, hub shutdown or global illumination lowered insulin/Zn²⁺ release to below the dissolution rate of the probe (i.e., Zn²⁺ binding is lower than Zn²⁺ removal) (Figure 5L).

While it was not technically possible to directly link hub activity with pulsatile insulin release, the acetylcholinomimetic carbachol (Zhang et al., 2008a) was able to accelerate β cell population activity (Figure S3E) without altering the proportion of links or hubs (Figures S3F and S3G). Moreover, rapid imaging performed over dozens of minutes—i.e., within the range of insulin pulses (Head et al., 2012)—revealed that hubs are also a feature of population behavior over longer periods (proportion hubs = 7.1% ± 1.3%; proportion links = 9.7% ± 2.0%). Since carbachol has been shown to phase-set activity between islets in vitro (Zhang et al., 2008a), parasympathetic neurons may plausibly target hubs in vivo to synchronize islet activity and generate insulin pulses.

Hubs Possess a Characteristic Metabolic Signature
We next sought to understand what makes a hub cell unique. Islet-wide Ca²⁺ signals were recorded before metabolic profiling of the hub population in the same islet using the mitochondrial potential dye tetramethylrhodamine ethyl ester (TMRE), which sequesters in active, hypopolarized mitochondria. Following stimulation at high glucose, mitochondria in hubs became more hyperpolarized versus those in non-hubs (Figures 6A and 6B), suggesting increased proton pumping, ATP synthase activity, and ATP generation (Tarasov et al., 2012). While the duty cycle (i.e., proportion of time the cell spends “ON”) was slightly increased in hubs compared to non-hubs (Figure 6C), other activity parameters including Ca²⁺-spiking amplitude and frequency were broadly similar (Figures 6D–6F). Spatially, hubs and non-hubs were intermingled, with no clear preference for the islet center or periphery detected for either population based on polar coordinates (angle and distance taken from the islet center) (Figures 6G and 6H).

Hubs Display Features of both Mature and Immature β Cells
Using photoactivatable Tag-RFP (PA-TagRFP) to photopaint single hubs within islets using a 405 nm laser (Figures S4A and S4B), post hoc immunostaining against a variety of markers of β cell “identity” (Rutter et al., 2015) could be performed (Figure 6I) without adversely altering Ca²⁺ dynamics (Figure S4C). These studies revealed reduced insulin content, increased glucokinase (Gck) levels, lowered expression of pancreatic duodenum homeobox-1 (Pdx1), but normal levels of the mitochondrial import receptor subunit TOM20 homolog (Tomm20) in hubs versus the rest of the population (Figures 6I and 6J) (Figure S5). The transcription factor NK6 homeobox-1 (Nkx6.1), recently shown to be required for insulin biosynthesis and β cell proliferation (Taylor et al., 2013), was almost absent from hubs (Figures 6I and 6J). Suggesting that hubs are unlikely to represent a multihormonal (e.g., Glu+, Ins+) population, no co-localization with glucagon was detected (Figure 6K). Likewise, neurogenin 3 (Ngn3), a β cell precursor marker, was undetectable at the protein level in the adult islet, implying that hubs are unlikely to be trapped in a progenitor state (Figure 6K). Inspection of oversampled and deconvolved superresolution confocal images revealed no differences in mitochondrial distribution/shape or endoplasmic reticulum content in hubs (Figures 6L–6P), although expression of the sarco(endo)plasmic reticulum Ca²⁺/ATPase, SERCA2, was markedly reduced (Figures 6O and 6P).

Suggesting a hyposecretory (or degranulated) nature, insulin granule numbers were lower in hubs versus non-hubs, despite a similar distribution (Figures 6Q and 6R). Furthermore, the area of individual hub cells was comparable to the rest of the population (range = 122–381 µm² and 194–355 µm², non-hubs versus hubs, respectively), and their shape appeared to be normal. Consequently, hubs constitute a metabolically adapted, repurposed subpopulation of β cell that displays features of immature cells.

