

# Innervation Patterns of Autonomic Axons in the Human Endocrine Pancreas

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## SUMMARY

The autonomic nervous system regulates hormone secretion from the endocrine pancreas, the islets of Langerhans, thus impacting glucose metabolism. The parasympathetic and sympathetic nerves innervate the pancreatic islet, but the precise innervation patterns are unknown, particularly in human. Here we demonstrate that the innervation of human islets is different from that of mouse islets and does not conform to existing models of autonomic control of islet function. By visualizing axons in three dimensions and quantifying axonal densities and contacts within pancreatic islets, we found that, unlike mouse endocrine cells, human endocrine cells are sparsely contacted by autonomic axons. Few parasympathetic cholinergic axons penetrate the human islet, and the invading sympathetic fibers preferentially innervate smooth muscle cells of blood vessels located within the islet. Thus, rather than modulating endocrine cell function directly, sympathetic nerves may regulate hormone secretion in human islets by controlling local blood flow or by acting on islet regions located downstream.

## INTRODUCTION

The body is able to maintain strikingly constant levels of plasma glucose thanks to hormone secretion from the endocrine pancreas, the islets of Langerhans. A failure to do so leads to potentially life-threatening dysregulation of glucose homeostasis. A close relationship between the nervous system and the regulation of plasma glucose was revealed early by Claude Bernard (Bernard, 1855), and Paul Langerhans recognized a rich innervation of the pancreatic islets since their discovery (Langerhans and Morrison, 1937). Many studies have implicated the autonomic nervous system in islet function and glucose homeostasis (reviewed in Åhrén, 2000; Brunnicardi et al., 1995; Gilon and Henquin, 2001; Havel and Taborsky, 1989; Satin and

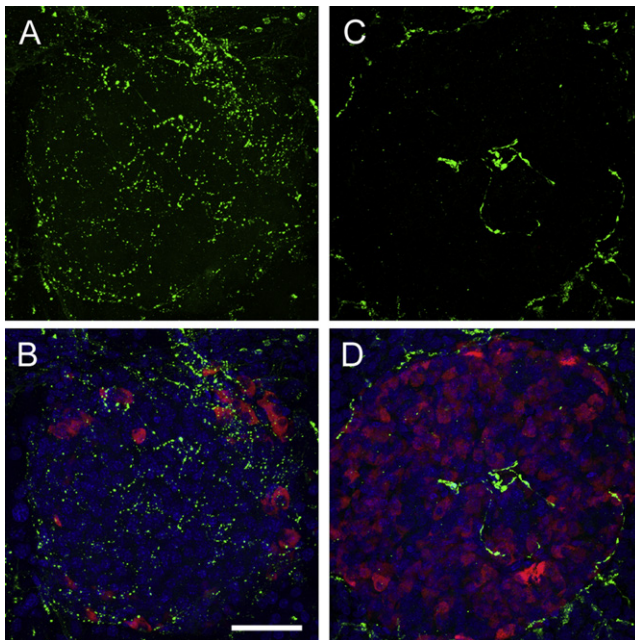
Kinard, 1998; Woods and Porte, 1974). Few studies, however, have described the structural innervation of the human islet, and these studies are not comprehensive (Åhrén et al., 1991; Amenta et al., 1983; Bishop et al., 1980; Ding et al., 1997; Shimosagawa et al., 1993). Importantly, human islets have not been examined for the presence of classical markers of the parasympathetic and sympathetic systems. We also do not know how and where neuronal axons terminate within the islets and what particular cells are innervated. Because the autonomic nervous system is thought to play an important role in islet function in several species, the precise organization of human islet innervation needs to be assessed.

Here we systematically investigated the innervation patterns in human islets and compared them to those in mouse islets, a species commonly used in islet research. We performed immunohistochemical studies on pancreatic sections with a battery of antibodies recognizing markers for neuronal axons, parasympathetic and sympathetic axons, endocrine cells, and vascular cells. The parasympathetic innervation was identified by the presence of cholinergic markers and the sympathetic innervation by the expression of catecholaminergic markers. The density of axons and their proximity to putative targets were detected and quantified in Z stacks (40  $\mu$ m) of confocal images. The paths of axons within islets were examined in three-dimensional reconstructions of the images. This approach allowed us to obtain a detailed, objective picture of the complexity of islet innervation, revealing that human and mouse islets greatly differ in their innervation patterns. In mouse islets, parasympathetic and sympathetic axons innervate beta, alpha, and delta cells, but in human islets endocrine cells are barely innervated. Instead, sympathetic axons in human islets preferentially contact smooth muscle cells of the vasculature. Thus, the autonomic nervous system may regulate human islet function via sympathetic input acting on contractile cells of the vasculature within the islet and altering blood flow locally or by using the vascular route to influence endocrine cells located downstream from the release sites.

## RESULTS

### Human Islets Are Sparsely Innervated

Because little is known about the innervation of human islets, we first examined the general innervation pattern in human islets



**Figure 1. Human Islets Are Sparsely Innervated**

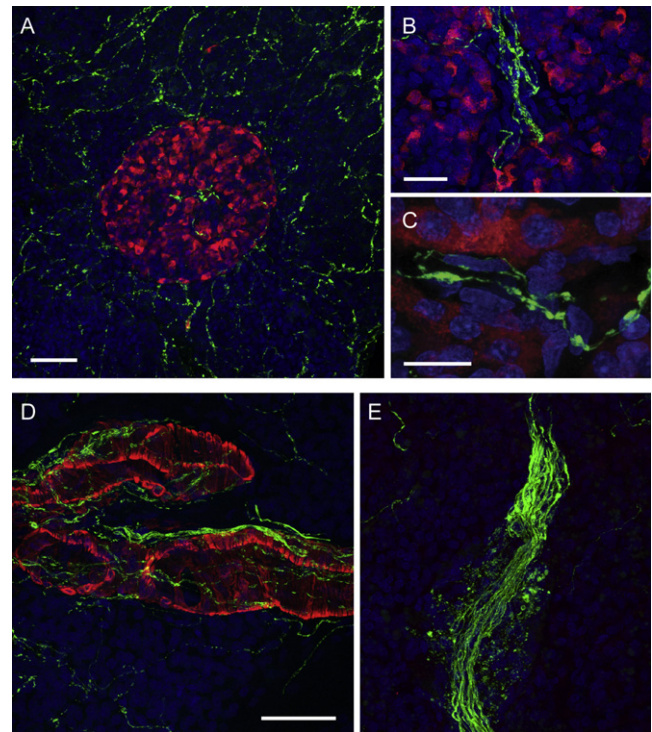
(A and B) A mouse pancreatic section showing immunostaining for synapsin I/II (green) within an islet. Numerous axonal varicosities are present throughout the islet. Glucagon immunostaining (red) and nuclear counterstaining (DAPI, blue) are merged with synapsin labeling in (B) and (D). Unless otherwise stated, all figures in the article show maximal projections of Z stacks of 40 confocal images. Scale bar, 50  $\mu$ m.

(C and D) Synapsin immunostaining (green) in a human pancreatic section. Few immunostained axons can be seen in the islet, and these are localized to discrete regions.

and compared it to that in the commonly studied mouse islets. We used a marker found in all axon terminals of vertebrate neurons, synapsin I/II (De Camilli et al., 1990), which labels all axonal terminals containing conventional transmitters stored in small synaptic vesicles. Mouse islets were densely innervated and could be readily distinguished from the surrounding exocrine tissue by the neuronal plexus of synapsin-labeled axons and axonal varicosities (Figure 1; see Movie S1 and Movie S2 available online). By contrast, human islets were mostly devoid of synapsin-labeled axons. Only a few large axons were present in distinct areas of the islet (Figures 1 and 2). Neuronal axons were strongly labeled in the exocrine tissue of the human pancreas, confirming specific staining and ruling out a lack of antibody penetration (Figure 2). We obtained similar results with another antibody for synapsin as well as with the axonal markers PGP 9.5 and acetylated alpha tubulin (Figure S1). Thus, only few neuronal axons penetrate human islets, and these innervate discrete regions within the islets.

