Pancreatic Islets Communicate with the Brain via Vagal Sensory Neurons

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UNIVERSITY OF MIAMI

PANCREATIC ISLETS COMMUNICATE WITH THE BRAIN
VIA VAGAL SENSORY NEURONS

By
Madina Makhmutova

A DISSERTATION

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VIA VAGAL SENSORY NEURONS

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Sensory denervation affects pancreatic islet function, glucose metabolism and diabetes onset, but how islet endocrine cells interact with sensory neurons has not been studied. In this thesis work we attempted to anatomically and physiologically characterize vagal islet-brain pathway in search of the sensory modality that islet is transmitting to the brain. Here we show that the pancreatic islets are innervated by vagal sensory axons expressing substance P, calcitonin-gene related peptide, and serotonin receptor 5HT3R. Vagal neurons projecting to the pancreas terminate in the commissural nucleus of the solitary tract. These neurons respond to chemical but not mechanical stimulation of the pancreas. By recording activity from nodose neurons in vivo and from sensory axons in living pancreas slices, we show that sensory nerves respond to serotonin secreted from stimulated beta cells. Beta cell serotonin is co-released with insulin, in normal physiology promotes glucose uptake, and in conditions of increased beta cell activity, promotes insulin secretion. Hence, islet secretion of serotonin carries information on islet secretory state and capacity to accommodate appropriate glucose clearance, which fits in the bigger picture of peripheral serotonin role in energy metabolism. Our study thus establishes that pancreatic islets communicate with the brain using the neural route and identifies serotonin signaling as a peripheral transduction mechanism.
This work is dedicated to:

*The strongest women I know, my grandmother and my mother.*
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Chapter 1. Introduction

1.1 The biology of visceral sensory innervation

Interception, the ability to sense the internal state of the body, is an essential component of homeostasis maintenance. Interoceptive inputs are represented by a wide range of stimuli from all internal organs, and are transmitted to the brain via nervous, circulatory, and endocrine systems. Sensations of thirst, hunger, satiety, and nausea are just a few examples of the conscious processing of visceral sensory inputs, which motivates our behavior to fulfill bodily needs. Most of the time, however, visceral stimuli are processed unconsciously and trigger autonomic responses that maintain metabolic equilibrium. Analogous to exteroception, where five senses (vision, hearing, taste, smell, and touch) bring awareness of the surroundings, interoception informs the brain about the internal milieu.

The field of study of visceral sensations arose over a century ago infused by the seminal works of Claude Bernard, Charles Sherrington, and Ivan Pavlov, who demonstrated the importance of interoception on physiology, homeostasis, and behavior. However, studies of visceral sensations encountered many challenges. First, the measurement of visceral experience is something ephemeral, since the majority of interoceptive information is processed unconsciously. Second, physiological feedback loops triggered by visceral sensations are not easily distinguishable within the complex machinery of homeostatic maintenance. Third, the discovery of the efferent autonomic branches - parasympathetic and sympathetic systems - that trigger robust physiological effects, overshadowed more ephemeral afferent neighbors that were difficult to study (Gaskel, 1917; Langley, 1921). As a result, despite its physiological importance, the field of interoception over the years,
has not gained a lot of attention, and is still not widely recognized as a separate entity in sensory biology.

Viscera contains three types of sensory innervation: enteric, splanchnic (also referred to as “sympathetic sensory”), and vagal-pelvic (also referred to as “parasympathetic sensory”). Enteric sensory fibers are involved in the maintenance of the enteric nervous system autonomy and do not have central projections. Splanchnic and pelvic sensory fibers project to the brain in ascending spinal pathways, and are responsible mostly for unpleasant sensations induced by noxious stimuli (Ádám, 1998; Cervero, 1982, 1994; Grundy, 2002). Sensory fibers of the vagus nerve terminate in the nucleus of the solitary tract of the brainstem and are thought to contribute to autonomic regulatory functions and homeostasis maintenance (Ádám, 1998; Andrews, 1986; Cervero, 1982, 1994; Grundy, 2002). This study focuses on the role of sensory innervation in glucose metabolism, and therefore, it will also preferentially target vagal sensory system responsible for the autonomic regulatory functions.

1.2 Vagal sensory system

The vagus nerve projects from the brain stem all the way to the lower viscera, sending branches to most major organs in the thorax and the abdomen. The vagus is a mixed nerve that has both motor (efferent) and sensory (afferent) divisions and is mainly known for its parasympathetic motor branch. The cell bodies of these two divisions are spatially segregated into sensory nodose ganglion (NG), situated at the base of the scull and parasympathetic dorsal motor nucleus of vagus (DMV), located in the brainstem. Unlike the well explored “rest and digest” effect of the parasympathetic nervous system, the role
Figure 1. Autonomic innervation of visceral organs. Vagal afferent nerves (black) bring information from all internal organs through the nodose ganglion (NG) into the nucleus tractus solitarius (NTS). Descending projections from the NTS project to the dorsal motor nucleus of vagus (DMV), which brings parasympathetic efferent innervation (green) to all internal organs. Ascending projections from the NTS, among others, target parabrachial nucleus (PBN) and Hypothalamus (Hpy). PBN ascends further into Hpy and Thalamo-Cortical tract terminating in insular cortex. **Figure modified from Williams et al., (2016). Sensory Neurons that Detect Stretch and Nutrients in the Digestive System. Cell, 166(1), 209–221.**
of the afferent supply, which contains nine times more neurons, still remains elusive. This prevalent afferent component of the vagus nerve suggests that visceral sensory innervation significantly impacts homeostatic processes.

Indeed, nearly century old discoveries find support in modern studies, showing the role of vagal afferent innervation in physiology of visceral organ systems. Vagal afferents of the cardiovascular system have a strong influence on blood pressure and baroreceptor reflex (Aviado and Schmidt, 1955; Heymans, 1951; Heymans and Neil, 1958; Paintal, 1953; Whitteridge, 1948; Zeng et al., 2018); in the respiratory tract, vagal sensory neurons mediate breathing patterns (Adrian, 1933; Chang et al., 2015; Nonomura et al., 2017; Widdicombe, 1954); in the gastrointestinal (GI) tract, they are involved in modulation of gastric motility (Berthoud, 2008; Serdyukov, 1899; Williams et al., 2016). In addition to mediating these autonomic responses, vagal sensory nerves are implicated in the higher order cognitive circuits such as neuropsychological reward processing (Han et al., 2018). Besides highlighting the importance of vagal sensory innervation in the homeostasis maintenance, these studies also shine a light on the mechanisms of peripheral and central signal transduction.

Unlike exteroceptive sensory systems, that have specialized structures for stimuli detection (e.g. photosensitive cells, taste cells, hair cells, olfactory neuroepithelium, specialized skin receptors), vagal afferent fibers receive their input through the free nerve endings, and are generally classified into two major categories based on their sensitivity: mechanosensors and chemosensors (Berthoud and Neuhuber, 2000; Paintal, 1973). Visceral mechanosensitivity is primarily attributed to the mechanoreceptors Piezo1 and Piezo2 (in the respiratory and cardiovascular systems), and intricate branching structures
of intraganglionic laminar endings (IGLES) and intramuscular arrays (IMA) of the GI tract, receptive properties of which are still not fully understood (Nonomura et al., 2017; Powley and Phillips, 2002; Umans and Liberles, 2018; Zeng et al., 2018). Visceral chemosensation is very diverse based on the variety of chemoreceptors expressed by the nodose ganglion neurons (Kupari et al., 2019). In the hollow organs, free nerve endings can either be stimulated directly by the substances in the lumen (blood pH, inhalants in the respiratory tract, intestinal nutrients), or indirectly through the paracrine and synaptic interactions with cells facing the lumen, such as endocrine cells of the intestinal mucosa that synaptically couple to the vagal afferents via glutamatergic and serotonergic transmission (Bellono et al., 2017; Kaelberer et al., 2018). Sensory transduction pathways in the solid organs (pancreas, liver, and spleen), lacking luminal stimulation, have not yet been explored. Altogether, peripheral receptive properties segregate sensory vagus nerve into populations with distinct physiological functions, thus, implying some degree of coding at the level of the primary sensory neuron.

Internal organs are very diverse in their functions and chemical composition, yet, a single nerve is able to collect sensory information from all of this diversity. This means that the vagus nerve is either a mix of extremely diverse neuronal populations that are specifically tuned to sense different organs, or that the diverse organs evolved with a similar conduction mechanism that can be sensed by the same machinery of the vagus nerve. These theories are not contradicting and have support in the literature. A recent single cell RNA sequencing study of the nodose ganglion neurons divided vagal afferents into 18 clusters (Kupari et al., 2019), which reflect diversity of its innervation targets. However, 11 out of the 18 clusters represent only ~20% of vagal afferents, whereas the
majority of the nerves were distributed into 7 clusters, suggesting that sensory neurons expressing the same neuronal marker or receptor, might receive sensory input from very different tissues. Therefore, the coding capacity at the level of the primary sensory neuron exists, but is very limited and alone, cannot fully reflect the full spectrum of visceral sensory inputs, thus requiring higher order of coding for the brain to be able to make sense of this information.

Indeed, the terminal point of the vagal afferents in the brain, the caudal nucleus of the solitary tract (cNTS), contains subnuclei that receive information from the visceral organs in rostro-caudal topographical order, thus forming a map of internal organs, which is somewhat similar to the homunculus of the somatosensory cortex (Figure 1; Powley, 2000). In addition to the rostro-caudal axis, recent molecular studies of the GI vagal afferents determined that mechanosensory neurons terminate in the medial subnucleus of the cNTS, whereas chemosensory neurons project to the commissural cNTS, thus suggesting potential medio-lateral axis of coding mechano- versus chemoperception (Williams et. al., 2016). cNTS sends descending projections to the autonomic centers (parasympathetic dorsal motor nucleus of vagus and sympathetic spinal cell columns) which directly feedback to organ physiology (Powley, 2000). Ascending projections of cNTS travel primarily through parabrachial nucleus (PBN) to reach metabolic and emotion control regions of the brain (hypothalamus, stria terminalis, reticular formation, periaqueductal gray, amygdala), as well as serotonergic (raphe nuclei) and adrenergic (locus coeruleus) systems (Kawai, 2018). PBN also gives rise to thalamo-cortical projections that terminate in the insular and entorhinal cortex (Figure 1 and Figure 2). Interestingly, in primates, neurons from the NTS have direct thalamo-cortical projections,
that bypass parabrachial nucleus (Figure 2). This pathway is especially well developed in humans and is thought to strongly contribute to emotional and cognitive properties of human self-awareness (Craig, 2003; Mayer, 2011; Pritchard et al., 1986).