Hubs Are Targeted by Diabetic Milieu
Lastly, the robustness of diabetics was determined by challenging islets with cytokine cocktails (IL-1β/IL-6 or IL-1β/TNF-α) to re-create the pro-inflammatory milieu thought to be associated with diabetes (O’Neill et al., 2013). Acutely, the application of cytokines led to a large ramp-up in Ca²⁺ spiking activity in the presence of high glucose (Figure S6). However, after only 2 hr incubation, a collapse in hub cell number was apparent (Figures 7A and 7B), and this could be viewed in real-time by recording the same islet left in situ before and during exposure to cytokine (IL-1β/IL-6) (Figures 7C and 7D). The cytokine-induced disruption to hub cell function was further evidenced by a reduction in the number of cells occupying the upper or “high connectivity” region of the link-probability distribution (Figures 7E and 7F), as shown by a decrease in the exponent value of the power law fit. This resulted in a dramatic decline in correlated β cell population function (Figure 7G) due to the presence of fewer and less well-connected hubs. The actions of cytokines were not explained by effects on cell viability, as assessed using indices of necrosis (Figures 7H and 7I) and apoptosis (Figure 7J). However, 2 hr cytokine exposure decreased mRNA levels of the major islet gap junction isoform connexin-36 (Gjd2) 3-fold (Figures 7K and 7L), and this was already associated with a substantial reduction in gap junction plaque number (Figure 7M), in line with that recently reported using a similar paradigm (Farnsworth et al., 2015). Likewise, preferential hub failure was detected in both rodent and human tissue in response to gluco(lipo)toxic insults (Figures 7N and 7O).

DISCUSSION

β cells are a phenotypically diverse population, presenting a mosaic of metabolic and electrical activity patterns (Pralong et al., 1990), which is mirrored at the level of insulin secretory capacity (Katsuta et al., 2012; Kiekens et al., 1992; Li et al., 2011; Salomon and Meda, 1986). When viewed as a population, β cells are often termed a functional synctium, although a role for cell heterogeneity in generating multicellular dynamics has been invoked repeatedly (Benninger and Piston, 2014; Stoezer et al., 2013). Indeed, it has been shown that a subset (~10%–15%) of β cells may exert a disproportionate influence over islet dynamics (Hraha et al., 2014). By combining large-scale functional cell mapping with optogenetics and photopharmacology, we
provide here a revised blueprint for islet function whereby a few pioneer hubs with reduced β cell identity dictate emergent population behavior in response to glucose. Importantly, hub topologies are a feature of dynamical systems, including cell networks in the brain and pituitary (Bonifazi et al., 2009; Hodson et al., 2012), since they are functionally robust at a low wiring cost (Bullmore and Sporns, 2009) (i.e., the chances of randomly hitting a hub are low). However, should a hub be specifically targeted,
the effects on cell population function are far reaching, as observed in the islet during exposure to cytokine or glucolipotoxicity.

The present study used a single-photon-based confocal system to control the activity of individual hubs or followers within isolated islets. While two photon approaches in theory increase the accuracy of cell targeting by restricting the beam to within a few microns of the focal point, there are drawbacks when used with optogenetics. First, a diffraction-limited two-photon laser spot (i.e., ~500 nm) is insufficient to reliably activate optogenes, and the long excited state halftime can quickly saturate the rhodopsin (Rickgauer and Tank, 2009). Second, commercial lasers are unable to deliver the >1,100 nm excitation required for eNpHR3.0 activation without an optical-parametric oscillator (Andersen et al., 2009). By contrast, a single-photon diffraction-limited laser spot (~500 nm) of known absorbance cross-spectrum can be introduced to the surface of the sample, with minimal aberration and steep power drop-off as a function of 1/distance^2. Demonstrating the high degree of localization of the effective beam, we were clearly able to photopaint single cells within an islet and did not see any population silencing when a follower cell was targeted.