### Most Axons Innervating Human Islets Are Sympathetic

We next sought to identify the nature of the neuronal axons innervating human islets. We focused on markers of the autonomic nervous system because autonomic innervation has been widely reported to influence islet function. We labeled parasympathetic axons with antibodies for the vesicular acetylcholine transporter



**Figure 2. Axon Immunostaining in the Pancreas Shows Tissue-Specific Staining Patterns**

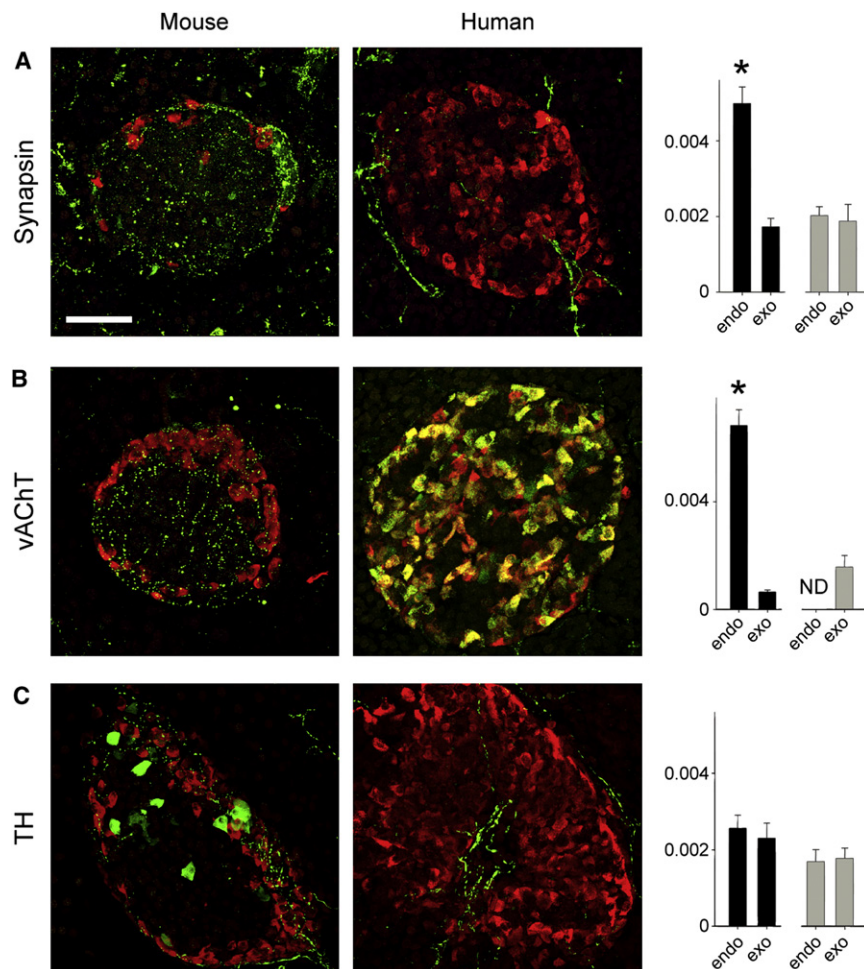
(A) A human pancreatic section immunostained for synapsin (green) and glucagon (red in A–C) shows many synapsin-labeled axons in the exocrine tissue, but only few penetrating the islet.

(B and C) Strong synapsin staining (green) can be seen inside the islet in large axons running along elongated cell nuclei. These axons do not enter the endocrine tissue (glucagon staining shown in red).

(D and E) Strong synapsin staining (green) can be seen inside around large arteries (D, alpha smooth muscle actin in red) or in nerves traversing the exocrine pancreas (E). Scale bars, 50  $\mu$ m (A, D, and E; in D also applies to E), 20  $\mu$ m (B), and 10  $\mu$ m (C).

(vAChT), a marker for cholinergic neurons and axons, and found that the core of mouse islets was densely innervated with vAChT-labeled axons and terminals (Figure 3B). In the mouse pancreas, the innervation density in the islet was much higher than in the surrounding exocrine tissue (Figures 3B and 4A). vAChT-labeled axons innervated both beta and alpha cells (Figure 4C). A few vAChT axons were found along blood vessels. Human islets showed a remarkably different staining pattern: we examined 15 human pancreata and found that islet cells, but few neuronal axons or terminals, were labeled for vAChT in the islet. In the human islet much of the vAChT staining colocalized with glucagon (see yellow in Figure 3B). A quantification of the density of vAChT-labeled axons in human islets was not possible because of the strong staining in endocrine cells. Nevertheless, labeled axons could not be seen in endocrine regions devoid of vAChT-labeled cells, whereas labeled axons were clearly seen in the exocrine tissue (Figures 4F and 4G). Similar results were obtained with choline acetyltransferase (ChAT), another marker of cholinergic neurons and axons (Figure S2).





**Figure 3. The Innervation of the Human Islet Is Different from that of the Mouse Islet**

(A) Synapsin I/II immunostaining (green) differs qualitatively and quantitatively between mouse and human islets. Maximal projections of confocal images show numerous axonal varicosities in mouse islets but few long axons in human islets. Quantification (right) shows that the density of immunostained axons is much higher in mouse (black bars) endocrine (endo) regions than in exocrine regions (exo, -). There is no increased innervation density in the human endocrine pancreas (gray bars). Innervation density is expressed as the ratio of axonal labeling to total examined islet volume (see the [Experimental Procedures](#)). One-way ANOVA followed by multiple comparisons (Student-Newman-Keuls). Data are presented as mean  $\pm$  SEM. Glucagon staining (red) in all panels shows the extent of the islet. Scale bar, 50  $\mu$ m (applies to all panels).

(B) The patterns of vAChT immunostaining (green) are completely different in mouse and human islets. Axonal varicosities fill the core of the mouse islet, whereas axons are absent in human islets. Notice that many cells are labeled in human islets. Quantification of axons (right) shows that the innervation density is highest in the mouse islet (endo). A quantification of vAChT-labeled axons in human islets was not possible due to strong cellular staining (ND, not determined).

(C) TH immunostaining (green) is very different in mouse and human islets. TH-labeled fibers are localized to the mantle of the mouse islet. Large TH-labeled axons penetrate the human islet where they localize to discrete regions in the core of the islet. Notice that some mouse endocrine cells are also TH-immunostained. Quantification of the innervation densities (right) shows no differences between pancreatic regions and species.

