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**REGULATION OF HUMAN PANCREAS HORMONE
SECRETION BY AUTONOMIC INNERVATION**

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To my son Pablo de Jesus

ABSTRACT

Diabetes mellitus is a silent killer doing away with one person every 10 seconds. We speak of diabetes when the organism cannot control the right level of glucose in the blood. The hormones insulin and glucagon secreted by the islets of Langerhans are the major players maintaining glucose homeostasis. In the living organism, the function of the islets is orchestrated by their interaction with other organs through the vasculature and with the nervous system. Most of our current knowledge of islet biology has been obtained by using mouse models, but caution is needed, as mice are not simply small humans. Indeed, recent studies have revealed that the cell composition and architecture of the human islet are different from that of mouse islets. Thus, other important features such as nervous regulation of islet function may also be different.

The work in this thesis aimed to identify the role of innervation for islet function. Our hypothesis is that autonomic and paracrine signals are involved in islet function and that the relative role of these components varies among species. To identify the sympathetic and parasympathetic components of innervation as well as their cellular targets we used immunohistochemical staining of human and mouse pancreatic sections. In contrast to mouse, human islets are devoid of parasympathetic innervation. Instead, human alpha cells possess the machinery for exocytosis of acetylcholine, the major parasympathetic neurotransmitter. Our findings suggest that human islets depend less on neural cholinergic input than mouse islets. Alpha cells secrete acetylcholine as a paracrine signal priming the human beta cell to respond optimally to subsequent increases in glucose concentration. In addition, noradrenergic fibers contact few endocrine cells in the human islet and preferentially innervate smooth muscle cells of the islet vasculature. This suggests that sympathetic innervation regulates hormone secretion by controlling the blood flow rather than modulating endocrine cell function directly.

By taking advantage of our recently developed noninvasive anterior chamber of the eye imaging platform we were able to study the role of innervation in the maintenance of glucose homeostasis *in vivo*. We studied the process of reinnervation and revascularization of intraocular islet grafts and showed that islets orchestrate the process of engraftment to restore their original microenvironment. Islet grafts from two different mouse strains and human xenografts showed innervation patterns similar to those in pancreatic sections *in situ*. Islet grafts displayed the characteristic fenestrae of the pancreatic vascular endothelium independently of the origin of the new vessels. In addition, the model allowed controlling the fraction of the graft vasculature that is contributed by the donor islet endothelial cells to the point that the original donor vasculature of the islet is restored. Recording graft function while manipulating the eye's neural input through the pupillary light reflex revealed functional differences in parasympathetic innervation between the two mouse strains. The eye platform also allowed us to follow cell dynamics during immune responses, which will enable investigations aimed at clarifying the role of innervation in the pathogenesis of autoimmune diabetes.

To study human islet biology *in vivo* we further adopted the eye model by transplanting human islets into the eye of diabetic immune compromised mice. Human xenografts reversed diabetes and tightly controlled plasma glucose concentrations. Moreover, our results provided the first real time monitoring of revascularization and blood flow inside human islets and graft function could be modulated by local drug administration. Our findings establish a "humanized" mouse model to investigate human islet biology *in vivo* that will allow addressing how nervous input affects endocrine function or blood flow in human islets. The physiological relevance of the anterior chamber of the eye model is further underscored by the therapeutic potential as a novel transplantation site to treat type 1 diabetic patients.

LIST OF PUBLICATIONS

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- VIII. **Rayner Rodriguez-Diaz**, R. Damaris, Molano, Midhat H. Abdulreda, Camilo Ricordi, Antonello Pileggi, Alejandro Caicedo, Per-Olof Berggren. A new experimental platform to study human islet cell biology in vivo. *In manuscript*.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
AOTF	Acousto-Optic Tunable Filter
ATP	Adenosine Triphosphate
CGRP	Calcitonin Gene-Related Peptide
CCD	Charge Couple Devise
ChAT	Choline Acetyl Transferase
ChT	Choline Transporter
DAPI	4,6-diamidino-2-phenylindole
DIEC	Donor Islet Endothelial Cell
GFP	Green Fluorescent Protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IPGTT	Intraperitoneal Glucose Tolerance Test
NG-2	Chondroitin sulfate proteoglycan neuron-glial 2
NOD	Non-obese diabetic
NPY	Neuropeptide Y
PBS	Phosphate Buffer Solution
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PGP 9.5	Protein Gene Product 9.5
SMA	Smooth Muscle Actin
STED	Stimulated Emission Depletion
TH	Tyrosine Hydroxylase
TPLSM	Two-photon laser scanning microscopy
vAChT	Vesicular Acetylcholine Transporter
VEGF	Vascular endothelial growth factor
VIP	Vasoactive Intestinal Peptide
vMAT	Vesicular Monoamine Transporter

INTRODUCTION

A well functioning endocrine pancreas, the islets of Langerhans, is crucial for survival. The hormonal secretory activity of pancreatic islets efficiently maintains constant levels of plasma glucose. A failure to do so leads to potentially life-threatening fluctuations in plasma glucose levels. Dysfunction or death of the insulin-secreting beta cells is a major cause of *diabetes mellitus*, a devastating disease affecting millions worldwide. Understanding the biology of the islets of Langerhans is crucial for any type of therapy for *diabetes mellitus*. A great deal is known about the physiology of isolated rodent islets because several physiological parameters can be readily recorded. The signal transduction pathways that lead from glucose stimulation to insulin secretion in beta cells have been dissected out in great detail. Unfortunately, it is not well known how human islets behave, either in their native environment or after transplantation, a procedure intended to cure type 1 *diabetes mellitus*. It is possible that the biology of human pancreatic endocrine cells in vascularized and innervated islets differs from that observed in mice.

A close relationship between the nervous system and the regulation of plasma glucose was revealed early by Claude Bernard¹; Paul Langerhans then recognized a rich innervation of the islets since their discovery². Many studies have implicated the autonomic nervous system in metabolic control and islet function³⁻⁷. However, the importance of the autonomic innervation of the islets in the maintenance of glucose homeostasis remains controversial since it has been impossible to discern the effects autonomic fibers have specifically on islet cell physiology from those on many other organs. Because of these limitations, the relative role of innervation in islet hormone secretion has been a matter of a long-standing debate. There is strong evidence that autonomic activation plays a major role in several animal species, but it is unclear whether this is the case in humans. Without this knowledge, the model for the regulation of glucose homeostasis is incomplete.

Autonomic innervation in islets of Langerhans

Based on numerous studies, the endocrine pancreas is richly innervated by the autonomic nervous system³⁻⁷. Parasympathetic and sympathetic nerves travel to the islet through the neurovascular stalk. Within the islets, the nerves follow the blood vessels and terminate within the pericapillary space, within the capillary basement membrane, or closely apposed to the endocrine cells⁸. Autonomic axons innervating endocrine cells do not display structural features of classical synapses such as synaptic clefts or postsynaptic densities but rather have release sites near islet cells. It has been proposed that neurotransmitters are released into the interstitial space to affect a group of adjacent islet cells. The distribution and density of the different types of nerve fibers have been examined in several species. A dense sympathetic innervation, as identified by the presence of catecholamine-forming enzymes, has been reported in rodents, dogs, and cats⁹⁻¹². Studies based on the cholinesterase technique have revealed parasympathetic innervation in the cat, rat, and rabbit¹³⁻¹⁶. Fibers containing neuropeptides have also been reported, but it is unclear whether these represent unique fiber populations or whether these peptides are localized in the autonomic fibers¹¹. In

addition, there is a network of sensory fibers containing calcitonin gene-related peptide (CGRP) and substance P neuropeptides. Thus far, the innervation of human islets has been the subject of only a few studies¹⁷⁻¹⁹.

Several neurotransmitter receptors are expressed on the plasma membrane of islet cells. Activation of M3 muscarinic receptors stimulates insulin and glucagon secretion²⁰⁻²². Release of Ca^{2+} from intracellular stores in response to muscarinic receptor activation has an important role for the insulinotropic action, but the signaling underlying the enhanced glucagon secretion is still unknown. Activation of α_2 -adrenergic receptors inhibits glucose-induced insulin secretion via hyperpolarization of the beta cell. Activation of β_2 -adrenergic receptors stimulates insulin and glucagon secretion. Thus, the actions of a neurotransmitter vary according to the activated receptor type. To add to this complexity, neuropeptides, including VIP, NPY, and, in some species, galanin also have effects on islet cells^{5,23-27}.

The precise organization of islet innervation is still unknown. Only a few markers have been used, and because these markers have not been combined in immunofluorescence studies, it is unclear how the different fiber systems relate to each other. For instance, it is not known whether the patterns of the parasympathetic and sympathetic innervation are complementary or if they overlap in specific regions of the islet. There have been few attempts to identify peptides as co-transmitters in cholinergic and adrenergic fibers. Furthermore, relying on the cholinesterase technique may be misleading because esterases do not map exclusively to parasympathetic nerves. Other important prototypical cholinergic markers for the parasympathetic system (e.g. ChAT, vAChT) have not been examined. There are no systematic analyses on how and where the fibers terminate within the islets, and what particular cells they innervate. It is further noteworthy that our current view of islet innervation is mainly based on rodent studies, and that, given their unique cytoarchitecture, the situation in human islets may be very different^{28,29}.