Using patch-clamp recordings of dissociated β cells, eNpHR3.0 activation hyperpolarized membrane potential by ~60 mV, in line with previous reports (Mattis et al., 2012). While photocurrent size may be underestimated due to the presence of an electrochemical gradient, it should be noted that halorhodopsin derives...
energy from photons rather than the ion gradient itself (Pfisterer et al., 2009), and the photocycle is unaffected even in the presence of high Cl⁻ concentration (Váró et al., 1989). In any case, it is unlikely that hyperpolarizing spread throughout the islet per se could account for these observations, since (1) only 30% of voltage spreads to an immediately coupled cell and an 86 mV depolarizing step is required for activation via gap junctions (Zhang et al., 2008b); and (2) stimulation of follower cells was without effect. We prefer an explanation whereby large changes in conductance attributable to the hub cell or its very close neighbors are removed through eNpHR3.0-mediated silencing, leading to impaired propagation of Ca²⁺ waves (Benninger and Piston, 2014; Benninger et al., 2008; Zhang et al., 2008b). Although membrane potential was slightly more depolarized following cessation of illumination, this is also seen in neurons (Mattis et al., 2012) and may reflect the reversal potential of Cl⁻. We did not notice significant effects on hub indices during the Ca²⁺ imaging studies here due to use of a 5–10 min “rest” period to allow Cl⁻ re-equilibration.

Experiments in which hub cells were stimulated revealed that hubs and followers are unlikely to form local syncytia. While the exact mechanisms for antipodal signal propagation are difficult to determine precisely, a role for 3D chains of electrically coupled cells is plausible, given that entrainment was markedly blunted by both gap junction blockade and perfusion with 1 mM glucose. Other communication possibilities include autonomic neurons, which possess >100 µm axonal arborizations in pancreatic slices (Rodríguez-Diaz et al., 2012), and cilia, which provide a restricted signaling corridor due to their presence in only ~25% of β cells (Gerdes et al., 2014). Along these lines, the effect of hub silencing on islet function was surprisingly strong, given the relatively mild phenotype of animals deleted for the gap junction protein connexin36 (Ravier et al., 2005). However, considerable redundancy exists in the latter model with connexin-30.2 (Cx30.2) and ephrins providing alternative signaling routes (Farnsworth and Benninger, 2014; Konstantinova et al., 2007).

An intriguing possibility is that hubs are related to the previously described Pdx1+, Ins(low) β cell subpopulation (Szabat et al., 2009), albeit distinct in their low levels of both markers. Impaired identity, while conceivably restraining stimulus-induced secretion, may also limit GK-induced proliferation (Porat et al., 2011; Stolovich-Rain et al., 2015) to maintain the role of these cells as specialized pacemakers. Indeed, high levels of GK expression may sensitize hubs cells to increases in glucose concentration, allowing these cells to respond earlier and more robustly than their neighbors. By contrast, the failure of hubs when faced with a glucotoxic/pro-inflammatory milieu indicates that these cells are metabolically fragile. This vulnerability might reflect high Gck/Gck (Roma et al., 2015) expression coupled to low Pdx1 and SERCA2 levels (Fonseca et al., 2011; Fujimoto et al., 2009), which ultimately lead to ER stress and cell dysfunction.

We acknowledge that the hub protein characterization performed here constitutes a biased screen, but it nonetheless provides a strong foundation for understanding the biology of these unusual cells. In the future, unbiased multiplex approaches, including massive parallel sequencing (RNASeq) and CyTOF (single cell mass cytometry) (Proserpio and Lonnberg, 2015), will help define the hub signature. Although attempts were made to obtain dissociated cells/cytoplast for these purposes, PA-TagRFP fluorescence disappeared following dissociation of islets, possibly reflecting either the fragility of these cells, or the fluorophore itself. Similar problems were encountered with electron microscopy, where available antibodies cannot differentiate between activated and non-activated PA-TagRFP.