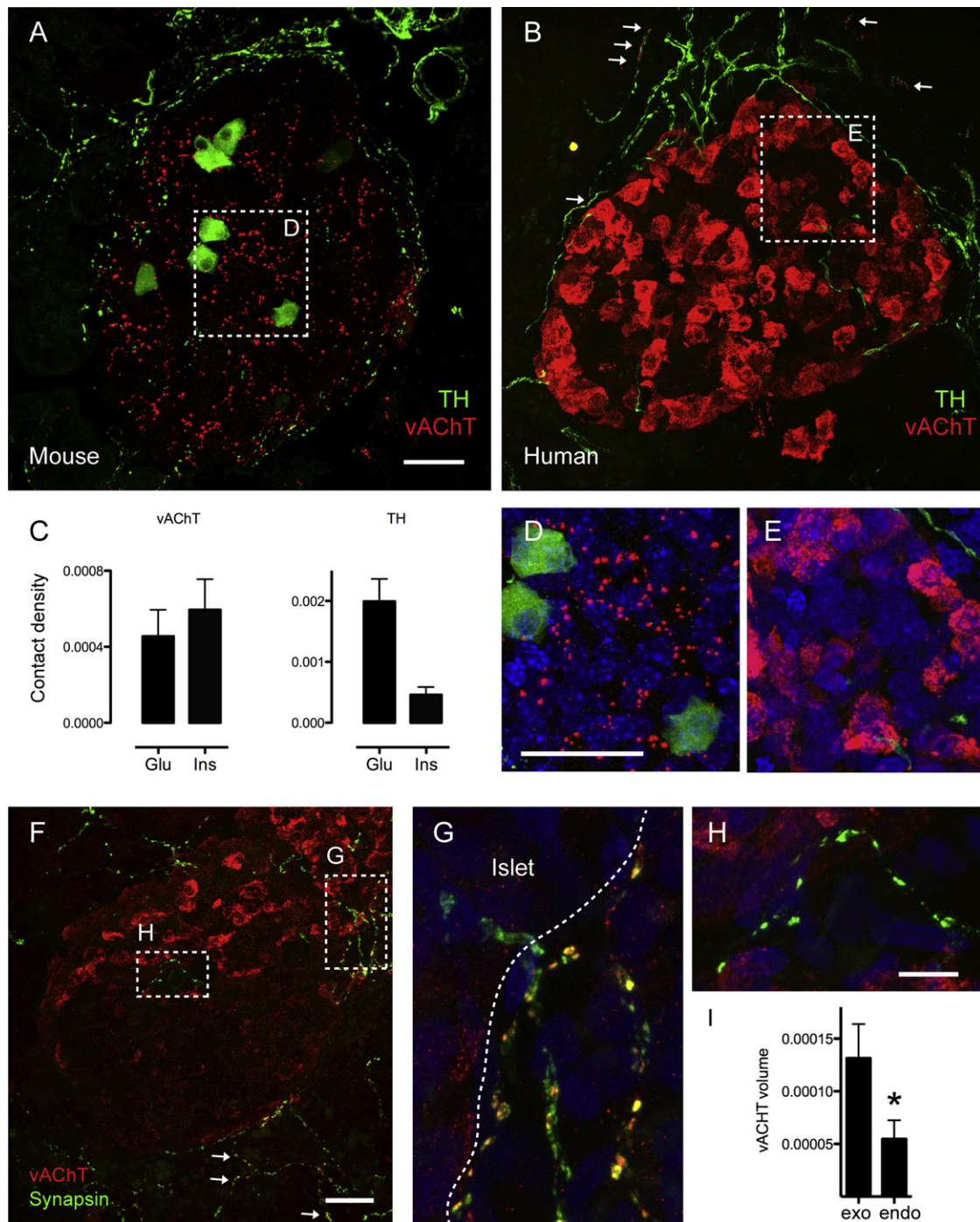
To distinguish axonal vAChT labeling from cellular vAChT labeling, we costained human pancreas tissues for vAChT and the axonal markers synapsin or acetylated alpha tubulin (Figures 4H and 4I; [Figure S3](#)). Because all vAChT-labeled varicosities were also labeled for synapsin in exocrine regions, double-stained varicosities in the human islet likely represented bona fide cholinergic axon terminals. By using this approach we found cholinergic axons within the human islet ([Figure S3](#)), but their density was lower than in the exocrine tissue ([Figure 4I](#)). Unlike cholinergic axons in mouse islets, those in the human endocrine parenchyma were sparse and appeared localized to discrete regions ([Figure 4](#); [Figure S3](#)).

To label the sympathetic component of the autonomic innervations, we immunostained pancreatic sections for a common sympathetic marker, tyrosine hydroxylase (TH). The distribution in mouse islets of TH-labeled axons did not overlap with that of vAChT-labeled axons ([Figure 4A](#)). That is, the periphery, but not the core, of the islets was densely innervated with TH-labeled axons (Figures 3C and 4A; [Movie S3](#)). TH was also present in a small subset of endocrine cells of the mouse islet ([Figure 4A](#)). By contrast, TH-labeled axons in human islets were present in the core of the islet, where they were localized to discrete areas (Figures 3C and 5; [Movie S4](#)). Immunostaining for the vesicular

monoamine transporter 2 (vMAT2), another marker of sympathetic axons, produced comparable results ([Figure S4](#)). Because the fraction of TH staining within synapsin-labeled axons was higher than that of vAChT staining ( $32\% \pm 5\%$  versus  $14\% \pm 3\%$ ), it is likely that sympathetic axons predominate in human islets.

### Sympathetic Axons Barely Innervate Endocrine Cells in Human Islets

What are the targets of the sympathetic innervation in human islets? We first examined if sympathetic axons or terminals contacted endocrine cells. TH-labeled axons were inspected for their proximity to insulin, glucagon, or somatostatin-labeled endocrine cells ([Figure 5](#)). In the autonomic nervous system, effector cells are considered innervated if they form close relationships with axonal varicosities ([Burnstock, 2009](#)). We quantified these contacts in Z stacks (40  $\mu$ m) of confocal images using Velocity software (see the [Experimental Procedures](#)), which automatically detected close proximity ("intersect") between the immunostaining for neuronal axons and that for endocrine cells. Using this approach we found that in mouse islets most TH-labeled axons contacted glucagon-labeled alpha cells or somatostatin-labeled delta cells (Figures 5B and 5C). Few axons



**Figure 4. Cholinergic Innervation of Mouse and Human Islets**

(A and B) Maximal projection of confocal images of a mouse (A) and a human pancreatic section (B) immunostained for the cholinergic marker vAChT (red) and the catecholaminergic marker TH (green). Notice the complementary staining patterns in the mouse islet. Arrows in (B) point at vAChT-stained axons in the human exocrine tissue. Scale bar, 20  $\mu$ m (applies to A and B).

(C) Quantification of contacts between vAChT-labeled axons and endocrine cells in mouse islets. Cholinergic axons targeted glucagon-labeled alpha (Glu) and insulin-labeled beta cells (Ins) alike, whereas catecholaminergic axons preferentially contacted alpha cells. Contacts were automatically detected in three dimensions by Volocity software as “intersects” and were expressed as the ratio of the intersect volume to the volume of the respective endocrine cell immunostaining (contact density; see the [Experimental Procedures](#)). The ratio is equivalent to the innervation density of each cell population. Data are presented as mean  $\pm$  SEM.

(D and E) Higher magnification images of islet regions delimited by the boxes shown in (A) and (B). Whereas vAChT-labeled varicosities were dense in the mouse islet parenchyma (D), these could not be seen in regions devoid of cellular vAChT staining in the human islet (E). Scale bar, 20  $\mu$ m (applies to D and E).



contacted insulin-labeled beta cells. In human islets, endocrine cells were barely innervated (Figure 5E). Most of the TH-labeled fibers were present in regions devoid of endocrine cells (Figures 5A and 5D).

### Sympathetic Axons in Human Islets Primarily Innervate Contractile Vascular Cells

Because sympathetic axons were localized to regions lacking endocrine markers, we examined if these axons contacted another major cell population in the islet, namely vascular cells. The vascular bed in the mouse islet is mainly composed of endothelial cells forming capillary tubes, but the structure of the human islet vasculature has not been studied in detail. We therefore examined the islet vasculature for the presence of endothelial cells and smooth muscle cells by respectively labeling these cells with antibodies against platelet endothelial cell adhesion molecule (PECAM) and alpha smooth muscle actin (SMA). As widely reported, blood vessels in mouse islets were mainly composed of endothelial cells, with one or two feeding arterioles containing contractile smooth muscle cells (Figures 6A and 6B). The vasculature in human islets was different. The content of smooth muscle cells was much higher in human islets than that in mouse islets (Figure 6C), and most of the blood vessels contained endothelial cells and smooth muscle cells (Figure 6D). Some of the SMA-labeled cells were also immunoreactive for the pericyte marker NG-2 (data not shown), suggesting that there are at least two populations of contractile cells, namely one (SMA-labeled only) that may be predominantly present in larger vessels (arterioles) and one (NG-2 labeled) that may be associated with capillaries. Thus, in human islets the vasculature has a larger proportion of contractile elements, which are putative targets for sympathetic innervation.