The overall effect of parasympathetic stimulation is an increase in insulin secretion^{15,16,30-33}. Several studies support the view that acetylcholine is released to stimulate insulin secretion. Exogenous treatment with acetylcholine or other muscarinic agonists stimulates insulin secretion *in vivo*. This effect can be blocked with the muscarinic antagonist atropine^{15,16}. *In vivo* studies in dogs and baboons reported that stimulation of the vagus nerve increases insulin secretion³⁰⁻³² but this stimulation also increases the secretion of other islet hormones such as glucagon, somatostatin, and pancreatic polypeptide^{15,34,35}.

The net effect of sympathetic nerve stimulation seems to be a lowering in plasma insulin concentration. Exogenous treatment with catecholamines and electrical activation of sympathetic nerves inhibits insulin secretion *in vivo*^{34,36-38}. Alpha-adrenergic receptor blockade counteracts this inhibition of insulin secretion^{37,39}, suggesting noradrenaline as the mediator. However, several other direct and indirect mechanisms could contribute to the effects of noradrenaline on insulin secretion. Noradrenaline may activate β_2 -adrenergic receptors on beta cells and adrenergic receptors on alpha cells. This makes it difficult to interpret the results. Moreover, this effect cannot be attributed solely to the release of noradrenaline from nerve fibers

close to beta cells because other tissues innervated by the activated nerves are also stimulated. The sympathetic nervous system further exerts profound effects on the secretion of the other islet hormones. Splanchnic nerve stimulation and noradrenaline application increases glucagon secretion and decreases somatostatin secretion^{35,37,39-43}. To our knowledge, similar studies have not been performed in human beings.

In addition to the parasympathetic and sympathetic efferent fibers, a network of autonomic afferent sensory fibers also innervates the islets. These fibers leave the pancreas along the sympathetic fibers within the splanchnic nerves and transmit information about noxious stimuli. Fibers containing the sensory neuropeptides CGRP and substance P have been observed in the endocrine pancreas. Vanilloid receptors are localized in sensory fibers and generally report pain information. Whether these fibers are involved in the regulation of islet hormone secretion remains to be determined. Intriguingly, recent papers suggest that molecular defects in the pancreatic sensory innervation dramatically affect autoimmune diabetes in NOD mice^{44,45}. These studies raise the possibility that signals derived from the nervous system can alter inflammation and insulin resistance and thus indirectly affect the development of autoimmunity. It is unclear, however, to which extent human islets are innervated by sensory fibers. Finding out which receptors are expressed on these fibers is crucial to be able to propose that a similar mechanism provides a link between the nervous system and autoimmunity in the natural history of type 1 diabetes in human beings.

The importance of autonomic innervation of the islets for glucose homeostasis is controversial

Many physiological events under parasympathetic and sympathetic control can indirectly interfere with insulin or glucagon secretion. It has been difficult to discern the direct effects of autonomic terminals in the islet from the confounding effects of the autonomic nervous system elsewhere (e.g. incretin secretion and activation of the adrenal medulla). Selective stimulation of islet innervation is difficult. For instance, to achieve a specific activation of the pancreas, investigators have to use electrical activation of the mixed autonomic nerves along a pancreatic artery with a concomitant blockade of the joint preganglionic cholinergic nerves^{37,43}. Furthermore, if not applied locally, exogenous application of neurotransmitters can influence multiple organs and tissues and, as a result, the effects are the sum of a multitude of activities. An additional limitation is that islet cell responses to nerve stimulation *in vivo* can only be measured indirectly in the systemic circulation (e.g. hormone plasma levels). It is not possible to record receptor activation directly in the postsynaptic cells.

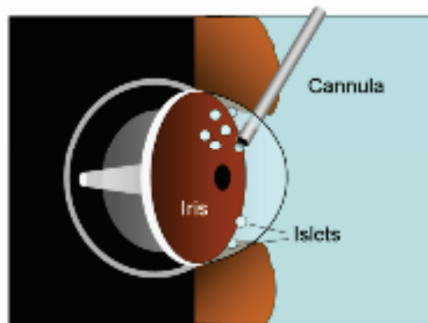
Although several studies suggest the involvement of islet innervation, the importance of the autonomic nervous system in regulating islet hormone secretion is unclear. The list of neurotransmitters that may modulate islet function is long and confusing because of species differences and uncertainty about the physiological relevance of the effects observed with *in vivo* models. In human subjects, for instance, decreased glucose tolerance after vagotomy has been reported^{46,47}, but patients who have undergone pancreas transplantation (and thus may have denervated islets) remain euglycemic without therapy⁴⁸⁻⁵⁰. For the same reason, there are doubts about whether the sympathetic nerve fibers exert a major influence on the basal and postprandial

insulin secretory responses. The contribution of autonomic signals to the glucagon secretory response *in vivo* is also unclear. Autonomic activation most likely plays a major role in dogs⁵¹ and in monkeys⁵², but combined adrenergic and muscarinic cholinergic blockade has no effect on the glucagon response to hypoglycemia in humans⁵³⁻⁵⁵. Furthermore, this glucagon response is not reduced in adrenalectomized, spinal-cord-sectioned, or vagotomized humans⁵⁶⁻⁵⁸.

The lack of experimental tools has prevented critical demonstrations of the effects of autonomic innervation^{5,23}. A successful new approach has been to generate mutant mice lacking a particular neurotransmitter receptor in a particular islet cell (e.g. M3 muscarinic receptor²¹). This strategy eliminates potential confounding factors caused by the widespread distribution of autonomic neurotransmitter receptors. This approach, however, is too cumbersome to allow a large-scale screening of the role of the many putative receptors on islet cells and it certainly cannot be used to examine the physiological role of islet cell receptors in humans.

An experimental platform to dissect out the effects of autonomic innervation on islet function *in vivo*

To dissociate the neural effects on islet function from hormonal and other confounding effects, we propose to use our new experimental platform in which islets are transplanted into the anterior chamber of the eye (from this point referred to as “the eye”) for functional monitoring^{59,60}. We expect *in vivo* imaging to show that islet cells behave in ways not predicted by *in vitro* experiments, in analogy to *in vivo* studies that



have revealed unique features of neurons embedded in their native environment^{61,62}. In addition, native islets are innervated and surrounded by a dense basement membrane. Unfortunately, imaging islets in the living organism is very challenging, and non- or minimally-invasive technologies to monitor islet cell function at the cellular level are needed.

Methods for *in vivo* imaging of pancreatic islet function are scarce. In an attempt to study beta cell function in a relatively intact environment, organotypic pancreatic slices containing islets were used for electrophysiological recordings⁶³, but in this preparation, islets are isolated from the organism. Islets have been imaged after transplantation under the kidney capsule, where it has been possible to visualize the revascularization process⁶⁴⁻⁶⁶. However, the instability and inaccessibility of this transplantation site makes this approach challenging, in particular for functional or longitudinal studies. Other emerging efforts to image beta cells *in vivo* include magnetic resonance imaging and position emission tomography⁶⁷. Such methods will likely improve quantifying transplanted islet mass but have low spatial resolution and do not allow functional monitoring of islets at the cellular level.

In the early 1870s, van Dooremaal made the extraordinary observation that tumor cells injected into the eye formed progressively growing tumors⁶⁸. Since then, the eye has been widely used as a transplantation site to study the biology of several tissues. Ovaries have been transplanted into the anterior eye chamber to study the physiology of ovulation^{69,70} and a wide variety of peripheral and central nervous tissues have been shown to proliferate and mature histologically after intraocular transplantation^{71,72}. This site has also been extensively used to study the survival and growth of pancreatic tissue grafts^{73,74}. Human xenografts survive in the eye of athymic nude rodents, where they become strongly and appropriately innervated^{72,75-77}.

The main advantage of the anterior chamber of the eye over other transplantation sites is that biological phenomena can be monitored repeatedly and noninvasively by *in vivo* microscopy because the cornea is transparent. The grafts might be easily vascularized and innervated owing to the rich blood and nerve supply of the iris that forms the bed of the anterior chamber of the eye. Noradrenergic and cholinergic nerve fibers in the iris control pupillary diameter. These fibers have been shown to invade the intraocular grafts and can be specifically activated by changing the illumination⁷¹ or by topical drug administration. Therefore, autonomic input on the grafted tissue can be modulated noninvasively and locally, providing an ideal tool for the study of islet function.

Reinnervation and revascularization of islet grafts

Ultimately, an important question to be addressed is whether intraocular islet grafts would restore their native morphology and physiology after transplantation in the eye. To pursue the use of intraocular grafts as experimental platform for islet functional studies we will need to demonstrate that islets recreate their native environment after transplantation. The intra- and inter-species differences with respect to islet architecture and functional regulation add more challenges to the model^{28,29,78}. Among the complex biological processes following islet engraftment, revascularization and reinnervation processes are best described. Pancreatic islet innervation and vasculature greatly differ among species⁷⁸⁻⁸⁰ and even strains (unpublished data). Such features need to be mimicked in any experimental platform to study islet function and, more specifically, to study the role of innervation *in vivo*, a main goal in this thesis.

Some studies have described the process of islet reinnervation after transplantation in different tissues⁸¹⁻⁸³, including the eye⁸⁴. Islet graft innervation patterns in different transplantation sites seem to differ only slightly from that of islets in the pancreas^{81,83}. The host tissues seem mainly to determine the reinnervation density of the islet grafts, while islets establish their own pattern of innervation after transplantation⁸². Considering the innervation density of the iris, reinnervated intraocular islet grafts might be the ideal tools to study the role of innervation in islet function *in vivo*. Intra-islet blood flow can be regulated by the nervous system⁸⁵⁻⁸⁸ and innervation and vascularization are likely connected. It has been shown that axons penetrate the graft in close association with the ingrowing blood vessels⁸¹⁻⁸³. Therefore, we cannot study islet reinnervation without concomitantly addressing vascularization processes.