Afferent vagus is a complex sensory system that delivers to the brain variable sensory modalities from a diverse pool of organs. The visceral sensory input feeds back to the organ physiology and also contributes to more complex cognitive functions. Decoding visceral code has proven to be rather challenging, due to great variability of peripheral stimuli and unconscious information processing. By studying mechanisms of vagal nerve sensitivity in
different organs, we can gain a better understanding of visceral sensory modalities and visceral information processing.

1.3 Pancreatic sensory innervation

Recent neurobiological studies are revealing how the brain communicates with visceral organs. This includes the pancreatic islet of Langerhans, a mini-organ whose endocrine cells secrete insulin and glucagon, two hormones that are crucial for glucose homeostasis. At their discovery in the late 19th century, Paul Langerhans described that pancreatic islets are richly innervated (Langerhans and Morrison, 1937). Claude Bernard had reported earlier that puncturing the floor of the fourth ventricle induces diabetes (Bernard C, 1849). Since then, it is generally assumed that the brain helps control glucose homeostasis, presumably via autonomic nerves that regulate pancreatic endocrine function. It is not until recently, however, that detailed neuroanatomical studies revealed how the efferent branches of the autonomic nervous system, the sympathetic and parasympathetic nerves, innervate distinct cell populations within the islet (Almaça et al., 2018; Rodriguez-Díaz et al., 2011; Taborsky, 2011). By contrast, little is known about the sensory innervation of the islet. Because visceral sensory innervation is a crucial component of homeostatic regulatory circuits (Chang et al., 2015; Li and Owyang, 1993; Nonomura et al., 2017; Williams et al., 2016), there is a need to understand how islets signal to sensory fibers.

A wide body of literature shows that chemical and genetic sensory denervation affects physiologic and pathophysiologic processes in the pancreas, including insulin secretion and diabetes onset (Bou Karam et al., 2018; Gram et al., 2007; Ikeura et al., 2007; Karlsson et al., 1992; Li et al., 2013; Liddle, 2007; Motter and Ahern, 2008; Noble et al., 2006; Razavi et al., 2006; Riera et al., 2014; Warzecha et al., 2001). Little is known, however,
about the basic patterns of pancreatic sensory innervation and its transduction mechanisms. Tracing studies showed that the pancreas is innervated by vagal and spinal afferent axons (Berthoud and Neuhuber, 2000; Carobi, 1987; Fasanella et al., 2008; Neuhuber, 1989; Sharkey and Williams, 1983). In rodents, sensory fibers are localized preferentially to the periphery of the islet forming a dense superficial network (Gram et al., 2007; Hannibal and Fahrenkrug, 2000; Karlsson et al., 1992; Lindsay et al., 2006; Pettersson et al., 1986; Seifert et al., 1985; Sternini et al., 1992). Functional studies found that dispersed sensory afferent neurons of the vagal nodose ganglion respond to pancreatic and gastrointestinal stimuli, as well as to islet-derived stimuli (Ayush et al., 2015; Iwasaki et al., 2013, 2017; Peters at al., 2004; Simasko & Ritter, 2003). These findings suggest that the vagus nerve could sense the islet microenvironment, yet this notion has not been explored. We still don’t know what activates the vagal islet-brain axis and how changes in the islet microenvironment affect vagus activity.

1.4 Physiology of pancreatic islet and intra-islet signaling

Pancreatic islet represents a unique microenvironment with complex signaling circuits that are tuned to regulate adequate hormone secretion. Islets are embedded into the acinar tissue of the exocrine pancreas, and represent less then 2% of the entire pancreatic mass. Islets are composed of three primary cell types: alpha cells that produce glucagon, beta cells that produce insulin and delta cells that produce somatostatin (Figure 3). Insulin lowers blood sugar levels as it facilitates glucose transport into cells via the GLUT4 transporter and inhibits glucose release from internal storages. Glucagon increases plasma glucose levels by targeting glycogen breakdown in the liver and muscles, and fatty acid breakdown in the adipose tissue. Secretory products of endocrine cells engage in complex
paracrine interactions, and both, alpha and beta cells, activate themselves via positive autocrine loops, thus making hormonal secretion synchronous and effectively fast. Somatostatin by itself does not directly influence blood glucose levels, but it inhibits secretion of both insulin and glucagon, thus introducing negative feedback regulation of otherwise dangerous autocrine and paracrine loops (Caicedo, 2013; Gromada et al., 2018; Rodriguez-Diaz et al., 2018; Rorsman and Huising, 2018).

We wondered whether vagal afferents could be sensitive to hormonal secretion in the islet, however several reasons argue against this hypothesis. First of all, islet endocrine hormones upon secretion enter the bloodstream and immediately reach brain centers through circulation, thus the brain awareness of the pancreatic endocrine secretion comes from glucose and hormonal levels in circulation. Second, local concentration of hormones inside the islet is continuously high, and glycemia is regulated not by a single hormone at a time but by a hormonal ratio, which is not the most adequate stimulus for a nerve. Third, insulin receptor signaling is coupled to the PI3K/AKT pathway, barely affects the membrane potential, and is therefore difficult to reconcile with quick activation of neurons. Although somatostatin, could modulate pancreatic vagal afferents via its inhibitory action, insulin and glucagon are not the likely candidates for excitatory sensory nerve stimulation as they will not make this conduction informative for the brain.

Production and secretion of insulin and glucagon is coupled to production and secretion of several other signaling molecules that play a role in autocrine and paracrine regulation of endocrine cells. Insulin secretion is accompanied by secretion of C-peptide, neuropeptide Y (NPY), islet amyloid polypeptide (IAPP), ATP, GABA, and serotonin (Caicedo, 2013; Ekholm, Ericson, & Lundquist, 1971; Wang, Bennet, Wang, Ghatei, &
Figure 3. Blood glucose regulation and intra-islet signaling. Pancreatic islet orchestrates regulation of blood sugar levels by lowering insulin and increasing glucagon secretion in conditions of hypoglycemia, while increasing insulin and lowering glucagon secretion in conditions of hyperglycemia. Plasma insulin and glucagon trigger a cascade of events in the peripheral tissues, returning blood sugar levels to equilibrium.
In addition to glucagon, alpha cells also secrete glucagon-like peptide 1 (GLP1) and glutamate (Caicedo, 2013; Sandoval and D’Alessio, 2015). Serotonin, is one of the most interesting targets from this list as, in addition to its essential role in islet physiology, it is also one of the most important regulators of energy metabolism in the central and peripheral nervous systems. Thus, we decided to explore properties of serotonin in yet unexplored context of islet-brain communication. Although our attention is focused on serotonin, it is important to keep in mind that, other by-products of hormonal release could still have an effect on sensory fibers in the islet.

1.5 Serotonin and its role in regulation of islet secretory state

5-Hydroxytryptamine (5HT), widely known as serotonin, is a versatile signaling molecule that has potent roles in the central nervous system (CNS) and in the periphery. Serotonin is produced from essential amino acid tryptophan via two-step process of hydrolysis and subsequent decarboxylation, catalyzed by rate-limiting enzyme tryptophan hydroxylase (TPH) and enzyme aromatic amino acid decarboxylase (AADC). Serotonin signal transduction pathway is rather complex due to great variety of functionally diverse types of serotonin receptors, and additional receptor-independent intracellular signaling mechanisms (Paulman et al., 2009). Seven families of serotonin receptors are known, six of which (Htr1, Htr2, Htr4, Htr5, Htr6, Htr7) are G-protein coupled receptors that induce various excitatory and inhibitory downstream circuits, and one excitatory ionotrophic receptor Htr3 (El-Merahbi et al., 2015).

Within the pancreas, the insulin-producing beta cell is likely the sole source of serotonin, as it is the only pancreatic cell type that has the full machinery for serotonin
production, vesicular transport, and reuptake (Teitelman et al., 1987). Rate-limiting enzyme in serotonin production (TPH) shares parts of the transcriptional processing with insulin (Ohta et al., 2011). Moreover, serotonin is packaged into insulin vesicles and co-released together with insulin in glucose-dependent manner (Ohta et al., 2011). In normal physiology, local serotonin inhibits glucagon release through Htr1f receptor and affects glucose-stimulated insulin secretion via Htr3 receptor (Almaça et al., 2016; Kim et al., 2015). In conditions that involve change in beta cell mass, such as pregnancy, serotonin levels in the islet increase more than hundred-fold. This local serotonin increase serves as a paracrine signal to promote beta cell proliferation and to reduce threshold for beta cell glucose sensitivity trough Htr2b and Htr3 receptors, respectively (Goyvaerts et al., 2016; Kim et al., 2010, 2017; Ohara-Imaizumi et al., 2013). Thus, islet secretion of serotonin carries information on islet insulin secretory state and capacity to accommodate appropriate glucose clearance. But serotonin also carries a message beyond the islet biology, and to understand this message, we need to step back from the islet microenvironment and look at a big picture of peripheral serotonin signaling and its role in metabolism.

1.6 Peripheral serotonin as a signal of energy intake and appropriate capacity of peripheral tissues to process energy

Mammalian serotonergic system is widely known for controlling emotions and mood, and serotonin is frequently called “happiness hormone”. Yet, it is one of the most ancient and evolutionary conserved signaling pathways, essential functions of which go beyond modulation of emotional aspects and include regulation of feeding and maintenance of energy homeostasis (Donovan and Tecott, 2013; Gillette, 2006; Tecott, 2007; Weiger, 1997).
Interestingly, as organismal feeding behaviors evolved, so did the serotonergic system. In invertebrates, serotonergic neurons are spread throughout the nervous system. Their stimulation triggers feeding behavior through activation of multiple physiological mechanisms including foraging, ingestion, gut expansion, release of digestive enzymes, and energy storage. In line with that, in many invertebrates appetite and meal size are determined by the food availability and animal physical capacity to intake food (Donovan and Tecott, 2013; Gillette, 2006; Tecott, 2007; Weiger, 1997). Vertebrates, on the other hand, have more complex control of appetite, and evolved two separate serotonergic systems: central and peripheral. Central serotonergic system starts with Raphe nuclei of the brainstem that send widespread projections throughout the central nervous system (CNS), including the major metabolic centers (hypothalamus, parabrachial nucleus, and vagal nuclear complex). Peripheral serotonergic system is composed of serotonergic neurons of the enteric nervous system (ENS) but also extends into some non-neuronal tissues: enterochromaffin (EC) cells of the GI tract, adipocytes, and pancreatic beta cells, all of which are important regulators of energy metabolism (El-Merahbi et al., 2015; Gershon and Tack, 2007; Tecott, 2007). While activation of peripheral serotonergic system promotes feeding, just like in more primitive invertebrates, activation of the central serotonergic system inhibits appetite and reduces feeding behavior. Having opposing physiological effects, the two serotonergic systems are anatomically uncoupled from one another, as serotonin cannot cross blood-brain barrier. Therefore, the only way for the brain to be aware of the peripheral serotonin is through visceral sensory nerves.