We examined if sympathetic axons contacted vascular cells and found that most TH-labeled axons in human islets traveled along blood vessels, ran parallel to PECAM-labeled endothelial cells, and contacted cells stained for SMA (Figure 7A; Figure S5; Movie S5 and Movie S6). Quantification of these results showed that SMA-labeled smooth muscle cells inside and encircling the islet were strongly innervated by sympathetic axons (Figure 7B). Like endocrine cells, PECAM-labeled endothelial cells were scarcely contacted by TH-labeled axons. Thus, contractile cells of blood vessels in the human islet were the major targets of sympathetic innervation. The feeding arterioles in mouse islets were also strongly innervated by TH-labeled axons (Figures 6A and 6B).

## DISCUSSION

Our results demonstrate that human islets are sparsely innervated by the autonomic nervous system. Most of the neuronal axons penetrating the human islet likely are sympathetic and contact contractile cells of blood vessels within the islet. Endocrine cells are sparsely innervated; that is, they usually lack the

close associations with axons and axonal varicosities that define autonomic effector junctions (Burnstock, 2009). Whereas it is generally assumed that the autonomic nervous system strongly affects islet hormone secretion, our findings suggest that there is no anatomical correlate for a direct neuronal influence on endocrine cells in the human islet. Instead, autonomic effects may be mediated indirectly by changing the vascular tone inside the islet and altering local blood flow or by neurotransmitters spilling over into the islet microcirculation and affecting downstream hormone secretion.

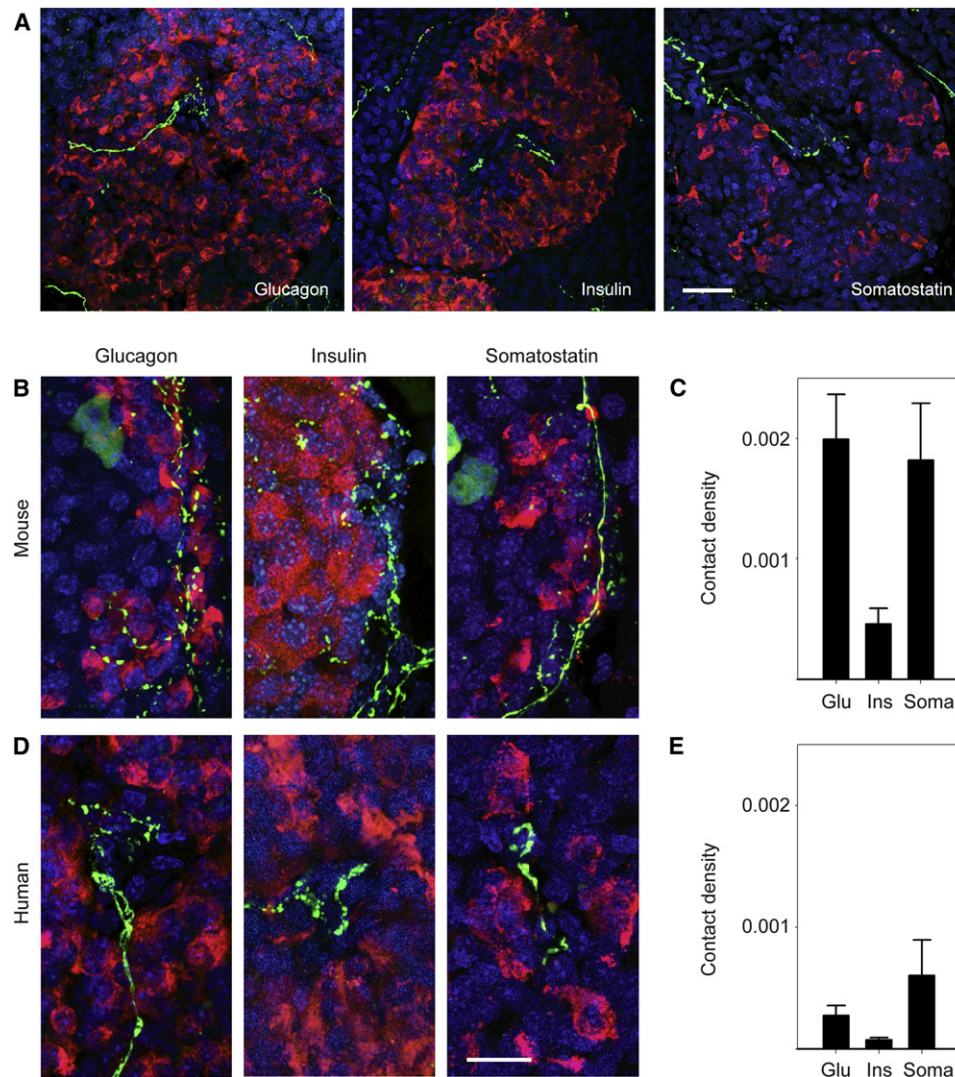
Previous studies reported the presence of neuronal axons expressing acetylcholinesterase or diverse neuropeptides such as galanin or vasoactive intestinal peptide in human islets (Ahrén et al., 1991; Amenta et al., 1983; Bishop et al., 1980; Ding et al., 1997; Shimosegawa et al., 1993). Based on these papers, however, it is difficult to obtain a cohesive picture of human islet innervation. Different markers were used in different studies, it was unclear which regions of the islet are innervated, and there was no attempt at identifying cell targets. Human islets have not been examined using markers for acetylcholine or noradrenaline, the classical neurotransmitters of the parasympathetic and sympathetic systems. Acetylcholinesterase is not considered a reliable marker for cholinergic cells (Mesulam et al., 1984). By conducting a comprehensive survey with numerous markers, using confocal microscopy and multiple immunofluorescence on thick pancreatic sections, and quantifying axonal densities and axonal contacts with target islet cells, we have now defined the innervation patterns in human and mouse islets in a way that will likely revise models about the anatomical innervation of the pancreatic islet.

A major finding of our study is that the innervation of the human islet is qualitatively different from that of the mouse islet. We established that in the mouse islet sympathetic axons primarily innervate alpha cells, whereas both beta and alpha cells receive parasympathetic input. By contrast, the human islet innervation is sparse and most human endocrine cells lack conventional autonomic innervation. Cholinergic innervation likely is minor in human islets, and the sympathetic axons penetrating the islet contact smooth muscle cells of blood vessels. Our results are in line with the notion that there are considerable species variations in the mechanisms of autonomic neurotransmission (Lundberg, 1996), and they reiterate that results from rodent studies should be extrapolated with caution to the human situation (see also Bosco et al., 2010; Brissova et al., 2005; Cabrera et al., 2006; Fiaschi-Taesch et al., 2010). Importantly, the autonomic nervous system may use different mechanisms to regulate islet function and glucose metabolism in different species.

Although we cannot completely rule out cholinergic innervation of endocrine cells because the strong cellular staining may mask cholinergic axons, our results suggest that direct cholinergic innervation of endocrine cells in the human islet is sparse. Axons containing vasoactive intestinal peptide, however, have been shown to innervate human islets (Bishop et al., 1980;

(F–H) Double labeling of vAChT (red) with synapsin (green) revealed the presence of cholinergic axons in the human pancreas. Arrows point at vAChT-stained axons in the exocrine tissue. All vAChT-labeled axons were also stained for synapsin (colabeling appears yellow in F and inset G). Most synapsin-labeled axons inside the islet did not contain vAChT (H). Scale bars, 20  $\mu$ m (F) and 5  $\mu$ m (in H, also applies to G).