Revascularization of islets of different species has been extensively studied in iso-, allo- and xeno-transplantation settings⁸⁹⁻⁹¹. Parameters including vasculature density, blood flow, capillary branching index and oxygen tension revealed differences in the dynamics and efficiency of the revascularization process depending on the transplantation sites⁹²⁻⁹⁷. The time to restore normoglycemia and glucose tolerance can serve as functional readouts of islet revascularization in transplanted mice^{66,95,98}. Several components are implicated in blood vessel formation including vascular endothelial growth factor (VEGF)^{94,95,99-101}. Islets secrete VEGF¹⁰², which can partially explain the rapid revascularization of transplanted islets. However, other cell types are also crucial for vessel formation. Recently, neutrophils have been implicated in the functional restoration of intranslet blood perfusion after transplantation¹⁰¹. It also has been shown that donor islet endothelial cells (DIEC) survive the transplantation procedure and form a functional part of the revascularized islet graft^{65,66,103}. Sustaining DIEC before and after transplantation may not only facilitate the islet revascularization but may also recreate the original microvasculature environment with the appropriate signals allowing a proper reinnervation process.

Central hypothesis

The collective evidence presented shows that there is a need to identify the innervation patterns and their functional roles in mouse and human islets. Four important observations have to be considered: 1) Autonomic activation plays a major role in glucagon secretion in animals^{52,104-107} but not in humans^{54-58,107-113}; 2) humans have a smaller cephalic-phase insulin response than animals^{7,114}; 3) patients subjected to pancreas transplantation remain euglycemic without therapy^{48-50,110}; and 4) acute insulin responses to intravenous glucose in patients with chronic high cervical cord transection are not altered¹¹⁵.

We therefore formulated the central hypothesis that mouse islets are heavily dependent on nervous input, whereas human islets regulate hormone secretion almost autonomously using paracrine and autocrine mechanisms.

However, we would like to emphasize that this hypothesis does not preclude the involvement of autonomic and intranslet (paracrine and autocrine) signals in islet function. Rather, it states that the relative role of these components varies with the species and that human islets may rely more on intranslet signaling. This view is in line with recent results showing a human islet cytoarchitecture that predisposes islet cells for paracrine signaling^{28,29} and new glutamate and ATP positive autocrine feedback loops in human alpha and beta cells^{116,117}.

AIMS

Identify species differences in the innervation patterns of mouse and human pancreatic islets that might explain the discrepancy in the role of the autonomic innervation in islet function for both species

Look for unidentified intraislet paracrine or autocrine signaling mechanisms supporting the functional independence of human islet of Langerhans from the autonomous nervous system

Establish that intraocular islet grafts orchestrate the engraftment process to restore their native morpho-physiological environment by studying the processes of reinnervation and revascularization

Investigate and manipulate the role of neural input on glucose homeostasis *in vivo* using mouse and human islet transplantation in the eye as a suitable experimental platform in a living organism

Explore the potential of the transplantation into the eye to answer “state of the art” questions or needs. In this thesis, we examine the potential of the model to study dynamic behaviour of immune cells to be able to ultimately explore the claimed pathophysiological link between sensory fibers and autoimmune diabetes. We further evaluate the eye as a site for therapeutic implantation of islets in a relevant preclinical animal model

MATERIAL AND METHODS

Tissue procurement for immunohistochemical studies

Human pancreata were obtained from multiorgan donors (n = 15, ages 18–63). Mice (C57BL/6, 129x1, NOD and Balb/c; n = 6 (per strain); 6 weeks old; three male and three female) were killed by exposure to a rising concentration of CO₂, 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 h. 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 x 1 x 0.5 cm to ensure quick fixation. We obtained best results with biopsies from living 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 cm³) were fixed in 4% paraformaldehyde for 4 h, cryoprotected in sucrose, and cut on a cryostat (40 µm). 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 h (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 acetyl transferase (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-glia 2 [NG-2; Millipore]). The antibodies used and their immunostaining patterns in the pancreas are summarized in Table 1. Immunostaining was visualized by using Alexa Fluor conjugated secondary antibodies (1:500 in PBS; 12 h 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 µg antigenic peptide to 1 µg antibody for 5 h). 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

Table S1. List of antibodies used and immunostaining patterns in the pancreas

Antibody to	Host	Company	Catalog No	Reactivity to Human			Reactivity to Mouse		
				varicosities	Axons	Others	shaft	Axons	Others
Synapsin I/II	Rabbit	SySy	106002	+++	+++	+++	+++	+++	Neuro-insular complex
	Mouse Mab	SySy	106011C5	+++	+++	+++	+++	+++	Neuro-insular complex
	Guinea Pig	SySy	106004	+	+		+	+	-
Tyroxine Hydroxylase	Rabbit	Chemicon	AB152	+++	+++	Beta cell subset*	+++	+++	Beta cell subset
	Sheep	Chemicon	AB1542	+++	+++	Beta cell subset*	+++	+++	Beta cell subset
	Mouse Mab	Sigma	T1299	-	-	-	-	-	-
	Chicken	Chemicon	AB9702	++	++	Beta cell subset*	++	++	Beta cell subset
Vesicular Acetylcholine Transporter	Rabbit	SySy	139103	+++	+	Alpha cells	+	+	Neuro-insular complex
	Rabbit	Sigma	V5387	+	+	Alpha cells	+	+	Neuro-insular complex
	Goat	BD-Pharmingen	556337	-	-	-	-	-	-
	Mouse Mab	Phoenix Pharm.	H-V005	-	-	-	-	-	-
Choline Acetyl Transferase	Goat	Chemicon	AB144P	++	+	Alpha cells**	+	+	Neuro-insular complex**
	Rabbit	Chemicon	AB143	+	+	-	+	+	-
	Rabbit	Pierce	OSC00008W	-	-	-	-	-	-
Choline Transporter	Rabbit	Chemicon	AB5966	-	-	-	-	-	-
Vesicular Monoamine Transporter 2	Rabbit	SySy	138302	++	++	-	++	++	Beta cell subset
	Rabbit	Phoenix Pharm.	H-V004	-	-	-	-	-	-
Dopamine β Hydroxylase	Sheep	Abcam	Ab19353	-	-	-	-	-	-
Neuron Specific Enolase	Chicken	Chemicon	AB9698	-	-	-	-	-	Endocrine cells
	Mouse Mab	Chemicon	MAB324	-	-	-	-	-	-
Protein Gene Product (PGP 9.5)	Rabbit	AbD Serotec	7863-0507	+	+	Endocrine cell nuclei	+++	+++	Endocrine cell nuclei, neuro-insular complex
Acetylated alpha-Tubulin	Mouse Mab	Sigma	T6793	+	+	Primary cilia, endocrine cells	+++	+++	Primary cilia, neuro-insular complex
β -Tubulin (Tub)	Rabbit	Covance	MRB4358	-	-	-	-	-	Neuro-insular complex
S100B	Rabbit	Abcam	ab14688	-	-	-	-	-	-
Neuro filament (NF-H)	Chicken	NeuroMics	CH22104	-	++	-	++	++	-
Neuro filament 200	Rabbit	Sigma	N4142	-	+	-	+	+	-
α -NG2 Condroitin Sulfate Proteoglycan	Rabbit	Chemicon	AB5320	-	-	Pericytes in islet	-	-	-
Human CD-31 (PECAM)	Mouse Mab	BD-Pharmingen	550389	-	-	Endothelial cells	-	-	Endothelial cells
Actin α -Smooth Muscle	Mouse Mab	Sigma	C6198	-	-	Smooth muscle cells	-	-	Smooth muscle cells
Cytokeratin-19	Mouse Mab	Bio-Genex	AM246-5M	-	-	Ductal cells	-	-	Ductal cells
Insulin	Guinea Pig	Dako	A0564	-	-	Beta cells	-	-	Beta cells
Glucagon	Mouse Mab	Sigma	G2654-5	-	-	Alpha cells	-	-	Alpha cells
Somatostatin	Rat Mab	Chemicon	MAB354	-	-	Delta cells	-	-	Delta cells

*In 1 out of 10 examined human pancreas samples; **Cell staining obtained with tyramide signal amplification

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. 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 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.

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 63x objective at 1024 x 1024 pixel resolution. The nerve fiber plexus was reconstructed in 3D stacks of images (step size = 0.7 μ m) and analyzed using Leica or Volocity 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 μ m) of confocal images using Volocity software. We developed a macro in Volocity 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 1 A,B,C). We further used Volocity 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 (Figure 1 D,E,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).