Pancreatic beta cells share multiple developmental features with serotonergic neurons. An ancient Pet1 transcriptional cascade drives differentiation of both cell types, making
serotonin and insulin an evolutionary partners in regulation of energy homeostasis (Ohta et al., 2011). While serotonergic neurons (both CNS and ENS) rely on the enzyme TPH2 for serotonin production, non-neural tissues use enzyme TPH1. Interestingly, unlike other non-neural tissues, pancreatic beta cells contain both enzymes, further supporting the idea of their partnership with serotonergic neurons (El-Merahbi et al., 2015; Tecott, 2007). Luminal nutrients and glucose activate serotonergic circuits of the ENS, inducing peristalsis and secretory reflexes in the GI tract (Gershon and Tack, 2007). As glycemia increases after the meal, islet serotonin signaling promotes condition of glucose clearance, by stimulating insulin and inhibiting glucagon secretion (Almaça et al., 2016; Zern et al., 1980). Thus, in normal physiology, serotonin signaling in the ENS and the islet, accommodates energy intake.

High fat diet increases ENS serotonin signaling, duodenal contractility, and accelerates colonal transit in mice (Reichardt et al., 2013). Analogously, in the islet, high fat diet promoted glucose-stimulated insulin secretion (Kim et al., 2015; Ohara-Imaizumi et al., 2013). Moreover, islet serotonin content is proportionally corelated with BMI of human patients, and increases tremendously in pregnancy (Almaça et al., 2016; Goyvaerts et al., 2016; Kim et al., 2015). It appears that islet and ENS serotonin signaling increases in conditions when the amount of energy available for uptake (nutrients and glucose) is chronically higher than normal physiological capacity of the tissues to uptake and store this energy. In other words, high peripheral serotonin is the message of “energy sufficiency” and a signal that the peripheral tissues are working at their maximum capacity to uptake and store excessive energy. Thus, peripheral serotonergic system could complement leptin and ghrelin signaling in regulation of appetite and energy balance.
The ability of the vagus nerve to sense peripheral serotonin has been demonstrated repeatedly, and recently a synaptic connection between vagal afferents and EC cells was identified (Bellono et al., 2017; Blackshaw and Grundy, 1993; Grundy, 2002; Hillsley and Grundy, 1998). Most neurons in the vagal afferents express Htr3 serotonin receptors and glucose modulates its density in diet-dependent manner (de Lartigue, 2016). Vagal sensitivity to serotonin further supports the notion that serotonin signaling is a common thread running through the physiological processes of digestion and energy homeostasis (Berthoud, 2008; Browning, 2015; Gershon and Tack, 2007).

Altogether, peripheral serotonin has been shown to modulate the process of nutrient and glucose metabolism via local paracrine interactions in the GI tract and pancreatic islets. It has also been shown to send long-distance messages to the brain through activation of vagal afferents in response to enterochromaffin cells stimulation. It is possible that the afferent vagus nerve is the wire connecting peripheral and central serotonergic systems, and communicating to the brain peripheral capacity to intake energy from nutrients. Thus, serotonergic connection between the islet and the vagus nerve, that conducts information about islet secretory state and capacity, fits into the bigger picture of energy processing in the periphery.

1.7 Serum serotonin – an elephant in the room

While talking about paracrine effects of serotonin and building beautiful theories around local serotonin signaling, we cannot continue this literature review without acknowledging serotonin circulating in the blood. Over 90% of the total body serotonin is produced by enterochromaffin cells (EC) of the GI tract in response to metabolites and irritants of the intestinal lumen. EC cells release serotonin into the loose connective tissue of the lamina
propria, where it stimulates sensory axons and gets immediately taken up by enterocytes and absorbed into the bloodstream (Bellono et al., 2017; Gershon and Tack, 2007). In the circulation, serotonin is picked up and stored by platelets, thus concentration of freely circulating serotonin in the blood is quite low (in lean mice, it stays below 5µM; Sumara et al., 2012) and is precisely maintained (Gershon & Tack, 2007). This portable storage of serotonin can be mobilized upon activation of platelets thus having a capacity for temporal and spatial control of serotonin levels in circulation. Platelets get activated primarily in response to vessel injury (including injury induced by thrombosis and atherosclerosis), but also in response to multiple chemical triggers, one of which is blood glucose (Sudic et al., 2006; Yun et al., 2016; Dandona et al., 2010). Serum serotonin increases moderately during fasting, and in conditions of starvation its levels can spike up to 30µM, which is above the Kd for activation of most serotonin receptor throughout the body and thus could be masking paracrine serotonin signaling in the peripheral tissues (Sumara et al., 2012). On the other hand, acute glucose challenge and high fat diet reduce serum serotonin levels (Ho et al., 2013; M. Kim et al., 2013; Park et al., 2014), thus potentially augmenting peripheral paracrine interactions.

Serum serotonin plays numerous roles in many physiological processes and fluctuates in multiple physiological and pathophysiological conditions. Therefore, it is an important variable to keep in mind when discussing paracrine serotonin signaling pathways, since serum serotonin can certainly affect and modulate these paracrine interactions.

1.8 Concluding remarks and hypothesis

Growing evidence stresses the importance of interoception on physiology and homeostasis maintenance, especially in the field of metabolism. Enteric and islet
Figure 4. Serotonergic gut-brain axis. Peripheral serotonin is a vagal signal of nutrients/glucose availability and local stimulator of digestion and insulin secretion.
serotonergic systems promote digestion and glucose uptake. Thus, making serotonin a potential transducer of peripheral nutrient availability and nutrient sufficiency. Vagal afferent neurons are very sensitive to serotonin and are in perfect position to transduce a message of a molecule that cannot reach the brain otherwise through circulation (Bellono et al., 2017; Hillsley & Grundy, 1998; Y. Li et al., 2001).

Within the pancreas, the insulin-producing beta cell is likely the sole source of serotonin. Beta cells secrete serotonin to communicate with neighboring cells and to promote glucose uptake (Almaça et al., 2016; El-Merahbi et al., 2015; Goyvaerts et al., 2016; Kim et al., 2015, 2017; Ohara-Imaizumi et al., 2013; Ohta et al., 2011; Teitelman et al., 1987). We therefore hypothesized that pancreatic islets use serotonin as a signaling molecule to communicate to the brain via vagal afferents the message of islet secretory state and its secretory capacity. We used anatomical and physiological tools to characterize the sensory innervation patterns in the islet, to identify brain regions that receive pancreatic input, to examine neuronal response profiles in the nodose ganglion during pancreatic manipulation in vivo, and to demonstrate that vagal sensory neurons, innervating the islet, respond to serotonin released from beta cells.
Chapter 2: Materials and methods

2.1 Mouse Models

For \textit{in vivo} $\text{Ca}^{2+}$ imaging experiments we used Snap25-GCaMP6s (The Jackson Laboratory, stock nr. 025111) and Pirt-Cre mice crossed to floxed GCaMP6s mice (The Jackson Laboratory, stock nr. 024106). For \textit{ex vivo} $\text{Ca}^{2+}$ imaging experiments in peripheral sensory terminals, we used Pirt-GCaMP3 knock-in mice. Pirt-Cre and Pirt-GCaMP3 knock-in mice were kindly donated by Dr. Stephan Roper, University of Miami (originally obtained from X. Dong, Johns Hopkins). It has been previously reported that in the these mouse models Pirt promoter drives expression of GCaMP in all sensory neurons (Kim et al., 2014; Wu et al., 2015). Snap25 promoter is reported to drive expression of GCaMP in most neuronal types throughout the brain (Madisen et al., 2015). We found that in these mouse models GCaMP was expressed in all neurons of vagal sensory ganglia. Peripheral nerve terminals, however, could only be seen in Pirt-GCaMP3 knock in mice. Only F1 heterozygous mice were used, from both sexes, 8-30 weeks old. We did not observe any gender or strain differences in our results.

For characterization of sensory fibers innervating the pancreas we used Htr3a-GFP mice [GENSAT, Tg(Htr3a-EGFP)DH30Gs]. In these animals, GFP is expressed in cells that endogenously express the ionotropic serotonin receptor 5Ht3a. The pattern of transgene expression was previously validated by overlap with endogenous gene products by \textit{in situ} hybridization and immunostaining (Chittajallu et al., 2013; Dvoryanchikov et al., 2017; Inta et al., 2008). Breeder mice were kindly provided by
Dr. Nirupa Chaudhari, University of Miami (originally obtained from Dr. Chris McBain, National Institutes of Health).

To compare sensory and parasympathetic innervation patterns in the pancreas we used ChaT-eGFP mice (The Jackson Laboratory, stock nr. 007902).

Experiments were conducted according to protocols and guidelines approved by the University of Miami Institutional Animal Care and Use Committee.

2.2 Surgical exposure of the vagal sensory ganglion (nodose/petrosal/jugular complex) for imaging and injections

We used the same surgical approach, with slight adjustments, for in vivo Ca\(^{2+}\) imaging and for ganglion injections. In all procedures we exposed the left vagal ganglion. For in vivo Ca\(^{2+}\) imaging, mice were anesthetized with a combination of isoflurane (1-1.5%) and ketamine (60 mg/kg) and xylazine (5 mg/kg) throughout the surgery. Anesthesia levels were monitored throughout the surgery by hind paw withdrawal reflex. The combined anesthesia was necessary to maintain blood glycemia levels within the physiological range and to stabilize breathing. Anesthetized animals were placed in supine position on an infrared surgical warming pad (DCT-15, Kent Scientific) and the head was fixed in the head holder (SG-4N, Narishige). Tracheotomy was performed to facilitate respiration. The hypoglossal nerve was bluntly dissected and pulled to the lateral side with silk suture.

To access the ganglion, tissues were bluntly dissected using custom microdissection hooks. A first hook was placed on top of the trachea and common carotid artery at the level of the carotid bifurcation. This hook was secured to pull the tissues medially. A second hook was placed on top of the posterior belly of digastric muscle and the internal carotid and occipital arteries. This hook was secured to pull the tissues rostrally. A third hook was
placed on top of the sternomastoid muscle and blood vessels supplying it. This hook was secured to pull the tissues laterally. Minimum tension was applied during retraction to minimize obstruction of blood flow. The animal was not allowed to recover from anesthesia and was euthanized by cervical dislocation at the end of the imaging session.