(I) Quantification of cholinergic axons (vAChT and synapsin double-labeled axons) shows that their density is lower in the islet than in the exocrine tissue of the human pancreas ( $p < 0.01$ ; Student's *t* test). Data are presented as mean  $\pm$  SEM.



**Figure 5. Endocrine Cells in the Mouse Islet Are More Densely Innervated by Sympathetic, TH-Labeled Axons Than Endocrine Cells in the Human Islet**

(A) Human pancreatic sections immunostained for TH (green) and glucagon (red, left), insulin (red, middle), or somatostatin (red, right). Notice that TH-labeled axons traverse the islet to reach regions devoid of endocrine cells. Scale bar, 50  $\mu$ m.

(B) Three maximal projections of confocal images showing immunostaining for TH (green) and glucagon (red, left), insulin (red, middle), or somatostatin (red, right) in mouse islets. TH-labeled axons localize to regions containing glucagon or somatostatin-labeled cells but avoid regions containing insulin-labeled cells. Scale bar, 10  $\mu$ m (applies to all panels).

(C) Quantification of contacts between TH-labeled axons and endocrine cells shows that sympathetic axons preferentially target glucagon-labeled alpha (glu) and somatostatin-labeled delta cells (soma) in mouse islets. Contacts were automatically detected in three dimensions by Volocity software as “intersects” and were expressed as the ratio of the intersect volume to the volume of the respective endocrine cell immunostaining (contact density; see the [Experimental Procedures](#)). The ratio is equivalent to the innervation density of each cell population. Ins, insulin. Data are presented as mean  $\pm$  SEM.

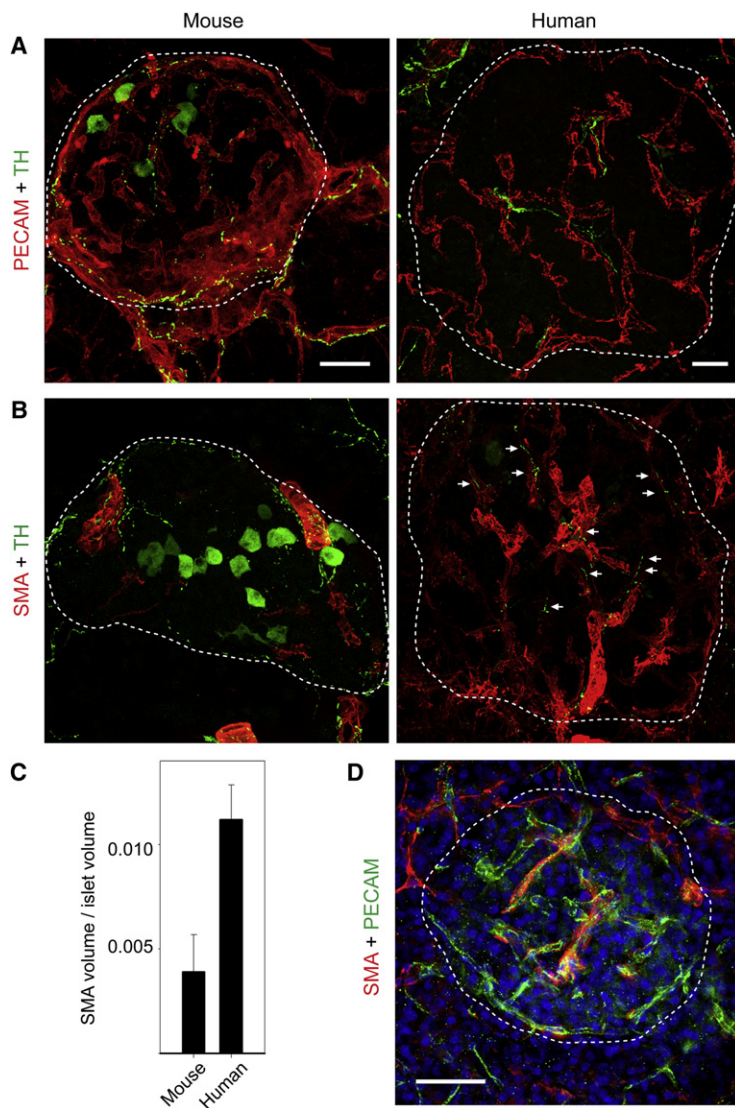
(D) Three maximal projections of confocal images showing immunostaining for TH (green) and glucagon (red, left), insulin (red, middle), or somatostatin (red, right) in human islets. TH-labeled axonal varicosities are present in regions devoid of endocrine cell immunostaining.

(E) Quantification of contacts between TH-labeled axons and endocrine cells in human islets shows that, compared to mouse endocrine cells (B), human endocrine cells are sparsely innervated. Data are presented as mean  $\pm$  SEM.

Fahrenkrug et al., 1987). Because this neuropeptide is a putative cotransmitter in parasympathetic nerves, human islets may receive noncholinergic parasympathetic input, as described for other organs (Mazzone and Canning, 2002). Indeed, studies show that the classical cholinergic nerve fibers cannot explain all of the vagal effects in the endocrine pancreas (Åhrén et al., 1986; Havel et al., 1997; Holst et al., 1981). Nonetheless, attrib-

uting these axons to the parasympathetic system is difficult and would require axonal tracing studies to rule out the possibility that they represent intrinsic axons or other axons from the enteric system (Kirchgessner and Gershon, 1990; Prinz et al., 1983). An arrangement in which neuropeptides are the sole transmitters of the parasympathetic system would have physiological implications because peptide neurotransmission is slow and easily





**Figure 6. Blood Vessels in Human Islets Show a Large Proportion of Smooth Muscle Cells**

(A) Two maximal projections of confocal images showing immunostaining for TH (green) and the endothelial cell marker PECAM (red) in mouse (left) and human islets (right). In islets from both species, most TH-labeled axons run parallel to the trajectories of PECAM-labeled blood vessels. Islets are outlined with dashed line. Scale bars, 50  $\mu$ m (left also applies to B, left; right also applies to B, right).

(B) Two maximal projections of confocal images showing immunostaining for TH (green) and the smooth muscle cell marker alpha smooth muscle actin (SMA, red) in mouse (left) and human islets (right). Notice that blood vessels in the human islet contain many more SMA-labeled structures. TH-labeled fibers in human islets (arrows) seem to be exclusively associated with SMA-labeled regions.

(C) Quantification shows a higher density of SMA-labeled smooth muscle cells in human islets ( $p < 0.05$ ; Student's *t* test). Data are expressed as the ratio of detected SMA-labeled volume to the examined islet volume (see the [Experimental Procedures](#)). Data are presented as mean  $\pm$  SEM.

(D) Maximal projection of confocal images of a human pancreatic section immunostained for alpha SMA (red) and PECAM (green). Notice that most vessels in the islet (dashed line) contain smooth alpha SMA-labeled vascular myocytes. Scale bar, 50  $\mu$ m.