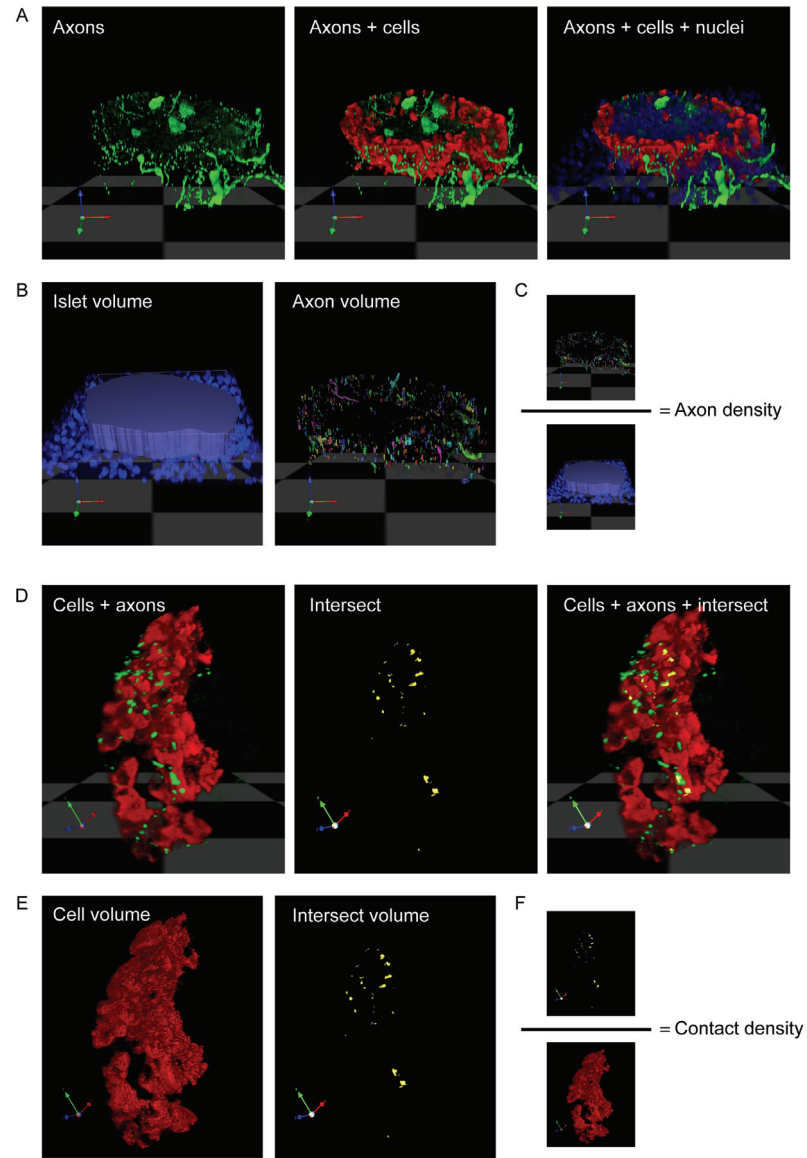


Figure 1. Representation of axon density and contact density quantification
 (A) Three dimensional rendering of a z-stack of confocal images ($n = 40$) of a mouse pancreatic section stained for TH (green), glucagon (red), and DAPI (blue). (B) Islet volume (in voxels) is determined by outlining the islet using DAPI staining. The volume of axonal staining is determined within this islet volume. (C) The axon (innervation) density is calculated as the quotient axon volume/ islet volume. (D) TH-labeled axons can be seen in close proximity to glucagon-labeled alpha cells. Shown is a higher magnification and rotated image of the islet in A. The proximity of axons to target cells can be detected using an algorithm in

Volocity software (“intersect). Intersect between axons and cells is shown in yellow. Notice that few axonal varicosities contact target cells (yellow and green overlap in the merged image on the right). (E) Cellular (cell) volume is determined based on cell immunostaining, intersect volume is determined as the close proximity or overlap of axon and cell immunostaining. (F) The contact density is calculated as the quotient intersect volume/ cellular volume.

Islet isolation

Human pancreata were obtained from heart-beating organ donors (donation after cerebral death). Islets from human pancreata were isolated at the Human Islet Cell Processing Facility of the Cell Transplant Center at the Miller School of Medicine, University of Miami. The glands were cold preserved in University of Wisconsin solution. Islets were isolated using a modification of the automated method using seven different lots of the enzyme Liberase HI (Roche, Indianapolis, IN) and a standard purification step, as described previously¹¹⁸. Human islet isolations were performed weekly at the Diabetes Research Institute. C57BL/6 mice were killed by exsanguination under general anesthesia. Mouse islet isolation is performed using collagenase digestion followed by purification on density gradients¹¹⁹.

Dynamic measurements of insulin and glucagon secretion

Our laboratory has developed a high-capacity, automated perfusion system (Biorep® Perifusion V2.0.0) to dynamically measure hormone secretion from pancreatic islets¹²⁰. In this system, a low pulsatility peristaltic pump pushes HEPES-buffered solution (100 μ L/min) through a sample container harboring 100 pancreatic islets immobilized in Bio-Gel P-4 Gel (BioRad, Hercules, CA). Glucose concentration was adjusted to 3 mM or 11 mM. Stimuli were applied with the perfusion solution. The perfusate was collected in an automatic fraction collector designed for a multi-well plate format. The sample container harboring the islets and the perfusion solutions were kept at 37°C in a built-in temperature controlled chamber, and the perfusate in the collecting plate was kept at < 4°C, to preserve the integrity of the analytes in the perfusate. Perfusates were collected every minute. Hormone release in the perfusate was determined with the human or mouse Endocrine LINCOplex Kit following manufacturer’s instructions (Linco research, St. Charles, MO). The biomolecular assays were performed on a Bio-Plex protein array system (Bio-Rad, Hercules, CA). This system is a flow-based dual laser system that simultaneously identifies and quantifies up to 100 different analytes in a single assay.

Islet transplantation into the anterior chamber of the mouse eye

Thirty to three hundred isolated islets were transferred from culture media to sterile PBS and aspirated into a 27G eye cannula (27 gauge anterior chamber cannula; Katena Products, Inc., Denville, NJ) connected to a 1 ml Hamilton syringe (Hamilton, Reno, NV) via a 0.4 mm polythene tubing (Portex Limited, Kent, England). Athymic nude mice were anesthetized with an intramuscular administration of ketamine hydrochloride (42.8 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA), xylazine (8.5

mg/kg; Phoenix Scientific, Inc., St. Joseph, MO), and acepromazine (1.4 mg/kg; Phoenix Scientific, Inc.). A topical anesthetic was also applied (proparacaine hydrochloride eye ophthalmic solution). Eyes were kept humidified (ophthalmologic eye drops) to avoid drying of the cornea. The cornea may otherwise become opaque. Under a stereomicroscope, the cornea was punctured close to the sclera at the bottom part of the eye with a 27G needle. Care was taken not to damage the iris and to avoid bleeding. The blunt eye cannula was gently inserted, and the islets were slowly injected into the anterior chamber where they settle on the iris. After injection, the cannula was carefully withdrawn and the animal was left lying on the side before awakening. Mice were then put back in the cages and monitored until full recovery, and observed daily thereafter. Analgesia was obtained after surgical procedures with buprenorphine (0.05-0.1 mg/kg s.c.).

Imaging of the mouse eye

Transplanted animals were anesthetized with a 40% oxygen and ~2% isoflurane mixture, placed on a heating pad. The head was restrained with a stereotaxic headholder (SG-4N, Narishige, Tokyo, Japan) and positioned with the eye containing the engrafted islets facing up. The eyelid was carefully pulled back and the eye was gently held at the corneoscleral junction with a pair of tweezers attached to a UST-2 Solid Universal Joint (Narishige). The tips of the tweezers were covered with a single piece of polythene tubing creating a loop between the two tips. This arrangement permitted a flexible but stable fixation of the head and eye without causing damage or disrupting the blood circulation in the eye. For fluorescence confocal imaging, an upright Leica DMLFSA microscope equipped with a TCS-SP5-AOBS confocal scanner (Leica Microsystems Heidelberg GmbH, Mannheim, Germany) and lasers for one- and two-photon excitation were used for imaging together with long distance water-dipping lenses (Leica HXC APO 10x 0.3W, 20x 0.5 W, 40x 0.8W and 63x 0.9W), using filtered saline solution as an immersion liquid. Widefield epifluorescence imaging was performed on the same microscope setup using an imaging system with a Xenon arc lamp for excitation, intensified CCD camera for detection, and motorized filter wheels for excitation and emission wavelength selection. Two-photon excitation was achieved using a tunable, femto-second Ti:Sapphire laser (Chameleon Ultra II, Coherent Inc., Santa Clara, CA) and emission light was collected and separated onto two non-descanned detectors. Other fluorophores were excited with their appropriate one- or two-photon wavelengths. In two-photon imaging, GFP and Texas Red were excited at 890 nm and emission light was collected and separated onto two non-descanned detectors using a dichroic mirror (RSP560) and emission filters (BP 525/50 and BP 640/20). The images captured with TPLSM were denoised using wavelet filtering. In one photon imaging, GFP is excited at 488 nm (35% AOTF) and emission light was collected between 495-530 nm. Reflected light was imaged by illumination at 546 nm (35% AOTF) and collection between 539-547 nm. Post-processing, analysis, and visualization of images were performed with a number of software tools including: Leica Confocal Software, Andor iQ software, Volocity (Coventry, UK), and Image J (NIH, USA).