The recording approaches used to monitor hubs were technically constrained to 2 to 3 hr. Indeed, such experiments necessitate leaving the islets in situ on the microscope, since the same field of view must be maintained for analysis purposes. Thus, it cannot be excluded that hubs may represent a transitory subpopulation that drifts over dozens of hours in line with transcriptional/translational processes. Indeed, modeling studies predict that “pacemakers” arise from the most excitable β cell, which is assumed to shift due to a random distribution of excitability as $K_{ATP}$ channel expression levels vary (Benninger et al., 2014). However, the possibility that such cells may arise during development could not be excluded (Benninger et al., 2014), and studies in FACS-purified GFP-labeled β cells suggest the presence of distinct transcriptional pools, with the proportions remaining similar between animals and days (Katsuta et al., 2012). Moreover, to the best of our knowledge, there is no evidence that $K_{ATP}$ channel changes over time, though the presence of a substantial proportion of channel subunits on internal membranes (Varadi et al., 2006) may complicate such measures.

Lastly, it should be noted that experiments in isolated islets may not necessarily reflect the situation in vivo, where blood flow direction (β cell $\rightarrow$ α cell) (Nyman et al., 2008) and molecule access dynamics (Michau et al., 2015) may all affect the role of hubs in dictating population dynamics and insulin secretion. This possibility might be tested in the future using in vivo imaging approaches (Nyman et al., 2008; Speier et al., 2008).

In summary, the present findings provide new insights into the regulation of islet function by individual β cells and the mechanisms that likely target and impair this during type 2 diabetes pathogenesis and treatment. More generally, the paradigm developed here to study the roles of individual cells within the functioning islet may be broadly applicable to other tissues or organisms.

**EXPERIMENTAL PROCEDURES**

**Animals, Glucose/Insulin Tolerance Testing and Insulin Measures**

β-cell-specific expression of halorhodopsin was achieved by crossing the Ins1Cre deleter strain (Thorens et al., 2015) with animals engineered to express eNpHR3.0:EFYP following excision of aloxP-flanked STOP cassette (B6;129S-Gt(Rosa)26Sortm39(CAG-hop/EYFP)Hze/J. Ins1Cre²⁻² and Ins1Cre²⁺² littersmates (i.e., derived from an Ins1Cre²⁻² × Ins1Cre²⁺² breeding pair) display similar glucose tolerance and growth curves (Thorens et al., 2015), as well as insulin tolerance, in vivo and in vitro insulin secretion, and β cell mass (Figure S7). For detailed information, see Supplemental Experimental Procedures.

**Islet Isolation**

Islets were isolated using collagenase digestion and cultured for 24–72 hr in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.
**Human Islet Culture**

Human islets were obtained from isolation centers in Italy and Switzerland, with necessary local and national ethical permissions, including consent from the next of kin. Islets were cultured in RPMI supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 mg/ml fungizone, supplemented with 5.5 mM D-glucose. Ethical approval was granted by the National Research Ethics Committee London (Fulham), REC #07/H0711/14.

**Calcium and Mitochondrial Potential Imaging**

Multicellular Ca²⁺ and mitochondrial potential imaging was performed as detailed in Hodson et al. (2013). Mitochondrial potential was monitored using TMRE. For detailed information, see Supplemental Experimental Procedures.

**Electrophysiology**

Pancreatic islets were dissociated into single β cells and plated onto glass coverslips. Electrophysiological recordings were performed in either perforated patch-clamp or whole-cell configuration using an EPC9 patch-clamp amplifier controlled by Pulse acquisition software (HEKA). For detailed information, see Supplemental Experimental Procedures.

**Dynamic Insulin Secretion Measures**

Zinc (Zn²⁺) co-released from insulin-containing granules was measured as a proxy for insulin secretion using the chemical probe JP-107 (300 µM), as described in Pancholi et al. (2014). For detailed information, see Supplemental Experimental Procedures.