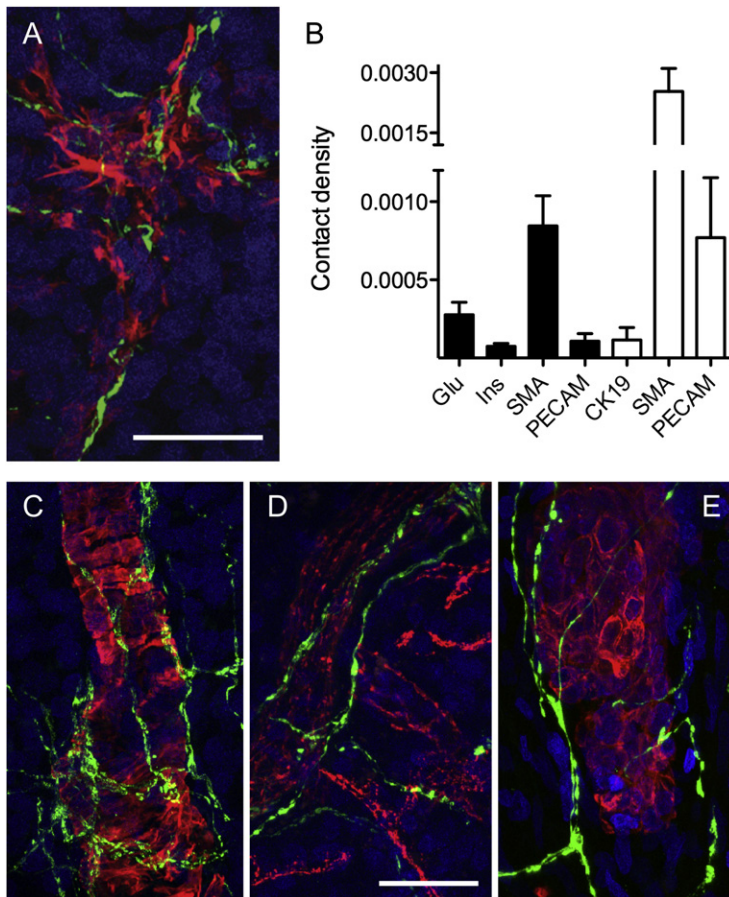
exhausted. The slow kinetics of a noncholinergic parasympathetic innervation may render this system less effective during vegetative responses to food ingestion. Thus, a direct neural influence on insulin secretion during feeding may be relatively small in humans (Ahrén and Holst, 2001; Teff et al., 1991). This interpretation of our results is supported by studies showing that patients who have undergone pancreas transplantation (and thus have denervated islets) remain euglycemic without therapy (Blackman et al., 1992; Diem et al., 1990; Madsbad et al., 1994; Pozza et al., 1985), although losses in fine-tuning of islet function may have escaped scrutiny.

Our findings further indicate that the innervation pattern of sympathetic axons in human islets could not have been predicted from rodent studies. Sympathetic axons innervate smooth muscle cells of blood vessels located around and deep within the human islet. This arrangement is unique. Smooth muscle cells in mouse islets are associated with one or two feeding arterioles, but we found that these contractile cells are

present throughout the vascular network in human islets. Because these cells are strongly innervated by sympathetic axons and likely contract in response to noradrenaline, the sympathetic input may modulate local blood flow. Sympathetic inhibition of insulin secretion in humans (Gilliam et al., 2007) may thus be mediated by noradrenaline-induced vasoconstriction and reduced perfusion of the islet, thereby diminishing insulin release into the circulation. Consistent with how secretion is regulated by sympathetic nerves in exocrine glands (Holst et al., 1989; Love et al., 2007; Proctor and Carpenter, 2007), sympathetic innervation may modulate insulin secretion not directly by acting on secretory cells but indirectly by controlling blood flow (Atef et al., 1992, 1996; Jansson and Hellerström, 1986). Nevertheless, it is also likely that in analogy to

the neuroendocrine route in the neurohypophysis, noradrenaline released from sympathetic nerves spills over into the arterial blood that perfuses the islet and activates adrenergic receptors on islet endocrine cells influencing their secretion.

How can our results be reconciled with previous physiological results? The consensus is that the autonomic nervous system affects meal-related insulin secretion and islet responses to stress (Ahrén et al., 1986; Havel and Taborsky, 1989). Most current physiological techniques, however, cannot distinguish the direct effects of autonomic axon terminals in the islet from the confounding effects of the autonomic nervous system elsewhere such as incretin secretion or activation of the adrenal medulla. Selective stimulation of islet innervation is difficult (Ahrén et al., 1987; Bloom and Edwards, 1984). Furthermore, exogenous application of agonist and antagonists can influence multiple organs and tissues, and as a result, the effects are the sum of many activities. Therefore, the local mechanisms by which the autonomic nervous system affects islet function



**Figure 7. Sympathetic Axons Preferentially Innervate Smooth Muscle Cells in Human Islets**

(A) TH-labeled axons (green) are localized to regions containing SMA-labeled smooth muscle cells (red) in human islets. Scale bar, 50  $\mu$ m.

(B) Quantification of cell contacts shows that TH-labeled axons preferentially contact smooth muscle cells inside the islet (SMA; filled bars) as well as in the exocrine tissue (empty bars). Few contacts with endocrine cells (Glu and Ins) and ductal cells (CK 19) could be detected. Contacts were expressed as the ratio of the “intersect” volume over the volume of the respective cell immunostaining (contact density; see the [Experimental Procedures](#)). The ratio is equivalent to the innervation density of each cell population. Data are presented as mean  $\pm$  SEM.

(C–E) TH-labeled axons and varicosities were seen contouring blood vessels in the exocrine tissue, where they are close to SMA-labeled smooth muscle cells (C) and endothelial cells (D). Th-labeled axons can be also seen in the vicinity of CK 19-labeled ductal cells (E).

donors ( $n = 2$ ), but most of the other samples (11 out of 13) showed similar preservation of tissue structure and antigenicity. Most human tissue samples were as well-preserved as mouse tissue samples, as shown by the similar overall immunostaining patterns.

#### Immunohistochemistry

Blocks of human or mouse pancreas (0.5  $\text{cm}^3$ ) were fixed in 4% paraformaldehyde for 4 hr, cryoprotected in sucrose, and cut on a cryostat (40  $\mu$ m). After a rinse with PBS-Triton X-100 (0.3%), sections were incubated in blocking solution (PBS-Triton X-100 and Universal Blocker Reagent; Biogenex, San Ramon, CA). Thereafter, sections were incubated 24 hr (20°C) with primary antibodies diluted in blocking solution. To visualize the general innervation of the islet, we used antibodies against synapsin I/II (Synaptic Systems, Goettingen, Germany) or PGP 9.5 (Millipore, Billerica, MA). To label parasympathetic axons, we used antibodies against vAChT (Synaptic Systems) and choline acetyltransferase (ChAT; Millipore); to label sympathetic axons, we used antibodies against TH (Millipore) and vesicular monoamine transporter (vMAT; Synaptic Systems). In addition, we immunostained beta cells (insulin; Accurate Chemical & Scientific, Wesbury, NY), alpha cells (glucagon; Sigma, St. Louis, MO), delta cells (somatostatin; Serotec), endothelial cells (PECAM; BD Biosciences, San Jose, CA), and vascular myocytes (alpha smooth muscle actin [SMA; Sigma] or cell surface chondroitin sulfate proteoglycan neuron-glial 2 [NG-2; Millipore]).

Immunostaining was visualized by using Alexa Fluor conjugated secondary antibodies (1:500 in PBS; 12 hr at 20°C; Invitrogen, Carlsbad, CA). Cell nuclei were stained with DAPI. Slides were mounted with ProLong Anti Fade (Invitrogen). In control experiments, primary antibodies were incubated with corresponding control peptides (50  $\mu$ g antigenic peptide to 1  $\mu$ g antibody for 5 hr). If peptide preadsorption was not possible (e.g., control peptides were not available for all antibodies used), we examined if the immunostaining for cell markers was tissue specific or cell specific, that is, if it corresponded to well-known staining patterns (e.g., TH-labeled axons around SMA-labeled blood vessels). The axonal and vascular staining patterns were very similar in the exocrine tissue in human and mouse pancreas, ruling out problems with antibody penetration, tissue preservation, and species cross-reactivity of the antibodies. Furthermore, we performed experiments with mouse tissues to mimic conditions of human pancreas handling. No differences were observed in immunostaining when mouse pancreata were left 6 hr in PBS before fixation.