Transplantation of islets for metabolic studies

Mouse (C57BL/6) pancreatic islets were isolated as described above. Diabetes was induced in athymic nude mice with a single intravenous injection of streptozotocin (200 mg/kg; Sigma, MO). To ensure that endogenous beta cells have been eliminated, only animals with nonfasting glycemic values >350 mg/dl were used. We transplanted 300-400 islet equivalents into the anterior chamber of the right eye of the mice. This provides an optimal beta-cell mass that we normally apply in the syngeneic transplantation setting either in the eye or under the kidney capsule. We ensured that the islets were evenly distributed across the iris. To avoid reflux of islets out of the anterior chamber, we let the intraocular pressure subside by waiting 10-20 seconds with the cannula in place and then resuming the injection. Thus, the 300-400 islet equivalents were injected in individual thrusts of 50-100 islets over 3 minutes. During the peritransplantation period mice were supported with insulin pellets or insulin injections. The procedure for human islets was different because approximately 1,000 human islet equivalents need to be transplanted to reverse diabetes in mice. We therefore transplanted both mouse eyes, with each eye receiving 500 human islet equivalents. Plasma glucose levels were monitored daily after transplantation. We considered transplantation successful and islet grafts functional once recipient mice reached glycemic values below 200 mg/dl. Our success rate for mouse to mouse transplantation was >90% and for human to mouse it was ~50% (human islet preparations are highly variable in terms of quality). Mice that did not revert to normoglycemia were not included in the studies.

Assessment of metabolic function

We monitored graft function by measuring nonfasting glycemia using a portable glucometer (OneTouch, LifeScan). We define normoglycemia as nonfasting blood glucose below 200 mg/dl. We performed glucose tolerance tests after 12–16 h of fasting by measuring glycemic values on peripheral blood following intraperitoneal injection of 2 g/kg glucose in saline for mouse transplanted with mouse islets or 4 g/kg for those mice transplanted with human islets and the respective control animals. Tail blood samples (50 μ l) were taken before the start of the experiment and at 0, 3, 10, and 30 min for determination of plasma glucagon and insulin secretion. Hormone plasma levels were determined with the human or mouse Endocrine LINCOplex Kit following manufacturer's instructions (Linco research, St. Charles, MO). The biomolecular assays were performed on a Bio-Plex protein array system (Bio-Rad, Hercules, CA). To exclude residual function of the native pancreas we removed the graft-bearing eye (enucleation) under general anesthesia (ketamine/xylazine) and followed the glycemia values for 2 days.

Statistical Analyses

Paper I. All data were presented as mean \pm SEM. For statistical comparisons of innervation densities and axons-endocrine cell contacts we used one-way ANOVA followed by multiple comparisons (Student-Newman-Keuls). Student's t test was used to compare the density of cholinergic axons and smooth muscle actin contents.

Paper II. All data were presented as mean \pm SEM. Association between vAChT and ChAT mRNA levels was assessed by linear regression. Colocalization of subcellular

staining was done by Pearson's correlation coefficient comparison by ANOVA followed by multiple comparisons. Biosensor Ca^{2+} responses and fluorescence assay data to estimate ACh secretion were compared by ANOVA followed by multiple comparisons. Pharmacological data showing that endogenous release of ACh amplifies glucose-induced insulin secretion were analyzed with Student's t tests for single comparisons for every particular drug as detailed in the results section of the paper. Paper III. Biosensor Ca^{2+} responses to estimate ACh secretion were compared by ANOVA followed by multiple comparisons (Student-Newman-Keuls). The differences between mouse and human islets in their ACh secretory profiles were tested by Fisher's exact test. Paper IV, V, VI, VII and VIII. All data were presented as mean \pm SEM. Differences in glycemia values and IPGTT were tested by ANOVA followed by multiple comparisons.

RESULTS AND DISCUSSION

Paper I

Innervation patterns of autonomic axons in the human endocrine pancreas

The endocrine pancreas is innervated by the autonomic nervous system, but the abundance and organization of this innervation are highly variable between species⁶. Several nerve fiber systems have been described for islets of different species using immunohistochemistry^{11,17,121-125}. Receptors for autonomic neurotransmitters are expressed on the plasma membrane of islet cells, and it is known that these neurotransmitters modulate islet hormone secretion^{5,23}. It is difficult, however, to obtain a coherent picture from the literature. There are no comprehensive studies that examine all innervation patterns and their targets in a single species. Comparisons between species are not possible because common techniques or markers have not been used. It is important to remember that most of the studies have been conducted on rodent islets and that few studies have examined the autonomic innervation and its effects in human islet cells. Because the cell composition and cytoarchitecture are different in rodent and human islets^{28,29}, it is very likely that the innervation patterns also differ.

This paper reassesses the innervation patterns in mouse and human islets by testing the working hypothesis that there are qualitative differences in the innervation patterns between mouse and human islets.

The experimental approach consisted of identifying the parasympathetic and sympathetic components of the innervation, as well as their cellular targets in the islet using immunohistochemistry on thick human and mouse pancreatic sections. As putative targets we examined alpha, beta, and delta cells, as well as endothelial and smooth muscle cells of the blood vessels. The nerve fiber plexus was reconstructed and analyzed in 3D stacks of images. We examined pancreatic tissues from 24 mice of different strains and 15 human biopsies from pancreas donations for islet isolation.

We found that, compared to mouse islets, human islets were sparsely innervated with most markers tested. In mouse islets, sympathetic fibers (TH) and parasympathetic fibers (vAChT) form a dense plexus in the islet periphery and core, respectively. In the human pancreas, neural fibers can be seen in the exocrine tissue, but only few penetrate the islets. Similar results were obtained with additional parasympathetic (ChAT), sympathetic (vMAT) and axonal markers (PGP 9.5, acetylated α -tubulin, Neurofilament 200 and synapsin). Immunostaining for the autonomic markers was very similar in nerves, major vessels and the exocrine tissue surrounding the islets in human and mouse pancreata, indicating that the observed differences were selective for the islets.

The results showed that most axons innervating human islets are sympathetic. Out of the total axonal labeling in the islets, few were reactive for vAChT. Instead, much of

the vAChT staining colocalized with glucagon. By contrast, TH-labeled axons in human islets were present in the core.

Characterization of the sympathetic innervation in human islets revealed that sympathetic axons barely innervate endocrine cells. This result was strikingly different to the pattern of sympathetic innervation within the mouse islets, where TH-labeled axons contacted glucagon labeled alpha cells or somatostatin-labeled delta cells. Because the sympathetic axons were localized in regions devoid of endocrine cells we examined vascular cells as potential targets of sympathetic axons. We observed that TH-labeled axons in human islets traveled along the vessels, ran parallel to PECAM-labeled endothelial cells, and contacted cells stained for smooth muscle actin (SMA). Thus the contractile cells of blood vessels in the human islets were the major targets of sympathetic innervation. The content of inraislet smooth muscle cells was much higher in human islets than in mouse islets.

In summary, 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. 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.

These findings are important because they demonstrate that it is problematic to extrapolate knowledge from mouse studies to humans. The results further suggest that innervation may have a strong influence on mouse islet function; it is however unclear how the sparse innervation in human islets should contribute to hormone secretion since parasympathetic and sympathetic fibers do not seem to innervate endocrine targets in human islets. This morphological discrepancy correlates to many of the physiological incongruities reported in the literature regarding the influence of autonomic innervation on human glucose homeostasis with respect to other species.

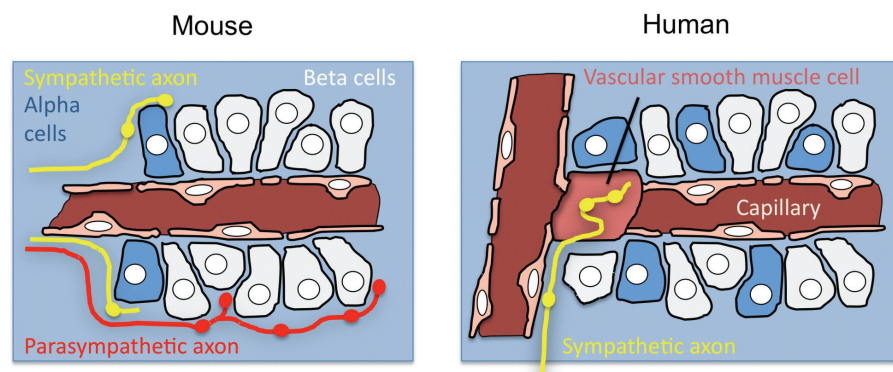


Figure 2. Compared to mouse, human endocrine cells are sparsely contacted by sympathetic and parasympathetic axons. Human inraislet smooth muscle cells of the vasculature are targets of sympathetic innervation.

Paper II

Alpha cells secrete acetylcholine as non-neuronal paracrine signal priming beta cell function in humans

Acetylcholine is a neurotransmitter that plays a major role in the function of the insulin secreting pancreatic beta cell^{6,21}. It is generally believed that the human endocrine pancreas is richly innervated by the autonomic nervous system^{3,5,6,126} and that acetylcholine is released from parasympathetic nerve endings in pancreatic islets during feeding^{5,6}. Parasympathetic innervation of the islets of Langerhans has been shown to provide cholinergic input to the beta cell in several species^{13,15,16,127}, but the role of autonomic innervation in human beta cell function is at present unclear. In paper I we showed that endocrine cells of the human islet receive little autonomic input. These findings are not consistent with the current view that the autonomic nervous system controls insulin secretion via parasympathetic fibers. Intriguingly, we observed that the cholinergic marker vAChT was consistently co-expressed with glucagon in alpha cells. Given the unique cellular arrangement of human islets, where most beta cells (70-80%) face alpha cells^{29,128}, it is conceivable that paracrine acetylcholine signaling occurs. Human alpha cells seem optimally placed to influence beta cells.