For tracer injections into the ganglion, the animal was anesthetized with 2-3% isoflurane throughout the surgery. The exposure of the ganglion was performed as described above, but without tracheotomy. Viral tracers were injected into the ganglion as previously described (Chang et al., 2015). Briefly, we used a pulled glass micropipette (30-80 µm diameter) connected to an automated nanoliter injector (2010, WPI) operated by a micromanipulator (MP-85, Sutter). The ganglion was pierced by the micropipette and 2-4 pulses of 69 nl tracer were injected into the ganglion. The wound was sutured, and animals were allowed to recover in a pre-warmed cage. Postoperatively, animals were given buprenorphin 5 µl/g subcutaneously, twice a day for three days, to minimize pain and discomfort. Animals were sacrificed for tissue collection 4 weeks post-surgery. Viral particles for anterograde neuronal tracing (AAV2 444-UbC-GFP titer 4.16×10^{12} and AAV8 733-mCherry titer 1.3×10^{14}) were obtained from the Miami Project to Cure Paralysis Viral Core facility. We observed higher transfection efficiency of the nodose neurons with the AAV8 serotype compared to the AAV2 serotype.

2.3 Confocal imaging of the vagal sensory ganglion (nodose ganglion) in vivo

Anesthetized animals with the exposed left vagal ganglion were placed under a Leica TCS SP5 upright confocal microscope. Anesthesia levels were checked by the hind paw withdrawal reflex throughout the imaging session. We used a 10x/0.3NA dry objective with 11 mm working distance (#11506505, Leica) and resonant scanner for fast image
acquisition. Because the anatomical position of the intact ganglion is not flat, we imaged in XYZT mode, spanning up to 500 µm in the z-plane to capture as much of the volume of the exposed ganglion as possible. The temporal resolution in all our experiments was 3 s at a digital resolution of 512x512 pixels. GCaMP fluorescence was recorded at 488Ex/510-550Em. The tissue did not dry out due to intact vascularization. We checked ganglion blood perfusion by injecting intravenously the mouse with DyLight 594 labeled lectin (DL-1067, Vector Labs, 594Ex/ 610-650Em) or 3kDa TRITC dextran (D3308, ThermoFisher, 568Ex/590-630Em).

2.4 Infusion of the pancreas through the common bile duct

We used a previously described surgical approach of pancreas infusion for tracer delivery to the pancreas as well as for pancreas stimulation during in vivo Ca$^{2+}$ imaging of the nodose ganglion (Xiao et al., 2014). After abdominal midline incision, the common bile duct was exposed, and a small incision in the duodenum just below the ampulla of Vater was made using 31G needle. A 31G catheter (CMF31G, WPI) was inserted through the incision and through the ampulla of Vater into the common bile duct and clamped from the duodenal and hepatic sides using bulldog clamps. The catheter was connected to the 1 ml luer lock syringe controlled by an automated injector (55-2222, Harvard Apparatus).

For tracer delivery into the pancreas via intraductal infusion, the animal was anesthetized throughout the surgery with 2-3% isoflurane. The pancreas was infused with tracer [CTB-594 (0.05%, Sigma # C22842), Fast Blue (0.5 mg/ml, Polysciences #17740), AAV-retro (AddGene # 44361 and # 44362: pAAVrg-hSyn-DIO-hM3D(Gq)-mCherry titer 7×10^{12} vg/mL and pAAVrg-hSyn-DIO-hM4D(Gi) titer 1.4 × 10^{12})] at a rate of 6 µl/min until a final volume of 7.5 µl/g of body weight was reached. After the infusion, the catheter was
removed, muscle and skin were sutured, and the animal was allowed to recover in a prewarmed cage. Postoperatively, animals were given buprenorphine 0.1 mg/kg subcutaneously, twice a day for three days, to minimize pain and discomfort. One-week post injection, we observed CTB and Fast Blue tracers throughout the pancreatic tissue but not in the duodenum. Altogether, these observations indicate that the tracer injection was specific. Although all three tracers provided similar tracing efficiencies at the level of the nodose ganglion, the AAV-retro was labeling the whole pathway from the pancreas to the brain stem.

For the in vivo imaging sessions, anesthesia was maintained as described in the “Surgical exposure of the vagal ganglion” section. To achieve pancreas distension, 300 µl of physiological buffer was infused at a rate of 300 µl/min. For chemical stimulation without pancreatic distension, 150 µl of stimulus was infused at a rate of 300 µl/min. Between stimuli, the pancreas was infused with physiological buffer at a rate of 5 µl/min for at least 5 minutes to wash out the remnants of the stimulus and to maintain the ductal tonus. The total volume infused into the pancreas per imaging session did not exceed 1 ml to eliminate pancreatic tissue damage and leakage of stimuli into the peritoneum. At the end of the imaging session, mice were not allowed to recover from anesthesia and were immediately euthanized by cervical dislocation.

2.5 Pancreas exteriorization and topical stimulation

To exteriorize the pancreas, a small vertical incision was made on the left side of the mouse at the level of the spleen. Using a cotton swab, the tail of the pancreas and the spleen were gently exteriorized and placed on a 22 x 40 mm coverslip. The wound was sealed with silicone (KWIK-SIL, WPI) and a silicon border was built on the glass coverslip
around the exteriorized portion of the pancreas, creating a chamber. It was essential to exteriorize the tissue very gently to avoid damage of the fine neuronal connections. The pancreas was submerged in physiological buffer inside the chamber. Solutions were added to the chamber with a pipette and removed by gentle aspiration.

2.6 Preparation and imaging of living pancreatic slices

Acute pancreatic slices were prepared from Pirt-GCaMP3 transgenic mice for imaging of sensory axonal terminals, as previously described (Almaça et al., 2018; Marciniak et al., 2014; Weitz et al., 2018). After euthanasia, the abdomen was exposed and the pancreas infused through the common bile duct with 1.2% low gelling temperature agarose (39346-81-1, Sigma) dissolved in physiological buffer without BSA. After injection, the pancreas was extracted, cut into pieces, further embedded in agarose, and allowed to solidify at 4°C for 10 min. Pancreatic slices were cut on a vibratome (VT1000S, Leica) and incubated in physiological buffer (125 mM NaCl, 5.9 mM KCl, 2.56 mM CaCl$_2$, 1 mM MgCl$_2$, 25 mM HEPES, 0.1% BSA, pH 7.4) containing 3 mM glucose. Living pancreatic slices were placed in a perfusion imaging chamber (Warner Instruments) and imaged on a Leica TCS SP5 upright confocal microscope under continuous perfusion. GCaMP3 fluorescence was excited at 488 nm and emission detected at 510–550 nm. To identify pancreatic islets, we used the backscatter of 647 nm laser light. We recorded changes in GCaMP fluorescence induced by serotonin and KCl.

2.7 Intraperitoneal glucose tolerance test

Intraperitoneal glucose-tolerance tests (IPGTTs) were performed after overnight fasting. Mice were injected with 200–300 ml glucose solution (2 g/kg body weight) and blood glucose (IPGTT) was monitored at predetermined time points after the injection.
2.8 Immunohistochemistry

Prior to tissue collection, animals were anesthetized with ketamine/xyazine (100/10 mg/kg, IP) and perfused transcardially with 4% PFA. Collected tissues (pancreas, duodenum, right and left vagal sensory ganglia, and the brain) were post fixed in 4% PFA overnight at 4°C, dehydrated in 30% sucrose overnight at 4°C, snap frozen and sectioned on a Leica CM-3050S cryostat. Sensory ganglia were sectioned at 15-30 µm, while pancreas, brain and duodenum were sectioned at 40-50 µm. Sections were rinsed with 0.3% PBS-Triton X-100 and incubated in blocking solution (Universal blocking Reagent, Biogenex) in 0.3% PBS-Triton X-100. Afterwards, sections were incubated for 24 h at 20°C with primary antibodies diluted in blocking solution, rinsed and incubated with Alexa-Fluor conjugated secondary antibodies (1:500 in PBS) for 12-24 h at 20°C. Refer to the antibody table for the full list of primary and secondary antibodies. Slides were mounted with the VectaShield mounting medium (H-1000, Vector Laboratories). Confocal images of immunostained sections were acquired on an inverted Leica TCS SP5 confocal microscope.

2.9 Chemogenetic inhibition of sensory nerves

Pancreatic sensory nerves were transfected with chemogenetic AAVretro viruses (AddGene # 44361 and # 44362: pAAVrg-hSyn-DIO-hM3D(Gq)-mCherry titer $7 \times 10^{12}$ vg/mL and pAAVrg-hSyn-DIO-hM4D(Gi) titer $1.4 \times 10^{12}$), via intraductal viral delivery as described above. Six weeks after the surgery animals were subjected to a series of chronic injections of clozapine nitric oxide (CNO, Tocris #4936) 5mg/kg twice a day for 11 days. On the 12th day animals were fasted overnight and received the final dose of CNO.
2.5 hours before the start of IPGTT. IPGTT and blood collection was performed on the fasted animals as described above.

2.10 Confocal imaging of neuropeptide Y-pHluorin: a technique to visualize insulin granule exocytosis in intact murine and human islet

Insulin secretion plays a central role in glucose homeostasis under normal physiological conditions as well as in disease. Current approaches to study insulin granule exocytosis either use electrophysiology or microscopy coupled to the expression of fluorescent reporters. However most of these techniques have been optimized for clonal cell lines or require dissociating pancreatic islets. In contrast, we developed the method which allows for real time visualization of insulin granule exocytosis in intact pancreatic islets (Almaça et al., 2015; Makhmutova et al., 2017).

Human pancreatic islets were obtained from the Integrated Islet Distribution Program (NIDDK, NIH). Upon arrival, islets were transferred into CMRL culture media (Connaught Medical Research Laboratories #1066, 10% (v/v) FBS and 2 mM L- Glutamine) at 37°C, 5%/95% CO2/O2 for 24h before viral infection. Mouse pancreatic islets were isolated following previously established protocols and handled identical to human islets.

Viral vectors were cloned in the laboratory of dr. Gaisano at the university of Toronto. The NPY-pHluorin fusion was cloned into the pcDNA3 vector and subcloned into an adenoviral vector for adenoviral production [adenovirus serotype 5 (DE1/E3)] by a recombinant adenovirus manufacturing company. The virus was aliquoted and stored at -80 °C. The viral stock is provided by the company at titers of $10^{12}$ to $10^{13}$ viral particles (~$3 \times 10^{10} - 3 \times 10^{11}$ PFU).
Islets were infected with adenoviruses containing NPY fused to the pH-dependent green fluorescent protein (GFP) pHluorin (5–10ul of viral stock added in 2ml CMRL culture medium containing ~200 islets) for 48 h and cultured at 37°C for 5–7 days in CMRL 1066 (Cellgro, Herndon, Virginia, USA), 10% (vol./vol.) FBS and 2 mmol/l L-glutamine. Approximately 20–40% of the islet cells were infected. Most of the infected cells showed no background fluorescence, with few granules visible at basal glucose concentration (3 mmol/l) in the absence of stimulation. Some cells had increased background fluorescence in the cytoplasm but did not show secretory events upon stimulation and were therefore not included in the analyses.