To rule out false negative results in the human tissue, our approach consisted of (1) using several antibodies for each axonal marker, (2) examining more than ten human pancreata for each antibody, (3) providing positive control staining in regions adjacent to the islet, and (4) mimicking human tissue handling conditions in mouse pancreas tissues. Results were only presented in

remain unknown. We found that most sympathetic axons are associated with blood vessels, and thus release of neurotransmitter into the local circulation may be one mechanism. Because contractile cells within blood vessels are innervated, it is also possible that sympathetic axons control blood flow within the human islet, thereby modulating hormone secretion. Indeed, manipulating adrenergic signaling has strong effects on islet blood flow and insulin secretion in rats ([Atef et al., 1996](#); [Jansson et al., 1989](#); [Pettersson et al., 2009](#)). The presence of many contractile elements in the vasculature of the human islet likely makes this mechanism highly efficient, and its derangement may contribute to the natural history of type 2 diabetes ([Pettersson et al., 2009](#)).

#### EXPERIMENTAL PROCEDURES

Human pancreata were obtained from multiorgan donors ( $n = 15$ , ages 25–63). Mice (C57Bl/6;  $n = 6$ ; 6 weeks old; three male and three female) were killed by overexposure to a rising concentration of  $\text{CO}_2$ , followed by cervical dislocation. All experimental protocols were approved by the University of Miami Care and Use Committee. We obtained tissue samples of human pancreata procured locally for islet transplantation from heart-beating organ donors (donation after cerebral death). These samples had ischemic times of <12 hr. In addition, we obtained pancreatic tissue samples from biopsies performed in living donors. These samples were fixed in 4% PFA with no delay. All pancreas samples were trimmed to a volume smaller than  $1 \times 1 \times 0.5$  cm to ensure quick fixation. We obtained best results with biopsies from living



the manuscript if the labeling pattern was consistent for most human pancreata inspected, if the overall staining pattern in pancreatic tissues (e.g., arteries, acini, nerves) was similar in human and mouse pancreata, and if the staining in mouse pancreas withstood the conditions under which human pancreata were processed (Table S1).

### Confocal Imaging

Confocal images (pinhole = airy 1) of randomly selected islets (three islets per section, minimum three sections per mouse or human) were acquired on a confocal laser scanning microscope (Leica SP5) with a 63 $\times$  objective at 1024  $\times$  1024 pixel resolution. The nerve fiber plexus was reconstructed in 3D stacks of images (step size = 0.7  $\mu$ m) and analyzed using Leica or Velocity software. Using confocal images, we established whether an individual axon contacted a particular cell by using these criteria: (1) the contacted cell should have clear cytoplasmic staining and the nucleus should be visible; (2) fibers should be thin, elongated, and strongly stained; and (3) the nerve fiber must contact the cytoplasm of a cell (as defined in 1) in three-dimensional planes. The proportion of contacted cells was expressed as a percentage of the total number of cells of the same type. In addition, we used an automated method to quantify these contacts (see below).

### Automated Quantification of Innervation Density and Nerve Contacts

Axon immunostaining was analyzed and quantified in Z stacks (40  $\mu$ m) of confocal images using Velocity software. We developed a macro in Velocity that automatically detected all the immunostained elements (in voxels) within a predefined volume (e.g., islet). The volume of immunostained elements is then expressed as a fraction of the examined islet volume. Using this macro, we calculated the fraction (i.e., density) of immunostained axons in mouse and human islets (Figure S6, Movie S7). We further used Velocity to quantify contacts between axons and islet cells. We developed a macro that automatically detects the "intersect" (i.e., proximity) between the immunostaining for axons and that for islet cells. The incidence of these nerve fiber-cell contacts was expressed as the fraction of the total volume of detected intersects/total volume of cell immunostaining, which gave us the innervation density (i.e., contact density) of a particular cell type. We obtained similar results using conventional methods (see above) and this automated method (Figure S6). The automated method, however, is more objective and less time-consuming. The figures show data obtained with this method. All analyses were performed blindly and independently by two investigators (I.G. and A.L.F.).

The overlap of vAChT immunostaining with synapsin immunostaining was quantified using the "intersect" macro described above. This allowed us to determine the volume of axonal vAChT staining as opposed to cellular vAChT staining. The volume of axonal vAChT staining was then expressed as the fraction of the examined tissue volume. We further quantified the fractions of synapsin-labeled axons that were also labeled for vAChT or TH (volume of detected double staining/volume of synapsin immunostaining).

### SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and seven movies and can be found with this article online at doi:10.1016/j.cmet.2011.05.008.

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### REFERENCES

- Ahrén, B. (2000). Autonomic regulation of islet hormone secretion—implications for health and disease. *Diabetologia* 43, 393–410.
- Ahrén, B., and Holst, J. (2001). The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia. *Diabetes* 50, 1030–1038.
- Ahrén, B., Taborsky, G.J., and Porte, D.J. (1986). Neuropeptidergic versus cholinergic and adrenergic regulation of islet hormone secretion. *Diabetologia* 29, 827–836.
- Ahrén, B., Veith, R., and Taborsky, G.J. (1987). Sympathetic nerve stimulation versus pancreatic norepinephrine infusion in the dog: 1). Effects on basal release of insulin and glucagon. *Endocrinology* 121, 323–331.
- Ahrén, B., Ar'Rajab, A., Böttcher, G., Sundler, F., and Dunning, B. (1991). Presence of galanin in human pancreatic nerves and inhibition of insulin secretion from isolated human islets. *Cell Tissue Res.* 264, 263–267.
- Amenta, F., Cavallotti, C., de Rossi, M., Tonelli, F., and Vatrella, F. (1983). The cholinergic innervation of human pancreatic islets. *Acta Histochem.* 73, 273–278.
- Atef, N., Ktorza, A., Picon, L., and Pénicaud, L. (1992). Increased islet blood flow in obese rats: role of the autonomic nervous system. *Am. J. Physiol.* 262, E736–E740.
- Atef, N., Lafontan, M., Doublé, A., Hély, C., Ktorza, A., and Pénicaud, L. (1996). A specific beta 3-adrenoceptor agonist induces increased pancreatic islet blood flow and insulin secretion in rats. *Eur. J. Pharmacol.* 298, 287–292.
- Bernard, C. (1855). *Leçons De Physiologie Expérimentale Appliquée À La Médecine* (Paris: Baillière).
- Bishop, A.E., Polak, J.M., Green, I.C., Bryant, M.G., and Bloom, S.R. (1980). The location of VIP in the pancreas of man and rat. *Diabetologia* 18, 73–78.
- Blackman, J., Polonsky, K., Jaspan, J., Sturis, J., Van Cauter, E., and Thistlethwaite, J. (1992). Insulin secretory profiles and C-peptide clearance kinetics at 6 months and 2 years after kidney-pancreas transplantation. *Diabetes* 41, 1346–1354.
- Bloom, S.R., and Edwards, A.V. (1984). Characteristics of the neuroendocrine responses to stimulation of the splanchnic nerves in bursts in the conscious calf. *J. Physiol.* 346, 533–545.
- Bosco, D., Armanet, M., Morel, P., Niclauss, N., Sgroi, A., Muller, Y., Giovannoni, L., Parnaud, G., and Berney, T. (2010). Unique arrangement of alpha- and beta-cells in human islets of Langerhans. *Diabetes* 59, 1202–1210.
- Brissova, M., Fowler, M., Nicholson, W., Chu, A., Hirshberg, B., Harlan, D., and Powers, A. (2005). Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. *J. Histochem. Cytochem.* 53, 1087–1097.
- Brunicardi, F., Shavelle, D., and Andersen, D. (1995). Neural regulation of the endocrine pancreas. *Int. J. Pancreatol.* 18, 177–195.
- Burnstock, G. (2009). Autonomic neurotransmission: 60 years since Sir Henry Dale. *Annu. Rev. Pharmacol. Toxicol.* 49, 1–30.
- Cabrera, O., Berman, D., Kenyon, N., Ricordi, C., Berggren, P., and Caicedo, A. (2006). The unique cytoarchitecture of human pancreatic islets has implications for islet cell function. *Proc. Natl. Acad. Sci. USA* 103, 2334–2339.
- De Camilli, P., Benfenati, F., Valtorta, F., and Greengard, P. (1990). The synapsins. *Annu. Rev. Cell Biol.* 6, 433–460.
- Diem, P., Redmon, J., Abid, M., Moran, A., Sutherland, D., Halter, J., and Robertson, R. (1990). Glucagon, catecholamine and pancreatic polypeptide secretion in type I diabetic recipients of pancreas allografts. *J. Clin. Invest.* 86, 2008–2013.