In this paper we put forward the new notion that human islets are less dependent of neural cholinergic input than mouse islets. We therefore tested the working hypothesis that acetylcholine is a paracrine signal in human islets, which is activated under circumstances that cannot be modeled with rodent studies.

We reassessed the presence of cholinergic markers in mouse and human islets. Using immunohistochemistry on human and mouse pancreatic sections we confirmed that, in contrast to mouse islets, human islets are scarcely innervated by cholinergic (vAChT-immunoreactive) parasympathetic nerve fibers. Instead, most human alpha cells were strongly vAChT and ChAT immunoreactive. The presence of vAChT, ChAT and ChT1 in isolated, denervated human islets was confirmed by Western blot analysis and mRNA expression analysis consistently showing that these cholinergic genes were expressed in the human islets with comparable or higher levels than in the brain.

Within the alpha cell, vAChT staining did not overlap with glucagon staining and appeared confined to distinct subcellular compartments. This agrees with studies showing that, in neuroendocrine cells, vAChT localizes preferentially in synaptic-like vesicles and is excluded from hormone granules. To strengthen this observation, we used STED microscopy imaging of similarly stained pancreatic sections after the publication of this paper. Our findings support our published results (Figure 3). That vAChT is localized in vesicles other than glucagon granules, however, needs to be confirmed by electron microscopy studies.

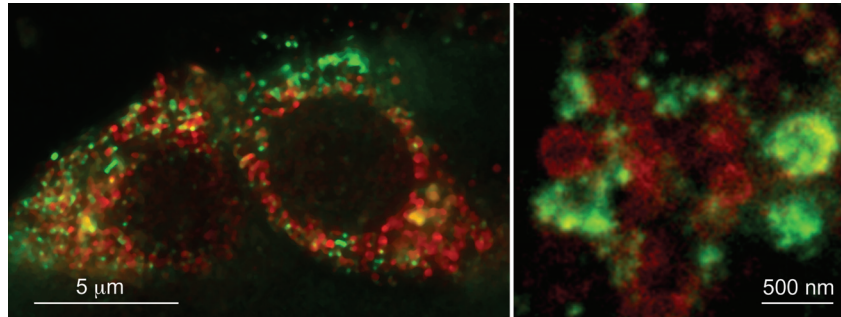


Figure 3. Human pancreatic alpha cells stained for glucagon (red) and vAChT (green) were visualized by STED microscopy at 60 nm of resolution (left panel). Glucagon granules and vAChT reactive structures are depicted in higher magnification (right panel).

After demonstrating that human alpha cells have the machinery for the exocytosis of acetylcholine, we tested if and when they secrete it using acetylcholine biosensor cells. We found that human islets release acetylcholine when stimulated with kainate or a lowering in glucose concentration, two specific stimuli for alpha cells. Thus, in marked contrast to the parasympathetic cholinergic control of insulin secretion described for rodents and other species, alpha cells of the human islet may provide paracrine cholinergic input to surrounding endocrine cells.

To infer the role of acetylcholine as a paracrine signal, we subjected isolated human islets to an experimental protocol in which beta cells and alpha cells were stimulated intermittently while modulating the intrinsic cholinergic signaling. Blocking acetylcholine degradation with the acetylcholinesterase blocker physostigmine increased insulin release during repeated exposure to high glucose (11 mM), whereas adding the M3 receptor-specific antagonist J-104129 reduced insulin responses. Thus, in the absence of any influence from the autonomic nervous system, endogenously released acetylcholine in human islets is able to sensitize the beta cell to subsequent increases in glucose concentration.

Our results demonstrate that human alpha cells secrete acetylcholine, the major parasympathetic neurotransmitter, as a paracrine signal that primes the beta cell to respond optimally to subsequent increases in glucose concentration. The nature of acetylcholine as a paracrine signal and not only a neural signal as in rodents, further implies that cholinergic signaling in the human endocrine pancreas is activated under circumstances that cannot be predicted from rodent studies, highlighting the importance of species divergence in the pancreatic islet. We anticipate these results to revise models about neural input and cholinergic signaling in the human endocrine pancreas.

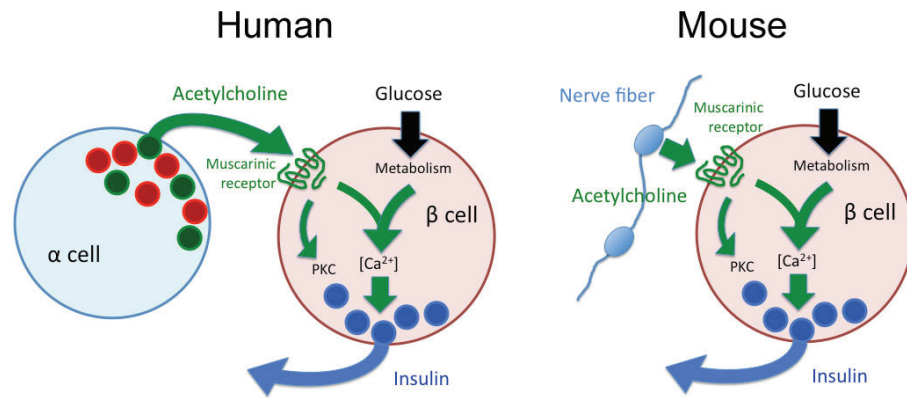


Figure 4. Human islets of Langerhans are less dependent on neural acetylcholine than mouse islets. Acetylcholine, the major parasympathetic neurotransmitter, is secreted as a paracrine signal by human alpha cells and primes beta cells for an optimal response to glucose.

Paper III

Real time detection of acetylcholine release from the human endocrine pancreas

Acetylcholine was the first neurotransmitter to be characterized. It is the main neurotransmitter in muscular synapses and plays an important role in the central nervous system associated with attention, memory, learning, consciousness, sleep and control of voluntary movements¹²⁹. However, the lack of simple methods to detect its secretion has limited studies where acetylcholine plays an important role, since estimating acetylcholine is fraught with difficulties related to selectivity, temporal and spatial resolution, and the rapid degradation of the molecule¹³⁰. Techniques most commonly used include dual enzyme biosensors¹³¹⁻¹³⁴, electrochemical detection^{129,135,136} and liquid chromatography/mass spectrometry¹³⁶⁻¹³⁹. The application of all these methods demands the investment in expensive and sophisticated equipment and highly specialized expertise of the personnel. In fact, the actual determination of acetylcholine release from human islet endocrine cells became the most challenging approach to evaluate that acetylcholine is a paracrine signal in human islets, which was the working hypothesis of paper II. We were able to record acetylcholine release from human pancreatic islets by applying a relatively simple and inexpensive approach using acetylcholine biosensors cells.

In this paper we describe a simple protocol, which allowed us to detect the release of the neurotransmitter acetylcholine from pancreatic human islets. Our aim was to show the advantages and simplicity of a method that can be easily applied to studies with other neurotransmitters without all the intrinsic technological and practical limitations associated with other methods.

In this protocol, we describe the use of biosensor cells to detect neurotransmitter release from endocrine cells in real time. CHO cells expressing the muscarinic acetylcholine receptor M3 were used as acetylcholine biosensors to record acetylcholine release from human pancreatic islets. We show how acetylcholine biosensors loaded with the Ca^{2+} indicator Fura-2 and put in close contact to isolated human pancreatic islets allow detection of acetylcholine release. We found that Ca^{2+} responses in biosensor cells could be evoked under conditions that stimulate human alpha cells but not beta cells. By contrast, we could not measure acetylcholine secretion from mouse islets. This led us to conclude that human alpha cells secrete acetylcholine.

The biosensor approach is simple. The Ca^{2+} signals generated in the biosensor cell reflect the presence (release) of neurotransmitter. The procedure has great sensitivity and temporal resolution because it detects the neurotransmitter near to its release site. A major limitation is that the technique is qualitative and results from different experiments cannot be compared quantitatively. Individual biosensor cells may have different sensitivities to the neurotransmitter. As with all microscopic approaches, the biosensor technique is also affected by spatial constraints such as distance and access to the release sites.

Biosensor cells are readily accessible. For instance, CHO cells stably expressing a wide variety of different transmitter receptors are commercially available and easily maintained in the laboratory, making these cells biosensors of choice for many applications. Furthermore, biosensor cells can be designed to express a combination of receptors to demonstrate co-release of neurotransmitters. Thus, the protocol can be used to define the secretory phenotype of cells in other tissues.

Paper IV

A simple noninvasive system for investigating the role of innervation in pancreatic islet function *in vivo*

Numerous studies have shown that the endocrine pancreas is richly innervated by the autonomic nervous system³⁻⁷. The role of islet innervation in the maintenance of glucose homeostasis is still poorly understood since autonomic input on islet physiology cannot be studied *in vivo*. There are no reliable methods for functional monitoring islet innervation due to the lack of experimental tools. The eye has long been used to study the mechanisms of reinnervation of tissue grafts, including pancreas, and the behavior of intrinsic nerves^{71,72,74,140-149}. Human brain and heart xenografts have been shown to survive for at least 15 months in the eye and the human grafts were functionally innervated by the autonomic nerve fibers of the host rat iris^{72,75-77}. Moreover, studies have shown that iris sympathetic fibers establish functional connections in intraocular brain grafts similar to the synapses normally formed *in situ*^{150,151}. Islets are reinnervated after transplantation, both in the eye⁸⁴ and elsewhere^{81-83,152}. Independently of the transplantation site, the innervation pattern of the islet grafts seems to differ only slightly from that of pancreatic islets *in situ*^{83,153}. The density of innervation, however, depends on the transplantation site. Islets transplanted under the kidney capsule are more densely innervated than islet grafts in the spleen and liver⁸², indicating that the host organ shapes the reinnervation density of the islet graft. If so, the highly innervated iris is an ideal transplantation site for reinnervation of the islets.