Islets were placed on a coverslip in an imaging chamber (Warner instruments, Hamden, CT, USA) for imaging on a Leica TCS SP5 upright laser-scanning confocal microscope (Leica Microsystems, Wetzlar, Germany). Islets were continuously perfused with extracellular solution (in mmol/l: 125 NaCl, 5.9 KCl, 2.56 CaCl2, 1 MgCl2, 25 HEPES, 0.1% BSA, 3 mmol/l glucose, pH 7.4, 37°C) and confocal images were acquired with LAS-AF software (Leica Microsystems) using a 63× water immersion objective (HCX APO L 63×/0.9 NA). We used a resonance scanner for fast image acquisition to produce time-lapse recordings spanning 50 μm of the islet (z-step: 5 μm, stack of ten confocal images with a size of 512×512 pixels) at 1.5 s resolution (XYZT imaging). NPY-pHluorin fluorescence was excited at 488 nm and emission detected at 535–550 nm. Insulin granule exocytosis was stimulated by increasing glucose concentration from 3 to 16 mmol/l, in the presence of cAMP-raising agents 3- isobutyl-1-methylxanthine (IBMX) (100 μmol/l) and forskolin (10 μmol/l) [5] or by KCl depolarization (30 mmol/l). cAMP-raising agents increased the consistency of secretory responses but did not change temporal patterns of
granule secretion so that secretory events appeared in discrete bursts synchronized with the average cell response (see below) and with similar burst periods (range 1.4–6.6 min with IBMX/forskolin vs 1.5–10 min without). Intracellular pH was increased by using 50 mmol/l NH₄Cl (to replace NaCl on an equimolar basis) and the plasma membrane was labelled with di-8-ANEPP (2 µmol/l for 1 h at 37°C; Invitrogen), excited at 488 nm and detected at 620 nm. ImageJ (http://imagej.nih.gov/ij/) was used to determine changes in fluorescence intensity over time in regions of interest (ROI) placed around single secretory events in confocal planes (see electronic supplementary material [ESM] Methods for a complete description of imaging data analyses). To identify sites of preferential secretion, we plotted fluorescence intensity along cell membranes and determined the number of secretory events that reappeared at the same site in two secretory bursts (adaptation from the birthday problem). Temporal stacks of images acquired during 30 s (~20 images) were used to calculate the CV of the fluorescence signal in the cell, according to the formula: CV = SD/mean. Higher CV values indicate higher concentration of fluorescence signals. Pearson’s correlation coefficient was used to determine colocalization of secretory events in 30 s temporal stacks from two bursts.

Infected islets were placed on a coverslip on an imaging chamber and imaged under an upright laser-scanning confocal microscope while being continuously perfused with extracellular solution containing stimuli, listed below. Confocal images spanning 50 µm of the islet were acquired as time-lapse recordings using a fast-resonant scanner. The fusion of insulin granules with the plasma membrane was followed over time. This procedure also allows for testing a battery of stimuli in a single experiment, is compatible with both mouse
and human islets, and can be combined with various dyes for functional imaging (e.g., membrane potential or cytosolic calcium dyes).
Chapter 3: Data presentation, analyses and statistics

3.1 Quantification of sensory innervation

Immunohistochemistry images are presented as z-stack of confocal images, 8-10 images in a 40 µm section, z step = 4 µm. We used ImageJ software (https://imagej.nih.gov/ij/) to quantitify the density of sensory innervation in the pancreatic islet and islet-surrounding exocrine tissue. For this quantification we used substance P immunostaining, which of all sensory markers tested provided the best signal to noise ratio with minimal background staining. We quantified mean gray value of substance P fluorescence in the maximal projections of confocal planes spanning the islet and surrounding exocrine tissue. Regions of interest in the islet were selected based on glucagon staining and dense DAPI staining. For the exocrine tissue, regions of interested were selected based on homogeneous DAPI staining of acini, avoiding endocrine, ductal, or blood vessel structures. Mean gray values of axon staining were calculated for several mouse strains (Figure 12). Average of mean gray values per animal are reported in Figure 5C.

3.2 Quantification of retrogradely traced neurons in the nodose ganglion

We used ImageJ software (https://imagej.nih.gov/ij/) to estimate the number of neurons labeled with the retrograde tracer. Data were calculated as percentage of nodose ganglion neurons stained with general neuronal marker NeuN in confocal planes.

3.3 Quantification of cytosolic Ca$^{2+}$ Levels

To quantify changes in intracellular Ca$^{2+}$ levels, we manually selected regions of interest (ROIs) around individual nodose ganglion neurons using the imageJ plugin Cell Magic Wand, developed by Fitzpatrick lab at the Max Planck Florida Institute. We only
included neurons that expressed either stimulus-evoked or baseline Ca\textsuperscript{2+} responses. Thus, ROI selection was biased towards bright and responding neurons.

We measured changes in mean GCaMP fluorescence intensity using ImageJ. We used custom MatLab script for data analyses. Changes in fluorescence intensity were expressed as percentage changes over baseline (dF/F). The baseline was defined as the mean of the intensity values during the non-stimulatory control period of each recording. Most neurons exhibited Ca\textsuperscript{2+} signals during the non-stimulatory control period (baseline activity) as well as stimulus evoked responses. The strong baseline activity made it difficult to distinguish responders from non-responders based on amplitude and area under the curve, giving many false negatives and not adequately reflecting the data. For this reason, data were displayed as heatmaps including all recorded neurons. In the heatmaps, the response incidences, magnitudes and kinetics are visualized simultaneously. We further reported percentage of responding neurons based on inspection of individual Ca\textsuperscript{2+} traces by three blinded independent observers.

To analyze response kinetics, we took the average of the 15 top responding neurons for each stimulus and normalized these averages to 100% (Figure 8E and 8F). Using GraphPad Prism, we then fitted a sigmoidal curve over each average trace. For each sigmoidal curve we calculated slope coefficient and time to peak (Table 2).

3.4 Data analyses

Sample sizes are indicated in main text, figure legends, or heatmaps (numbers in parentheses). Significance was determined by comparisons to the paired control group using paired t-test (Figures 5 and 9), multiple t-tests (Figure 15), or between indicated groups using a two-tailed Mann-Whitney test (Figure 11). All experiments involved
biological, not technical replicates. We considered statistical significance when P values were lower than 0.05.
Chapter 4. Pancreatic islets communicate with the brain via vagal sensory neurons

4.1 The mouse pancreas is innervated by vagal afferent neurons that project to the commissural nucleus of the solitary tract

We investigated the anatomical components of the vagal pancreas-brain axis (Figure 5A) using immunohistochemistry and neuronal tracing. To examine the pattern of sensory innervation of the mouse and human pancreas we used immunohistochemistry for the common sensory neuronal markers substance-P and calcitonin-gene related peptide (CGRP; Figures 5B-5E). Islets were innervated by substance P and CGRP immunoreactive axons. The density of innervation within the islet was significantly higher than in the surrounding exocrine tissue (Figures 5B and 5C). CGRP-labeled axons did not express choline acetyltransferase (ChAT), a marker for parasympathetic efferent terminals (Figures 5D and 5D’). We did not observe any gender or strain differences within the analyzed mouse strains (129, BALB6-C, C57Bl6; Figure 12). However, males of the non-obese diabetic (NOD) mouse strain had a significantly lower density of sensory fibers within the islet (Figure 12). Human pancreatic islets were similarly innervated by substance P immunoreactive axons (Figures 5E and 5E’).

To identify vagal sensory neurons projecting to the pancreas and examine their terminal fields in the brain, we performed retrograde tracing by infusing the pancreas with tracers through the common bile duct, as previously described (Guo et al., 2014; Figure 6). We compared tracing efficiencies of three tracers: fast blue (Figures 6A and 6B), cholera toxin subunit beta (CTB, Figure 6C), and retrograde AAV-hSyn-mCherry (Figures 6D and 6E). We obtained similar results with all three tracers at the level of the nodose ganglion (the
Figure 5. Mouse and human pancreatic islets are innervated by SP- and CGRP-positive sensory fibers

(A) Cartoon representation of the vagal islet-brain axis.
(B) Mouse pancreas section showing the innervation pattern of substance P-positive sensory axons (green) in an islet. Endocrine alpha cells labeled for glucagon are shown in red. Scale bar, 20 µM. All immunostaining figures are shown as z-stacks of confocal images.
(C) Quantification of the mean fluorescence intensity of substance P immunostaining in the islet and the surrounding exocrine tissue (n = 18 mice, 3-7 islets per animal; mean +/- SEM, paired t-test; for data showing innervation densities in mice of different genders and strains, see Figure 12).
(D-D’') Innervation pattern of CGRP-positive sensory axons (green) and cholinergic parasympathetic axons (red) in an islet of a ChAT-GFP reporter mouse. Scale bar, 20 µm.
(E-E’’) Innervation pattern of substance P-positive sensory axons in a human pancreatic islet (green). Arrows point at axon varicosities at the border of the islet. Scale bar, 20 µm.
vagal sensory ganglion), with CTB tracing showing a lower tracing efficiency (Figures 6B and 6C). Retrograde tracing from the pancreas to the nodose ganglion using CTB labeled 72 +/- 9 neurons in the right ganglion and 100 +/- 8 neurons in the left ganglion (n = 3 mice). In line with previous observations we could not find strict topographic organization of these neurons (Fasanella et al., 2008; Zhuo et al., 1997). We estimated that from 2–6% of nodose neurons were traced from the pancreas. By contrast, 50% of nodose ganglion neurons were traced when CTB was injected intraperitoneally (Figure 6C). Neurons in the trigeminal ganglion could not be traced from the pancreas (negative control, Figure 6C).

Although fast blue and CTB-traced neurons were seen in the nodose ganglion, we could not identify the terminal fields of these neurons in the brain. The retrograde AAV-hSyn-mCherry tracer, by contrast, worked efficiently to trace the whole pathway from the pancreas to the brain (Figures 6D and 6E). We found that the central projections of pancreas-innervating vagal sensory neurons terminated in the commissural regions of the caudal nucleus of the solitary tract.

We next sought to identify projections of vagal sensory neurons in the pancreas. We performed anterograde tracing by injecting AAV-CMV-mCherry into the nodose ganglion, as described (Chang et al., 2015). Four weeks after tracer injection, sensory fibers could be traced to the pancreas, thus confirming previous findings (Neuhuber, 1989). Traced fibers were also seen in the brain and duodenum, matching the pattern previously reported (Chang et al., 2015; Figure 13).
Figure 6

(A-A’) Section of a mouse nodose ganglion showing retrogradely traced neurons (green) 1 week after intraductal pancreas infusion of the neuronal tracer fast blue. NeuN staining (red) visualizes all neurons.

(B) Quantification of data as in A showing the percentage of neurons that is traced with Fast Blue in the left nodose ganglion and trigeminal ganglion (negative control); (n = 5 mice, 5-9 sections per ganglion).