- Ding, W.G., Kimura, H., Fujimura, M., and Fujimiya, M. (1997). Neuropeptide Y and peptide YY immunoreactivities in the pancreas of various vertebrates. *Peptides* 18, 1523–1529.
- Fahrenkrug, J., Pedersen, J.H., Yamashita, Y., Ottesen, B., Hökfelt, T., and Lundberg, J.M. (1987). Occurrence of VIP and peptide HM in human pancreas and their influence on pancreatic endocrine secretion in man. *Regul. Pept.* 18, 51–61.
- Fiaschi-Taesch, N.M., Salim, F., Kleinberger, J., Troxell, R., Cozar-Castellano, I., Selk, K., Cherok, E., Takane, K.K., Scott, D.K., and Stewart, A.F. (2010). Induction of human beta-cell proliferation and engraftment using a single G1/S regulatory molecule, cdk6. *Diabetes* 59, 1926–1936.
- Gilliam, L., Palmer, J., and Taborsky, G.J. (2007). Tyramine-mediated activation of sympathetic nerves inhibits insulin secretion in humans. *J. Clin. Endocrinol. Metab.* 92, 4035–4038.
- Gilon, P., and Henquin, J. (2001). Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. *Endocr. Rev.* 22, 565–604.
- Havel, P., and Taborsky, G.J. (1989). The contribution of the autonomic nervous system to changes of glucagon and insulin secretion during hypoglycemic stress. *Endocr. Rev.* 10, 332–350.
- Havel, P., Dunning, B., Verchere, C., Baskin, D., O'Dorisio, T., and Taborsky, G.J. (1997). Evidence that vasoactive intestinal polypeptide is a parasympathetic neurotransmitter in the endocrine pancreas in dogs. *Regul. Pept.* 71, 163–170.
- Holst, J.J., Grønholt, R., Schaffalitzky de Muckadell, O.B., and Fahrenkrug, J. (1981). Nervous control of pancreatic endocrine secretion in pigs. II. The effect of pharmacological blocking agents on the response to vagal stimulation. *Acta Physiol. Scand.* 111, 9–14.
- Holst, J.J., Orskov, C., Knuhtsen, S., Sheikh, S., and Nielsen, O.V. (1989). On the regulatory functions of neuropeptide Y (NPY) with respect to vascular resistance and exocrine and endocrine secretion in the pig pancreas. *Acta Physiol. Scand.* 136, 519–526.
- Jansson, L., and Hellerström, C. (1986). Glucose-induced changes in pancreatic islet blood flow mediated by central nervous system. *Am. J. Physiol.* 251, E644–E647.
- Jansson, L., Eizirik, D.L., and Sandler, S. (1989). Terbutaline decreases the blood flow of the pancreatic islets but does not reduce the diabetogenic action of streptozotocin in the rat. *Eur. J. Pharmacol.* 161, 79–83.
- Kirchgessner, A.L., and Gershon, M.D. (1990). Innervation of the pancreas by neurons in the gut. *J. Neurosci.* 10, 1626–1642.
- Langerhans, P., and Morrison, H. (1937). Contributions to the Microscopic Anatomy of the Pancreas (Baltimore: The Johns Hopkins Press).
- Love, J.A., Yi, E., and Smith, T.G. (2007). Autonomic pathways regulating pancreatic exocrine secretion. *Auton. Neurosci.* 133, 19–34.
- Lundberg, J.M. (1996). Pharmacology of cotransmission in the autonomic nervous system: integrative aspects on amines, neuropeptides, adenosine triphosphate, amino acids and nitric oxide. *Pharmacol. Rev.* 48, 113–178.
- Madsbad, S., Christiansen, E., Tibell, A., Tydén, G., Rasmussen, K., and Burchard, F. (1994). Beta-cell dysfunction following successful segmental pancreas transplantation. Danish-Swedish Study Group of Metabolic Effect of Pancreas Transplantation. *Transplant. Proc.* 26, 469–470.
- Mazzone, S.B., and Canning, B.J. (2002). Central nervous system control of the airways: pharmacological implications. *Curr. Opin. Pharmacol.* 2, 220–228.
- Mesulam, M.M., Mufson, E.J., Levey, A.I., and Wainer, B.H. (1984). Atlas of cholinergic neurons in the forebrain and upper brainstem of the macaque based on monoclonal choline acetyltransferase immunohistochemistry and acetylcholinesterase histochemistry. *Neuroscience* 12, 669–686.
- Pettersson, U.S., Henriksnäs, J., and Jansson, L. (2009). Reversal of high pancreatic islet and white adipose tissue blood flow in type 2 diabetic GK rats by administration of the beta3-adrenoceptor inhibitor SR-59230A. *Am. J. Physiol. Endocrinol. Metab.* 297, E490–E494.
- Pozza, G., Bosi, E., Secchi, A., Piatti, P., Touraine, J., Gelet, A., Pontiroli, A., Dubernard, J., and Traeger, J. (1985). Metabolic control of type I (insulin dependent) diabetes after pancreas transplantation. *Br. Med. J. (Clin. Res. Ed.)* 291, 510–513.
- Prinz, R.A., El Sabbagh, H., Adrian, T.E., Bloom, S.R., Gardner, I., Polak, J.M., Inokuchi, H., Bishop, A.E., and Welbourn, R.B. (1983). Neural regulation of pancreatic polypeptide release. *Surgery* 94, 1011–1018.
- Proctor, G.B., and Carpenter, G.H. (2007). Regulation of salivary gland function by autonomic nerves. *Auton. Neurosci.* 133, 3–18.
- Satin, L., and Kinard, T. (1998). Neurotransmitters and their receptors in the islets of Langerhans of the pancreas: what messages do acetylcholine, glutamate, and GABA transmit? *Endocrine* 8, 213–223.
- Shimosegawa, T., Asakura, T., Kashimura, J., Yoshida, K., Meguro, T., Koizumi, M., Mochizuki, T., Yanaiharu, N., and Toyota, T. (1993). Neurons containing gastrin releasing peptide-like immunoreactivity in the human pancreas. *Pancreas* 8, 403–412.
- Teff, K.L., Mattes, R.D., and Engelman, K. (1991). Cephalic phase insulin release in normal weight males: verification and reliability. *Am. J. Physiol.* 261, E430–E436.
- Woods, S., and Porte, D.J. (1974). Neural control of the endocrine pancreas. *Physiol. Rev.* 54, 596–619.