To specifically study the effects of parasympathetic and sympathetic innervation on islet function we used a new experimental model allowing *in vivo* imaging of vascularized and reinnervated islets. Mouse islets were transplanted into the eye and their function was recorded locally and systemically after manipulation of the eye's neural input. The goal was to establish an experimental platform that ultimately allows us to investigate the role of innervation on human islet function *in vivo*.

We first examined the time course of the reinnervation process and the extent to which the incoming fibers are of the appropriate type and whether they innervate the correct targets in intraocular islet grafts. Immunostaining showed growing fibers traveling first along blood vessels and then turning to their endocrine targets. Intraocular islet grafts were strongly innervated 90 days after transplantation in a pattern that closely resembled that of the native pancreas (Figure 5). It is important to state that the intraocular islet grafts might not be under the control of the autonomic nervous system as they are in the pancreas. The sympathetic and parasympathetic fibers in the eye are not likely to receive input from the brain regions regulating islet function; they are part of the circuits regulating pupillary size. However, endocrine cells in intraocular islet grafts attract fibers similar to the ones that innervate them in the pancreas.

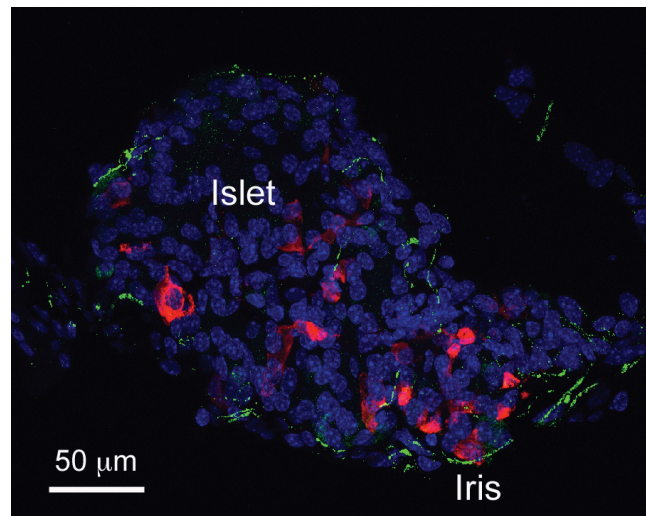


Figure 5. Mouse islets of Langerhans transplanted into the mouse eye are reinnervated with sensory fibers. Z-stack of confocal images of an intraocular islet graft showing sensory fibers reactive for CGRP and substance P antibody cocktail (green) and glucagon positive cells (red). Cell nuclei were counter-stained with DAPI (blue).

To image cholinergic fibers noninvasively, we transplanted islets into the eyes of mice expressing GFP under the control of the ChAT promoter. GFP-labeled, cholinergic axons and cell bodies of the neuroinsular complex could be seen associated both with the intraocular islet grafts and with pancreatic islets in sections from ChAT-GFP mice. This confirms that islets are similarly innervated in the native pancreas and after transplantation into the eye. The results show the feasibility of *in vivo* tracing of autonomic fibers using mice with GFP-labeled axons. In future studies, we will visualize plastic changes in the cholinergic innervation under physiological and pathophysiological conditions (e.g. diabetic neuropathy).

Having established that the islet innervation patterns after transplantation were appropriate, our model allowed a detailed dissection of the role innervation has on islet function *in vivo*. One feature of intraocular grafts is that their autonomic input can be regulated by light via the pupillary reflex. Given that light increases cholinergic input to the iris to constrict the pupil, while noradrenergic fibers are activated in the dark to dilate it, we expected that intraocular islets grafts secrete more insulin in ambient light compared to darkness. Indeed lower values of fed glycemia, higher plasma insulin levels and improved glucose excursion after IPGTT under light conditions confirmed our prediction. This indicated that light modulates islet graft function in the eye.

The eye has the great advantage that drugs can be applied locally in a noninvasive manner. Eye drops containing autonomic drugs are commonly and routinely used in

the ophthalmic clinic. We tested if islet graft function, as measured by glucose tolerance tests, could be modulated directly with known autonomic modulators of islet function. Topical application of pilocarpine, a muscarinic agonist, improved glucose tolerance. By contrast, applying atropine, a muscarinic antagonist, partially inhibited the effects of light stimulation, indicating cholinergic mechanisms. These data are important because they show that with our model it is feasible to manipulate autonomic input noninvasively.

It has been shown that various mouse strains differ in their glucose tolerance and threshold glucose concentration for 1st phase insulin release, with 129x1 mice exhibiting lower blood glucose levels than C57BL/6 mice¹⁵⁴. In earlier studies we found that C57BL/6 mouse islets have a higher density of parasympathetic innervation than 129x1. By using the eye imaging platform, we tested the hypothesis that the different sensitivities to glucose are related to their different degrees of parasympathetic innervation. We transplanted islets from both mouse strains into the eye of two groups of nude mice and performed glucose tolerance tests in the dark and in ambient light to manipulate the cholinergic input to the islets grafts. We found that parasympathetic innervation had a great impact on islet function in C57BL/6 but not in 129x1 mice, which might be related to the major difference in glucose homeostasis of both mouse strains.

Our study shows the versatility and simplicity of our experimental platform which enables noninvasive, local, and selective manipulation of the nervous input while islet function is recorded locally or systemically in real time or longitudinally under physiological conditions. Similar studies can be performed in transplanted mice under diabetic conditions.

Paper V

Donor islet endothelial cells in pancreatic islet revascularization

Within the native islets, the nerves follow the blood vessels and terminate within the pericapillary space, within the capillary basement membrane, or closely apposed to the endocrine cells⁸. Furthermore, glucose reaches endocrine islet cells in the native pancreas through the microvasculature. The vascular arrangement therefore surely has an impact on islet cell function. Transplanted islets are revascularized by blood vessels that grow into the islets from the host organ via angiogenesis, but donor islet endothelial cells (DIECs) can also form functional vessels within transplanted islets^{65,103}. The contribution of DIECs in the process of revascularization is highly dependent on the time frame between islet isolation and their transplantation in the eye since most of the DIECs disappear if islets are cultured^{65,103,155}. Therefore, it is important to determine the experimental conditions that contribute to establish an islet graft neovasculature that closely mimics that of islets in the pancreas.

In this paper we further characterized islet transplantation into the eye as an experimental platform to study islet biology *in vivo* by testing the hypothesis that the neovasculature in transplanted islets resembles that of the islets in the native pancreas, independently of the origin of the endothelial cells in the process of revascularization.

Freshly isolated mouse islets, which contain DIECs, and cultured mouse islets, which have lost DIECs, were transplanted under the kidney capsule and into the mouse eye. Intravital laser-scanning microscopy was used to monitor the revascularization process and DIECs in intact grafts. The grafts' metabolic function was examined by reversal of diabetes, and the ultrastructural morphology by transmission electron microscopy. We used revascularization rate, vascular density, and endocrine graft function after transplantation of freshly isolated and cultured islets as parameters to infer how DIECs contribute to neovascularization and impact graft function.

As previously reported^{65,103}, DIECs significantly contributed to the neovasculature of fresh islet grafts and remained in the graft vasculature over long time periods. The early participation of DIECs in the revascularization process correlated with a faster revascularization rate of freshly isolated islets compared with cultured islets. However, after complete revascularization, the vascular density was similar in the two groups. Surprisingly, grafts originating from cultured islets reversed diabetes more rapidly than those originating from fresh islets. This result could be explained by other parameters like lowering the pro-inflammatory profile of the freshly isolated islet or selection of the most viable islets after culture. In addition, the high oxygen tension of the anterior chamber of the eye could have masked the advantage of a higher revascularization rate by reducing hypoxia during engraftment.

One striking finding was that endothelial cells in intraocular islet grafts acquired the typical and native intraislet endothelial cell phenotype with thin cell bodies and abundant fenestrations. We could not find any morphological evidence that would suggest differences between host and donor endothelial cell ultrastructure.

It is conceivable that human DIECs will participate in the revascularization of human islet grafts in the eye of immune compromised mice, as reported in other sites of transplantation¹⁰³, and therefore acquire their native intranslet ultrastructure. Thus, functional human blood vessels may be reassembled in human xenografts in the mouse eye and persist for long times. This platform would enable physiological studies that recreate a vascular-endocrine environment connected to a living organism similar to the native human pancreas.

Paper VI

High-resolution, noninvasive longitudinal live imaging of immune responses

We have previously shown that sensory fibers reinnervated mouse islets engrafted in the eye. Mouse sensory afferent neurons are a critical component in prediabetes initiation, promoting islet inflammation through altered glucose homeostasis and progressive beta cell stress^{45,156}. Islet transplantation into the eye could be an excellent approach to dynamically evaluate the interface between the nervous and the immune system in the pathogenesis of autoimmune diabetes.