**Generation of Adenoviral PA-TagRFP and Photopainting**

cDNA encoding the photoactivatable fluorescent protein PA-TagRFP (Subach et al., 2010) was cloned into pShuttleCMV via Xho I and Xba I sites before recombination with pAdEasy1 and virus production as described in Luo et al. (2007). Islets were incubated for 48 hr with adenovirus harboring PA-TagRFP at a MOI = 100. For detailed information, see Supplemental Experimental Procedures.

**shRNA-Silencing of Connexin-36**

For detailed information, see Supplemental Experimental Procedures.

**Immunohistochemistry**

Islets were fixed at 4°C overnight in paraformaldehyde (4%, wt/vol) before permeabilization (PBS + Triton 0.1%) and application of primary and secondary antibody for 24–48 hr at 4°C. Connexin-36 staining was performed as above, but following 10 min fixation in ice-cold acetone. For detailed information, see Supplemental Experimental Procedures.

**β and α Cell Mass**

For detailed information, see Supplemental Experimental Procedures.

**Necrosis and Apoptosis Assays**

For detailed information, see Supplemental Experimental Procedures.

**Real-Time PCR**

Relative mRNA abundance was determined on an Applied Biosystems ABI 7500 Fast Real-Time PCR System using SYBR Green reagents and primers against connexin 36 (Gjo2) (GATTGGGAGGATCCTGTTGAC and AGG GCTAGGAGAAGACAGTAGAG). Gene expression was normalized to β-actin (CGAGTCGCGTCCACCC and CATCCATGGCGAACTGGTG) and fold-change in mRNA expression compared to control was calculated using the 2⁻ΔΔCT.

**Correlation, Similarity Analyses and Polar Coordinates**

Individual EYFP-expressing β cells were identified using an ROI to produce a mask overlay of the imaged population. Correlation analyses were then performed in MATLAB on Hilbert-Huang transformed Ca²⁺ signals using binarization and matrix analyses, and statistical significance assigned using non-deterministic (Monte-Carlo) methods, as described (Hodson et al., 2010, 2012). For detailed information, see Supplemental Experimental Procedures.

**Measurements of Insulin Secretion from Isolated Islets**

For detailed information, see Supplemental Experimental Procedures.

**Cytokines and Glucolipotoxicity**

Interleukin 1β (IL-1β), interleukin 6 (IL-6), and tumor necrosis factorα (TNF-α) (all from R&D Biosystems) were stored as stock solutions at −20°C and used at 20 pg/ml, 40 pg/ml, and 20 pg/ml, respectively (Maedler et al., 2004; O’Neill et al., 2013). For glucolipotoxicity studies, cells were exposed to 33 mM glucose and/or 0.5 mM BSA-conjugated palmitate for 48 hr.

**Statistical Analyses**

Data normality was assessed using the D’Agostino Pearson omnibus test. Pairwise comparisons were performed using paired or unpaired Student’s t test. Interactions between multiple treatments were determined using one-way or two-way ANOVA (adjusted for repeated-measures as necessary), followed by pairwise comparisons with Bonferroni’s or Tukey’s posthoc tests. Analyses were conducted using R (R Project), Graphpad Prism 6.0 (Graphpad Software), IgorPro (WaveMetrics), and MATLAB (Mathworks), and results deemed significant at p < 0.05. Unless otherwise stated, data are presented as the mean ± SEM.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures, six movies, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.06.020.

**AUTHOR CONTRIBUTIONS**

N.R.J., G.A.R., and D.J.H. conceived and designed the experiments. N.R.J., R.K.M., E.H., M.P.P., F.S., and D.J.H. conducted the experiments. J.F. provided reagents and intellectual input. L.P., P.M., M.B., D.E., and E.B. isolated and provided human islet of Langerhans. P.D., M.W., J.B., and D.T. designed, synthesized, and provided chemical reagents. N.R.J., G.A.R., and D.J.H. wrote the paper with input from all the authors.

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