(C) Quantification of the percentage of neurons traced with cholera toxin B (CTB) in the left nodose ganglion after IP injection of the tracer (positive control) and the left and right nodose ganglia and trigeminal ganglion (negative control) after intraductal pancreas infusion of CTB (n = 3 mice, 5-9 sections per ganglion).

(D-D”) Sequential images of mouse brainstem sections (rostral to caudal) 4 weeks after intraductal pancreas infusion of the neuronal tracer AAV-retro-hSyn-mCherry. Traced fibers are shown in green. Cytoarchitecture of the brainstem is identified with Nissl stain (blue).

(E) Section at the level of the caudal commissural NTS containing the final terminal field of neurons traced from the pancreas. Scale bars in all panels, 100 µm.
4.2 *In vivo* imaging of neuronal activity in the intact nodose ganglion in response to pancreas-specific stimulation

To characterize sensory innervation of the pancreas physiologically, we adapted a technique for *in vivo* imaging of the nodose ganglion (Williams et al., 2016). We developed a surgical approach that allowed imaging of cytoplasmic Ca\(^{2+}\) of neurons in the intact nodose ganglion without cutting the central branch of the vagus nerve (Figures 7A-7C). Using this method, we not only recorded stimulus-induced neuronal responses, but also observed that most vagal sensory neurons had a baseline activity that disappeared after cutting the central branch.

Specific sensory neuronal markers for the population of vagal neurons innervating the pancreas have not been identified yet. Thus, we recorded activity from all nodose neurons using mice expressing the Cre-dependent Ca\(^{2+}\) indicator GCaMP6 driven by the general sensory neuronal promoters Pirt or Snap25 (Madisen et al., 2015; Shin et al., 2014; Figure 7C). There was no difference in Ca\(^{2+}\) responses between the two strains. To stimulate the pancreas specifically and to control for non-specific responses induced outside of the pancreas, we infused stimuli either through the common bile duct (intraductally) or applied them topically to the exteriorized pancreas (Figure 7A). Intraductal administration of stimuli accesses the pancreas “inside out”. This procedure allows the stimuli to fill the pancreatic ductal system and diffuse into the periductal space, where the majority of the pancreatic islets are located. Topical stimulus administration, by contrast, accesses the pancreas “outside in” and primarily diffuses into exocrine tissues. Using this approach, responses could be clearly detected in the nodose ganglion neurons, with a strong signal to noise ratio (Figures 7D and 7E).
Figure 7. In vivo imaging of the intact nodose ganglion in response to pancreas-specific stimulation

(A) Cartoon illustrating the experimental setup, where the intact nodose ganglion is exposed for Ca\(^{2+}\) imaging under a confocal microscope and the pancreas is exteriorized for topical stimulus application. See Methods for detailed description of surgeries.

(B) Microphotograph of the exposed nodose ganglion.

(C) Confocal image of the nodose ganglion from a Pirt-GCaMP6 mouse, which expresses the Ca\(^{2+}\) indicator GCaMP6 in all nodose ganglion neurons.

(D) Sequential images of nodose ganglion neurons displaying Ca\(^{2+}\) responses (changes in GCaMP6 fluorescence, green) to topical stimulation of the pancreas with substance P (SP). Scale bars in C and D, 100 µm.

(E) Representative traces of neurons recorded in D responding to 5HT (1 mM), SP (100 µM), or both.

(F) Time point after topical application at which the mean fluorescence intensity was at least 3 SD above baseline fluorescence fluctuation (n = 3 animals).
To control for systemic distribution of topically applied substances, we applied a mixture of a fluorescent dextran (3 kDa) together with a chemical stimulus (substance P, 100 µM) to the exteriorized pancreas. We recorded neuronal Ca\textsuperscript{2+} responses and the appearance of the dextran fluorescence in the ganglion (Figures 7F and 7G). The average latency of the neuronal response was smaller than that of the appearance of a dextran in the circulation (32 ± 2 s versus 107 ± 25 s, Figure 7G). Cervical vagotomy eliminated responses to topical administration of substance P (Figure 7H). These observations indicate that topical administration of chemical substances to the pancreas elicits specific responses in peripheral axonal terminals. As shown below, the combination of two different stimulation approaches (intraductal and topical) together with proper controls made it possible to identify and study sensory mechanisms of vagal sensory transmission from the pancreas.

4.3 Vagal afferent neurons innervating the pancreas are chemosensors

Using \textit{in vivo} imaging of the nodose ganglion, we assessed the responsiveness of vagal sensory neurons to mechanical and chemical stimulation of the pancreas (Figure 8). Pancreata were stimulated mechanically either by light touch with forceps and gentle rinse.
**Figure 8. Pancreatic vagal afferents are chemosensors**

(A) Spatial map of GCaMP6 fluorescence signals in the nodose ganglion at the peak of the response to intraductal application of exendin-4 (Ex-4, 10 µM, red), serotonin (5HT, 1 mM, blue), and carbachol (CCh, 100 µM, green). Background fluorescence is shown in gray. Scale bar, 100 µm.

(B) Representative heatmap, showing color-coded dF/F Ca$^{2+}$ responses of 129 single nodose ganglion neurons to pancreatic stimulation with indicated stimuli. Each row is a single cell, x-axis is time, color scale is dF/F (%), where fluorescence intensity increases from blue to red.

(C) Series of heatmaps comparing nodose ganglion Ca$^{2+}$ responses to intraductal and topical administration of mechanical and chemical stimuli to the pancreas. Number of neurons/number of animals are indicated under each heatmap. dF/F color scale also applies to (B).

(D) Average traces of the top 15 responding neurons shown in (C). Ductal stimulation responders shown in black, topical stimulation responders shown in red.

(E and F) Average traces as shown in (D), normalized to 100%, of responses to topical stimulation (E) or intraductal stimulation (F) of the pancreas with indicated stimuli.
of the exteriorized portion of the pancreas (mimicking the conditions of topical stimulus application) or by stretching the pancreas via intraductal injection of saline at a rate of 300 µl/min for 30-60 s (twice the pressure at which chemical stimuli were applied intraductally). In contrast to what has been described for sensory neurons innervating the stomach where nearly 70% of neurons respond to stretch (Williams et al., 2016; Zagorodnyuk et al., 2001), few neurons responded to mechanical distention of the pancreas (~16%, 31/193, n = 3 mice; Figures 8B and 8C). The responses to mechanical stimuli were uncoordinated, had low amplitude, and were barely distinguishable from baseline neuronal activity. This lack of mechanical sensation is in line with the notion that the pancreas does not distend or contract physiologically.

We then explored the chemosensitive properties of pancreatic vagal afferents. We selected stimuli known to activate receptors on sensory axons directly (e.g. substance P and serotonin), activate pancreas tissues (e.g. carbachol), or both (e.g. cerulein and exendin-4). We tested a total of 15 compounds of which the aforementioned ones consistently elicited responses (Table 1). The route of stimulus administration (topical versus intraductal) affected the magnitude, kinetics, and incidence of responses to serotonin and carbachol (Figures 8C and 8D). Serotonin was more effective when applied intraductally, while carbachol was more effective when applied topically. Substance P, cerulein, and exendin-4, by contrast, elicited responses in a similar number of neurons regardless of the delivery route (Figure 8C).

Based on the response kinetics we could distinguish synchronized responses with short latency and fast rise times versus and non-synchronized responses with longer latencies and slower rise times (Figures 8E and 8F, Table 2). Responses to substance P and cerulein
were fast and had similar times to peak and slope coefficients, irrespective of the administration route. Carbachol, whether delivered topically or intraductally, elicited slow responses. The kinetics of the responses to serotonin and exendin-4 depended on the administration route. When applied intraductally, serotonin and exendin-4 elicited responses that were as fast as those to substance P and cerulein.

The numbers of responding neurons, the magnitude of the response, as well as the response kinetics suggest that serotonin, substance P, and cerulein directly activate their cognate receptors known to be expressed by vagal afferent neurons ($5HT_3R$, Tac1, and Cckar, respectively; Kupari, et al., 2019). The slower kinetics of the responses to carbachol suggest an indirect effect, for instance through activation of the exocrine tissue which then stimulates sensory nerves. Serotonin stimulated sensory neurons much faster and more efficiently when applied intraductally, indicating that serotonin-responsive axons were located closer to the pancreatic duct. Of note, the majority of pancreatic islets are aligned along the major pancreatic ducts.

4.4 *Mouse pancreatic islets contain serotonin*

Because vagal afferents in the pancreas were sensitive to serotonin, we searched for the endogenous source of serotonin that could activate these neurons. Although serotonin derived from enterochromaffin cells is released into the blood, most of it is stored in platelets and only a small fraction remains freely circulating (El-Merahbi et al., 2015). It is known that beta cells produce and secrete serotonin as a paracrine and autocrine signal (Almaça et al., 2016; Kim et al., 2015; Ohara-Imaizumi et al., 2013). Although serotonin levels are lower in mouse beta cells than in human beta cells (Almaça et al., 2016), basal beta-cell serotonin levels play a role in glucose stimulated insulin secretion, which is more
Figure 9. Mouse pancreatic islets contain serotonin
(A) Section of mouse pancreata showing that serotonin immunostaining (green) is limited to the islets and not detected in the exocrine tissue. Scale bar, 50 μm.
(B) Section of mouse pancreata shown in A, contains beta cell insulin immunostaining (red).
(B’) Enlarged view of the pancreatic islet shown in A and B, reflecting overlap in serotonin and insulin immunostaining (yellow).
(C and C’) Section of mouse pancreata showing islet immunostained with serotonin (green, C) and Tph1 (red, C’).
(D) Sections of mouse pancreata showing that serotonin immunostaining (green) varies between islets, Scale bar, 50 μm.
(E) Quantification of serotonin immunostaining as shown in (D). Immunostaining levels in endocrine regions (islet) were compared to those in exocrine regions (Exo) (n = 6 mice, 4-7 regions per animal; mean +/- SEM, paired t-test). Grey symbols represent values from all examined regions.
(E and F) Average traces as shown in (D), normalized to 100%, of responses to topical stimulation (E) or intraductal stimulation (F) of the pancreas with indicated stimuli.
evident under high fat diet-induced metabolic stress conditions (Kim et al., 2015). We therefore investigated if under normal physiological conditions, serotonin is produced and released from mouse pancreatic islets (Figure 9). Although variable, serotonin immunostaining in pancreatic islets was significantly higher than background levels in exocrine regions (Figures 9A and 9D, Figure 9). Serotonin was present primarily in the beta cells (Figures 9B and 9B’). No other region of the pancreas showed serotonin immunostaining (Figures 9A and 9D). We also detected the tryptophan hydroxylase-1 (Tph-1), the rate-limiting enzyme in serotonin synthesis, in islets (Figures 9C and 9C’). These data indicate that mouse pancreatic islets contain serotonin and machinery necessary for its production.