In this study we evaluated the anterior chamber of the eye as an experimental platform to study the dynamic processes of the immune response in the mouse eye with the ultimate goal to further evaluate how sensory fibers are linked to the autoimmune responses driving diabetes.

We noninvasively studied the *in vivo* dynamic behavior of green-fluorescent protein (GFP)-labeled T-lymphocytes in pancreatic islet allografts. MHC-mismatched DBA/2 mouse islets were transplanted without immunosuppression into the eye of recipient mice expressing GFP in effector and memory T-lymphocytes. Longitudinal *in vivo* imaging revealed a progressive accumulation of GFP+ T-lymphocytes, which paralleled loss of volume/function of allogeneic islets.

In vivo analysis of morphology and dynamic behavior of intraislet T-lymphocytes revealed a novel “ruffled” cell phenotype, which predominated during the effector phase of rejection. Ruffled cells extended pseudopodia in multiple directions simultaneously and engaged in contacts with other surrounding T-lymphocytes and target islet cells. The ruffled phenotype was characterized by a significantly increased but constrained dynamic behavior within the islet allografts, suggesting that cytotoxic T-lymphocytes engage in simultaneous contacts with multiple target cells ensuring effective killing during allorejection.

We demonstrate that transplantation into the eye provides a versatile experimental tool that enables longitudinal, noninvasive *in vivo* imaging of immune responses within target tissues with cellular resolution. It is noteworthy, that these data demonstrate that the immune-privilege, which is transiently provided by this transplantation site, is broken following revascularization of the graft, and hence immune reactions are feasible to study. This process allows studying cell–cell interactions within target tissues and visualizing immune cell motility *in situ*. By using our technological platform we can envisage studies extending beyond those that initially described the interactions between sensory axons and immune cells. We will perform experiments in which sensory axons innervating intraocular islet grafts are manipulated in transplanted NOD mice, while monitoring changes in the immune cell infiltration and behavior.

Paper VII

The anterior chamber of the eye as a clinical transplantation site for the treatment of diabetes: a study in a baboon model of diabetes

Pancreatic islet transplantation in patients with unstable type 1 diabetes has shown promise as a cure of the disease. Among the difficulties is the establishment of an appropriate transplantation site¹⁵⁷⁻¹⁵⁹. We have previously shown that transplanting pancreatic islets into the eye allowed for engraftment and adequate function along with noninvasive monitoring in mice^{59,60}. The eye as a transplantation site offers valuable advantages to be considered, including the claimed immune privilege properties and the immune-modulatory effects of the eye, the potential for noninvasive monitoring of the graft and local administration of drugs, e.g. immunosuppressant drugs.

The aim of this study was to provide evidence that the eye can serve as a novel clinical islet implantation site using a preclinical non-human primate model.

Allogeneic pancreatic islets were transplanted into the eye of a baboon model for diabetes, and metabolic and ophthalmological outcomes were assessed. Islets readily engrafted onto the iris along with a concomitant decrease in exogenous insulin requirements due to insulin secretion from the intraocular grafts. HbA1C levels were reduced to near pre-diabetes levels. No major adverse effects on eye structure and function could be observed during the transplantation period. Our study demonstrates the long-term survival and function of allogeneic islets after transplantation into the eye.

In this study, we provided a proof-of-concept that pancreatic islet transplantation into the eye of nonhuman primates significantly improves glycemic control and prevents serious hypoglycemic episodes in the recipient. The safety and simplicity of this procedure provides support for further studies aimed at translating this technology into the clinic. Remaining questions include whether accomplishing complete insulin independence is feasible with islet transplantation into a single eye versus both eyes and whether the procedure interferes with vision. Addressing these questions will help advance the anterior chamber as a clinical transplantation site for pancreatic islets in the treatment of type 1 diabetes.

Paper VIII

A new experimental platform to study human islet cell biology *in vivo*.

The ultimate goal of this thesis was to establish an experimental model to evaluate the role of neural input on human glucose homeostasis *in vivo*. Unfortunately, functional imaging of human islets *in situ* remains challenging since non- or minimally-invasive technologies to monitor islet cell function are not fully developed. We used transplantation of human islets into the eye of immune-compromised mice as a platform to visualize and follow up the engraftment and function of human islets in a living organism. It is very likely that human islets engrafted in the iris of immune-compromised mice retain their intrinsic nature and orchestrate the process of revascularization and reinnervation to reconstruct their natural environment (similar to the reinnervation observed for C57BL/6 and 129x1 transplanted islets in paper IV).

In this paper, we have explored islet transplantation into the mouse eye as an experimental platform to study human islet biology *in vivo*. Our aim was to establish a “humanized” mouse model in which human islets are maintaining glucose homeostasis. In view of the striking findings about human islet innervation and intraislet paracrine interactions described in paper I and II, this model may aid as a versatile tool to clarify the integration of complex signaling networks at the cellular level under *in vivo* conditions. With this platform, we can study the effects of modulatory inputs from hormonal and neuronal systems, as well as those of autocrine and paracrine signals of endocrine or vascular cells in the human islet of Langerhans.

We performed immunohistochemical studies to visualize endocrine cells in histological sections of the mouse eye containing human islet grafts. Intraocular human islet graft cytoarchitecture and cell composition resembled those of human islets in the pancreas. We further examined human islet grafts with axonal markers and found that sympathetic axons could be seen traveling along blood vessels inside islet grafts. There was no parasympathetic innervation of the islet graft, and similar to the native human pancreas some endocrine cells expressed cholinergic markers. These innervation patterns were similar to those of islets in the pancreas. Our results suggest that mouse and human islet grafts have the intrinsic capability to reproduce their endogenous innervation patterns in the eye. The lack of innervation could also be explained by the species mismatch that does not allow mouse axons to penetrate human tissues. These incompatibilities, however, can be ruled out because other human tissues have been successfully transplanted into the eye, where they become strongly and appropriately innervated^{72,75-77}.

We transplanted human islets into the eye of streptozotocin-treated athymic nude mice and the animals achieved and maintained normoglycemia for over 150 days. Human C-peptide and glucagon blood levels changed after 12h fasting or during IPGTT, indicating that function of intraocular islet grafts was regulated by glycemia. Our results indicate that human islets transplanted into the eye of immune-deficient mice engraft and are fully functional, achieving tight metabolic control.

Vasculature of human islet grafts in the eye was visualized by injection of fluorescein dextran. Islet neovascularization was completed within 30 days post-transplant and time to diabetes reversal paralleled the progression of vascularization. We further observed that blood flow in intraocular human islet grafts is highly regulated, as shown in time-lapse recordings of blood flow inside the intraocular human islet grafts. The speed of red blood cells could be effectively estimated. In addition, we visualized changes in regional vascular perfusion within the islet grafts that were most common at vessel bifurcations, suggesting that these might be sites of local blood flow regulation.

Transplantation of human islets into the eye is a suitable tool to study human islets in the living organism. With this approach, human islet biology can be studied at the cellular level and correlated with glucose homeostasis. This will allow measuring the effects of modulatory compounds on human islet health and survival *in vivo*. Our results thus establish the basis for noninvasive *in vivo* investigations of complex cellular processes, like beta cell stimulus-response coupling, which can be performed longitudinally under both physiological and pathological conditions, along with beta cell survival and/or proliferation.

CONCLUDING REMARKS

Autonomic innervation of pancreatic islets is qualitatively and quantitatively different between mice and human. The sparse innervation of human endocrine cells suggests that the autonomic nervous system does not contribute directly to hormone secretion as in mice.

In contrast to mouse islets, human islets do not receive a dense parasympathetic nerve supply. Parasympathetic innervation may play an important role in mouse islet function but human islets are self reliant in terms of acetylcholine signaling.

Human alpha cells secrete acetylcholine as a non-neuronal paracrine signal priming beta cells.

Sympathetic innervation may regulate human hormone secretion by controlling intraislet blood flow.

Mouse and human intraocular islets grafts determine their own pattern of innervation.

The study of the revascularization process in mouse intraocular islets grafts reveals that islets restore the features of the native vascular endothelium microenvironment independently of the origin of neovascularization.

It is possible to manipulate the lineage of intraocular islet graft vasculature through interventions that activate, amplify, or sustain donor intraislet endothelial cells before and after transplantation. Thus, islets grafts can reinstate their native vascular-endocrine microenvironment.

The anterior chamber of the eye can be used as an experimental platform to investigate and manipulate the nervous input on islets and its effect on glucose metabolism.

Transplantation of human islets into the mouse eye is a suitable method to study human islets in the living organism.

Transplantation into the anterior chamber of the eye provides a versatile tool that enables longitudinal, noninvasive *in vivo* imaging of immune responses within target tissues with cellular resolution.

The anterior chamber of the eye has therapeutic potential as a novel transplantation site to treat type 1 diabetes.

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This drawing by Alejo.com of myself while -frankly- sleeping in one of our weekly videoconference meetings Miami-Stockholm shows two things... first: the reality of “my meetings” especially when talking about the fantastic world of cell signal transduction; and second: how relaxed, warm, comfortable and satisfying it has been to work as part of our team in USA with Alejandro Caicedo, my boss, mentor and supervisor at University of Miami in addition to great painter and friend. Thanks Alejo for being that gift from GOD in my career, thanks for your invaluable help with the papers, this thesis, and thanks for making our group an authentic team. Thanks Alberto, Carol, Danusa, Judith, Midhat, Natalia and Joana for being part of this amazing group, thanks for your help and contributions. We rock together!

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