4.5 Serotonin activates axonal terminals in the pancreatic islet

The ionotropic 5HT₃ receptor is a prominent serotonin receptor in vagal afferent neurons (Browning, 2015; Browning and Mendelowitz, 2003; Lacolley et al., 2006). We used transgenic mice in which the promoter for the 5HT₃ receptor drives the expression of GFP (5HT₃R-GFP mice; Chittajallu et al., 2013; Inta et al., 2008) to assess the presence of this receptor in sensory axons innervating the pancreatic islet. We found a high density of GFP-labeled fibers around and inside most examined islets (Figure 10A). These fibers also expressed the sensory axon marker CGRP (Figure 10A’ and 10A’’). To directly determine whether serotonin elicited responses in sensory fibers we adapted a living pancreas slice approach that enables studying the function of different tissue compartments in a preserved pancreas environment (Weitz et al., 2018). We prepared pancreas slices from Pirt-GCaMP3 mice and recorded activity of sensory fibers in the islet (Figures 10B-10E).
Figure 10. Serotonin activates axonal terminals in the mouse pancreatic islet
(A-A”) Pancreatic section from a 5HT3r-GFP reporter mouse showing an islet innervated by 5HT3r-expressing fibers that also immunostain for CGRP (red). Scale bar, 20 μm.
(B) Z-stack of confocal images of a living pancreatic slice from a Pirt-GCaMP3 mouse. Sensory fibers (green) can be seen in an islet visualized by backscatter (red). Scale bar, 20 μm.
(C) Sequential images of sensory fiber shown in (B) displaying a Ca²⁺ response to serotonin (5HT, 50 μM) perfused over the slice. Arrow points to a region showing an increase in GCaMP3 fluorescence (from blue to red in pseudocolor scale).
(D and E) Representative traces of mean fluorescence intensity changes in the axonal terminals inside the pancreatic islet demonstrating that axonal terminals respond to an increase in glucose concentration (from 3 mM to 16 mM) and to 5HT (50 μM) stimulation.
We found that sensory fibers responded to stimulation of islet endocrine cells with high glucose concentration as well as to stimulation with serotonin (50 µM, Figures 10C-10E). We confirmed that vagal afferent neurons that express 5HT₃ receptors in the nodose ganglion project to the pancreas by retrograde CTB tracing in 5HT₃R-GFP mice (Figure 10F). Using in vivo Ca²⁺ imaging of the nodose ganglion, we further determined that topical application to the pancreas of the 5HT₃R antagonist Y-25130 (1 mM) diminished the spontaneous activity of a subpopulation of nodose neurons (~30%), but did not affect responses to substance P or cerulein (Figure 10G). These findings not only show that vagal sensory neurons innervating the islet express 5HT₃R and are able to sense serotonin directly, but also that their baseline activity partially originates from endogenous pancreatic serotonin signaling.

4.6 Vagal sensory neurons respond to serotonin secreted from activated beta cells

We next assessed if vagal sensory neurons respond to serotonin derived from stimulated beta cells. Using in vivo Ca²⁺ imaging of the nodose ganglion, we recorded neuronal activity in response to topical application of tolbutamide to the pancreas (5 mM; Figure 11). Tolbutamide is a sulfonylurea that closes K<sub>ATP</sub> channels, thus depolarizing beta cells and stimulating insulin secretion. Applying tolbutamide elicited small responses in a subpopulation of vagal sensory neurons (17%, n = 4 mice; Figures 11B, 11C, 11G, and 11H). Responses were delayed compared to those to substance P, suggesting that tolbutamide was acting indirectly via activation of beta cells. Topical application of the 5HT₃R antagonist Y-25130 inhibited responses to tolbutamide, but not to cerulein (Figure 11H).
We reasoned that the responses to tolbutamide were infrequent because islet endocrine cells represent a small fraction of the pancreatic mass (1-2%; Goodman, 2009). To amplify the effects of beta cell stimulation, we injected mice with the serotonin precursor 5-hydroxytryptophan (5HTP, 30 mg/kg; IP Zern et al., 1980). This treatment strongly and selectively increased serotonin levels in islets (Figures 11D and 11D’) but had only minor effects on glucose tolerance, as measured by intraperitoneal glucose tolerance test (12-24 hours after injection; Figure 15). In 5HTP-preloaded mice, the size and incidence of the responses to tolbutamide increased (>30% of vagal sensory neurons; Figures 11E-11G). 5HTP-preloading did not affect the magnitude and incidence of the responses to substance P and cerulein. These results demonstrate that beta cells use serotonin to communicate with vagal sensory neurons.

Figure 11. Vagal sensory neurons respond to serotonin secreted from beta cells
(A and D) z-stacks of confocal images of pancreatic sections from untreated mice (A) and mice preloaded with 5HTP (30 mg/kg; D). Serotonin immunostaining (green) is increased in islets (glucagon in red) from treated mice. (A’) and (D’) are higher magnifications of (A) and (D). Note that endogenous levels of serotonin in control tissue are not visible because imaging settings were adjusted for the increased serotonin levels in 5HTP-preloaded tissue. Scale bars, 1mm (A, D) and 50µM (A’, D’)
(B and E) Representative traces of Ca++ responses of nodose ganglion neurons to topical stimulation of the pancreas with tolbutamide (5 mM) and substance P (100 µM) in control (B) and 5HTP-preloaded (E) animals.
(C and F) Heatmaps showing responses of nodose ganglion neurons to pancreatic stimulation with tolbutamide and cerulein in control mice (C, 304 neurons, n = 4 mice) and mice preloaded with 5HTP (F, 325 neurons, n = 4 mice). Each row is a single cell, x-axis is time, dF/F is color.
(G) Quantification of the percentage of neurons responding to pancreatic stimulation in control and 5HTP preloaded animals (n = 4 for each group, Mann Whitney test, median +/- 95% CI).
(H) Average trace of 16 nodose ganglion neurons that show an increase in activity in response to tolbutamide stimulation of the pancreas. Antagonist of Htr3 receptor (Y-25130, 1mM) inhibited response to tolbutamide, and lowered overall neuronal baseline activity level. Response to cerulein was not affected.
4.7 Pancreas-specific inhibition of sensory nerves improves glucose tolerance

In the pilot study, that would require further follow up, we assessed the effect of pancreatic sensory nerves inhibition on glucose metabolism. To target sensory innervation of the pancreas specifically, we used Cre-dependent chemogenetic viral vector, which drives expression of an inhibitory designer receptor activated by designer drug (DREADD) under human Synapsin promoter (hSyn). Above mentioned virus was infused into the pancreas of Pirt-cre mice, through the common bile duct. Thus, we introduced three levels of control: intraductal infusion of the virus ensured pancreas specificity, hSyn promoter limited viral expression only to neuronal cells, while Pirt promoter and retrograde properties of AAVrg viral serotype, ensured infection of only sensory an not other types of neurons. Both virally-infected and sham-operated control animals were treated with specific DREADD ligand clozapine nitric oxide (CNO) for 12 days (5mg/kg injections twice a day), and we performed insulin glucose tolerance test (IPGTT) prior and after 12 days of CNO injections. CNO treatment improved glucose tolerance in virally-infected animals and did not affect glucose tolerance in control animals (Figure 16). However, we did not see any changes in plasma insulin levels (data not shown).

These data suggest that indeed pancreatic sensory innervation has a physiological role in the maintenance of glucose metabolism. But to be able to make more affirmative conclusions regarding the mechanism, this pilot study requires a follow up with increase number of animals, improved assessment of hormonal secretion, and quality control experiments to validate that DREAD induces functional inhibition of sensory neurons.
Figure 12

Figure 12. Levels of sensory innervation in the different mouse strains
Quantification of the mean fluorescence intensity of substance P immunostaining in the islet and the surrounding exocrine tissue (n = 24 mice total or 3 mice for each category, 3-7 islets per animal.)
Figure 13

(A) Section of the nodose ganglion with endogenous (non-amplified) expression of mCherry and GFP fluorescence 4 weeks after nodose ganglion transfection with AAV2 (red) and AAV8 (green). We observed better transfection and tracing efficiencies with AAV8 comparing to AAV2. Scale bar, 100µm.

(B) Section of the caudal brainstem with endogenous (non-amplified) fluorescence expression in the nerves traced from the nodose ganglion. Scale bar, 100µm.

(C) Section of the duodenum, with immunohistochemical amplification of the fluorescence in the vagal afferents traced from the nodose ganglion (green). Scale bar, 100µm.

(D and D’) Section of the pancreas, with immunohistochemical amplification of the fluorescence in the vagal afferents traced from the nodose ganglion(green), overlapping with CGRP staining (red). Scale bars, 100µm.
Figure 14. Chemical specificity of vagal sensory neurons during intraductal stimulation of the pancreas
Summary of the response profiles of 129 vagal afferents imaged in a single experiment as shown in Figure 8A and 8B. Each column indicates responses to a different compound specified at the bottom. Each row represents data from an individual nodose ganglion neuron. Most neurons responded to two stimuli: either 5HT and CCH or 5HT and Ex-4. Other neurons responded to one, three, and four stimuli.
Figure 15

Figure 15. Islet preloading with serotonin has a mild effect on glucose tolerance
Intraperitoneal glucose tolerance test in control (blue) and serotonin-preloaded (red) mice (n = 7 mice per experimental group, mean +/- SEM, multiple t-tests per timepoint, significant difference in glycemia is observed only at the 90 min readout).
Figure 16. Pancreas-specific chemogenetic inhibition of sensory nerves improves glucose tolerance

(A) Intraperitoneal glucose tolerance test (IPGTT) in Pirt-cre mice infected with AAVrg-HM4D(Gi). In these animals expression of inhibitory DREADD is specifically targeted to the pancreatic sensory neurons, thus we called this cohort of animals ‘inhibitory’ (inh). (B) IPGTT in sham-operated control animals (ctrl). IPGTT was performed in both cohorts of mice before before (black) and after chronic administration of clozapine nitric oxide (CNO) (red, 5mg/kg twice a day for 12 days). Area under the curve was quantified for the first 45 minutes of the IPGTT which reflect the progressive phase of insulin secretion (n = 3 and 4 mice per experimental group, mean +/- SEM, paired t-tests).
Table 1

<table>
<thead>
<tr>
<th>Stimuli</th>
<th>Concentration (µM)</th>
<th>Response strength</th>
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<tr>
<td>D-Glucose</td>
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<tr>
<td>Tolbutamide</td>
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<td>+</td>
</tr>
<tr>
<td>5HT</td>
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</tr>
<tr>
<td>ACh</td>
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</tr>
<tr>
<td>CCh</td>
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<td>+++</td>
</tr>
<tr>
<td>Kainate</td>
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</tr>
<tr>
<td>ATP</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>Fosf/IBMX</td>
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<td>+++</td>
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<tr>
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<tr>
<td>CGRP</td>
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<tr>
<td>Cycosomatostatin</td>
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</tr>
<tr>
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<tr>
<td>Glucagon</td>
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<tr>
<td>Cerulein</td>
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Table 1. Summary of the quantification of neuronal responses to all tested chemical stimuli applied to the pancreas topically.
A list of 15 tested compounds, with indicated concentrations. Response strength was coded based on the percentage of nodose ganglion neurons responding to the topical stimulation of the pancreas with the corresponding compound.
Table 2

<table>
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<th>Stimuli</th>
<th>Slope coefficient</th>
<th>Time to peak</th>
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<td>5HT_topical</td>
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<td>5HT_ductal</td>
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Table 2. Summary of the response kinetics of vagal sensory neurons stimulated from the pancreas
Chapter 5. Discussion

5.1 Overview

Our study establishes that the pancreatic islet uses serotonin as a signaling molecule to communicate with the brain via vagal afferent neurons. This mechanism is supported by our findings showing that (a) sensory axons innervating the islet express serotonin receptors and respond to serotonin, (b) inhibiting serotonin receptors in the pancreas reduces vagal sensory neuron responses to beta cell stimulation, and (c) increasing islet serotonin levels in beta cells amplifies neuronal responses to beta cell stimulation. By identifying serotonergic signaling between beta cells and sensory fibers as a peripheral transduction mechanism, our study reveals how beta cells activate vagal afferent neurons to make the brain aware of the process of insulin secretion and overall secretory capacity of the beta cell.

5.2 In vivo imaging of the nodose ganglion

One of the breakthroughs of our study was the ability to record neuronal activity in the nodose ganglion in vivo. The previously described approach of the nodose ganglion imaging was quite invasive as it required cutting of the central branch that connects ganglion neurons to the brain. As a consequence, neurons, even though elicited stimuli induced responses, were lacking their physiological baseline activity which normally allows the brain to monitor internal milieu. We developed a novel surgical approach that is less invasive, does not damage any of the vagal branches, and does not interfere with ganglion vascularization. Using this technique, we were able to demonstrate that even in the absence of exogenous stimuli, nodose ganglion neurons have enormous levels of baseline neuronal activity, which disappears after the vagus nerve is cut. This suggests that
the brain is continuously receiving substantial amount of information from the viscera. We think that this technique would gain vivid interest in the scientific community as it opens opportunities for decoding visceral code transmitted through the vagus nerve.

5.3 Islet serotonin and its message to the brain

Pancreatic beta cells share parts of differentiation pathway with serotonergic neurons, and have serotonin program linked to the ability to produce and release insulin (Ohta et al., 2011). As a consequence, serotonin and insulin are packaged in the same secretory vesicles and co-released in glucose dependent manner (Aspinwall et al., 1999; Ekholm et al., 1971). In normal physiology, serotonin contributes to paracrine control of hormone secretion, promoting the state of the islet which favors glucose clearance from the blood (via stimulation of insulin and inhibition of glucagon release; Almaça et al., 2016; K. Kim et al., 2015; Paulmann et al., 2009). Local islet serotonin is also known to facilitate islet adaptations to accommodate appropriate glucose clearance during pregnancy and high fat diet challenge (Goyvaerts et al., 2016; Kim et al., 2017; Ohara-Imaizumi et al., 2013). Our results indicate that regular levels of islet serotonin are physiologically relevant and sufficient to set the baseline activity in vagal afferent neurons. Activating sensory axons thus seems to be an important element in the functional repertoire of islet serotonin. Although, mechanisms regulating islet serotonin content and signaling pathways are not fully understood, it is clear that islet serotonin promotes glucose clearance in the blood. Thus, we propose that islet serotonin informs the brain about 1) islet secretory state in normal physiology and 2) overall secretory capacity of the beta cells in response to physiological challenges.
Because serotonin signaling is one of the most evolutionary conserved mechanisms of energy metabolism, serotonergic islet-brain communication not only conducts a message of the islet microenvironment, but also fits into the global context of glucose regulation. Serotonergic neurons of the enteric nervous system stimulate digestion and nutrient uptake, while serotonin signaling in the central nervous system inhibits appetite and food intake. These two serotonergic mechanisms complement each other, yet are uncoupled by the blood brain barrier (El-Merahbi et al., 2015; Gershon and Tack, 2007; Tecott, 2007). Vagal transduction of peripheral serotonin comes into play to transmit a message of a molecule that cannot reach the brain otherwise.

5.4 Decoding serotonin sensory modality of the vagus nerve

Recent studies are beginning to elucidate how vagal sensory neurons encode information originating from visceral organs (e.g. Bellono et al., 2017; Chang et al., 2015; Han et al., 2018; Kaelberer et al., 2018; Nonomura et al., 2016; Williams et al., 2016). A major challenge is that internal organs are very diverse in their functions and chemical composition, yet a single nerve collects sensory information from all of this diversity. It is still unclear if the vagus nerve is a mix of distinct, specifically tuned neuronal populations that possess unique strategies to sense the microenvironment in different organs (labeled lines) or if the diverse organs evolved similar and concerted transduction mechanisms across the vagus nerve (ensemble coding).

Our study shows that vagal afferents monitor insulin secretion by detecting serotonin, using a mechanism that is similar to that used by the vagus nerve to respond to input from the proximal intestine (Bellono et al., 2017; Blackshaw and Grundy, 1993; Grundy, 2002; Hillsley and Grundy, 1998). Moreover, we show that pancreatic afferents terminate in the
commissural subnucleus of the NTS, the same brain region reported to receive inputs from the chemosensitive fibers of the proximal intestine (Williams et al., 2016). Similarities in conduction properties could have a reason behind it, as nutrient uptake from the proximal intestine and glucose uptake from the blood are the subsequent physiological processes that target the same goal of energy uptake. These overlaps lead to an idea that perhaps both systems are transmitting the same sensory modality. In normal physiology, serotonergic accommodation of energy intake will transmit the message of “energy availability”, while high peripheral serotonin is the message of “energy sufficiency” and a signal that the peripheral tissues are working at their maximum capacity to uptake and store excessive energy. Thus, vagal serotonin conduction could be complementing leptin and ghrelin signaling in regulation of appetite and energy balance.

Internal organs are usually not stimulated individually, but rather are activated as a complex responsible for a common physiological function. In this regard, visceral sensory innervation is likely to be activated as a complex of receptive fields, where visceral serotonin could be a mediator-signal for the vagal afferents in several tissues involved in the physiological processes of energy uptake.

5.5 Specifics of islet sensory innervation

Unlike enterochromaffin cells, whose massive release can increase serotonin levels systemically, beta cells secrete serotonin most likely for local consumption only. Given that the islet is the only source of serotonin in the pancreas, sensory axons need to be close to the islet if they are to detect serotonin. Indeed, we found that islets have the highest density of sensory axons in the pancreas. These axons are peripheral terminals of sensory neurons expressing Htr3 receptors and respond to serotonin and activation of beta cells.
Thus, by reaching into and around the islet, serotonin-sensitive axons put themselves in an ideal position to be exposed to the spatially restricted intra-islet serotonin.

The production of serotonin in islets is remarkably variable. We found that even within the same pancreas islets had different serotonin levels. Under certain physiological conditions that involve changes in beta cell mass (e.g. pregnancy), serotonin synthesis increases tremendously, and serotonin serves as a paracrine signal that promotes beta cell proliferation (Kim et al., 2015; Ohara-Imaizumi et al., 2013). In the human islet, the levels of serotonin in beta cells also vary according to the BMI of the individual (Almaça et al., 2016). Moreover, some islets receive dense sensory supply, while others are innervated sparsely. This heterogeneity suggests that not all islets contribute equally or constantly to islet-brain communication.

5.6 Physiological effect of pancreatic sensory innervation on glucose metabolism

How sensory input from the islet affects responses in the central nervous system remains elusive. Whole body chemical and genetic sensory denervation affects physiologic and pathophysiologic processes in the pancreas, including insulin secretion and diabetes onset (Bou Karam et al., 2018; Gram et al., 2007; Ikeura et al., 2007; Karlsson et al., 1992; Li et al., 2013; Liddle, 2007; Motter & Ahern, 2008; Noble et al., 2006; Razavi et al., 2006; Riera et al., 2014; Warzecha et al., 2001). Yet we still don’t know what contribution, if any, pancreatic sensory innervation has to the metabolic effects observed in whole-body sensory sensory denervation studies. We approached this question in a pilot study, demonstrating that chemogenetic inhibition of the pancreas-specific sensory neurons improves glucose tolerance in mice. Our findings, combined with those of denervation studies, suggest that sensory information from the islet triggers a downstream effect on glucose metabolism.
Without this sensory input, the autonomic centers of the brain may lose information about the functional status of the islet that is crucial for glucose homeostasis.

5.7 Future directions

My work contributes to the field of islet biology by introducing the first functional investigation of islet sensory innervation. It also reports an additional role to the repertoire of islet serotonin functions. Future research could look into other components of islet microenvironment that could potentially have an effect on islet sensory innervation, especially in inflammatory conditions.

Effect of inflammation could be of a particular interest for sensory pathophysiology, in light of almost epidemic spread of diabetes. Emerging evidence suggests that metabolic stress and obesity lead to production of pro-inflammatory mediators by the islets. Islet inflammation is an attractive stimulus for sensory neurons. Sensory neurons however, are known not only for transmitting afferent signals, but they also contribute to inflammatory augmentation by releasing pro-inflammatory neuropeptides locally. Pancreatic islet inflammation is proposed to drive beta-cell failure and lead to diabetes. Yet little is known about the nature of neurogenic inflammation in visceral organs and specifically in the pancreas. As intra-islet serotonin signaling and activity is increased in response to physiological challenges, it could subsequently lead to neurogenic inflammation induced by chronically active sensory nerves. This is an interesting trajectory for future investigation.

The field of visceral neuroscience is going through a seminal and productive phase that is already changing how we view neural control of inner organs and its pathophysiological implications. My study is thought provoking as it starts a discussion about visceral
serotonergic sensory modality perceived by the vagus nerve in the pancreatic islet, proximal intestine and perhaps other tissues. I propose serotonin to be a message of peripheral “energy availability” in normal physiology, and “energy sufficiency” under physiological challenge, yet I recognize that this scenario is perhaps not so straightforward, as circulating serotonin levels can fluctuate in different physiological conditions, sometimes reaching the sufficient concentration for direct activation of vagal afferents. Central and peripheral serotonergic systems are essential for energy metabolism and are uncoupled by the blood brain barrier, placing vagus nerve, in a convenient position for serotonin-sensitive conduction. Serotonin is very abundant in the viscera and fluctuates in response to metabolic challenges. Thus, the question of vagal serotonin sensitivity is essential for understanding and decoding visceral code.
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