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Pancreatic Innervation in Mouse Development and β -cell Regeneration

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Abstract

Pancreatic innervation is being viewed with increasing interest with respect to pancreatic disease. At the same time, relatively little is currently known about innervation dynamics during development and disease. The present study employs confocal microscopy to analyze the growth and development of sympathetic and sensory neurons and astroglia during pancreatic organogenesis and maturation. Our research reveals that islet innervation is closely linked to the process of islet maturation—neural cell bodies undergo intrapancreatic migration/shuffling in tandem with endocrine cells, and close neuro-endocrine contacts are established quite early in pancreatic development. In addition, we have assayed the effects of large-scale β -cell loss and repopulation on the maintenance of islet innervation with respect to particular neuron types. We demonstrate that depletion of the β -cell population in the RIP-cmyc^{ER} mouse line has cell-type-specific effects on postganglionic sympathetic neurons and pancreatic astroglia. This study contributes to a greater understanding of how cooperating physiological systems develop together and coordinate their functions, and also helps to elucidate how perturbation of one organ system through stress or disease can specifically affect parallel systems in an organism.

There are three neuron types—sympathetic, parasympathetic, and sensory—that innervate the pancreas, in addition to an astroglial population. The sympathetic and parasympathetic branches of the autonomic nervous system are involved in maintenance of blood glucose homeostasis in response to changing energy demands. Sympathetic neurons mediate the so-called “fight or flight” response through stress-induced neural activity. They inhibit insulin secretion and up-regulate glucagon release by respective β - and α -cell populations in the pancreatic islets of Langerhans, the net physiological result of which is to convert glycogen stores to blood glucose to meet immediate energy demands (Munding et al. 2003). Through feeding-induced neural activity, parasympathetic neurons stimulate insulin secretion from insulin-producing β -cells to promote the removal of glucose from the blood into the liver for storage as glycogen, while repressing glucagon release (Bentham et al. 2001; Adeghate et al. 2000; Ahren 2000). Sensory neurons are involved in pain sensation; indeed, extreme pain is a well-documented concern in pancreatitis and pancreatic cancer patients (Wick et al. 2006a, 2006b). The function of pancreatic astroglia, which encapsulate the islets of Langerhans, is not definitively known, although there is increasing evidence that astroglia are involved in synaptic transmission in the brain, and thus may be more involved in neuronal signaling than previously speculated (Halassa et al. 2007).

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Anatomical and physiological characteristics of the pancreas pose technical challenges to the study of innervation. Many antigens that are considered dependable neural markers within the CNS are unsuitable for use in the pancreas because various pancreatic endocrine cells also display them. Furthermore, due to the irregular morphology of islets and the network of neurons that innervate them, thin-section immunofluorescence techniques miss important 3-dimensional information. Thus, previous developmental studies have been limited in their ability to distinguish between specific pancreatic nerve populations, and to obtain high-resolution images. In this study we employ confocal fluorescence microscopy, using neuronal subtype-specific antibodies on thick sections at particular stages in embryonic development, postnatal maturation, and synthetic pancreatic disease, to gain a greater understanding of the neuronal and glial populations associated with the pancreatic islets.

The mature pancreas is a dynamic organ; old endocrine cells die and new cells are born while endocrine innervation is maintained throughout the life of the organism. Pancreatic innervation is being viewed with increasing interest with respect to pancreatic diseases, yet relatively little is known about pancreatic innervation during development and disease (Saravia and Homo-Delarche 2003; Konturek et al. 2003; Yi et al. 2003; Persson-Sjogren et al. 2001; Rossi et al. 2005; Lindsay et al. 2006). Nonetheless, pancreatic nerves have recently been identified as a possible early target population in autoimmune diabetes, and there is increasing evidence that neuroendocrine remodeling does take place in the pancreatic islets of diabetic disease models (Persson-Sjogren et al. 2005; Saravia and Homo-Delarche 2003; Winer et al. 2003; Mei et al. 2002).

One goal of the present study was to perform a descriptive analysis of the growth and development of sympathetic and sensory neurons and astroglia during pancreatic organogenesis and maturation. In addition, we aimed to assay a synthetic pancreatic disease model for subtype-specific effects of large-scale β -cell loss and repopulation on the maintenance of islet innervation. To accomplish these goals we performed confocal analysis using single, double, and triple labeling immunohistochemistry. We included embryonic, neonatal, adolescent and adult wild-type mice in our developmental study. We also took advantage of the recent development of the RIP-cmyc^{ER} line, a transgenic mouse line that conditionally expresses cmyc specifically in β -cells. This enabled us to orchestrate the death and subsequent resurgence of this subpopulation of endocrine cells (Pelengaris et al. 2002). We used the RIP-cmyc^{ER} regeneration model to determine the effect on existing pancreatic neurons when the bulk of β -cells are ablated and replenished.

In contrast to previous research, this analysis concerns itself with the relationship of specific neural and glial pancreatic subpopulations to both the developing and mature endocrine pancreas. It explores these populations, at the level attainable by confocal fluorescent microscopy, in order to address both developmental and maturational aspects of islet innervation in the wild type mouse, as well as disease-related aspects of innervation maintenance in a synthetic adult disease model. We report that the sympathetic and sensory populations appear in the developing embryonic pancreas in a temporally discrete fashion. We show that islet innervation by distinct cell types is temporally integrated with the intrapancreatic endocrine cell migration and organization that characterize pancreatic embryonic development and postnatal maturation, respectively. Finally, we observe that the sympathetic, sensory and astroglial populations in the pancreas are affected differently by depletion and restoration of the β -cell population in RIP-cmyc^{ER} mice.

Experimental Procedures

Experimental Animals

Developmental experiments were performed on embryonic (e9.5-18.5), neonatal (p0-p7), adolescent (p10-28) and adult (3-6 month) wild type CD1 mice. Synthetic hyperglycemia and recovery experiments were conducted on RIP-cmyc^{ER/+} (C57BL/6) experimental mice and wild type littermate controls, aged 8-10 weeks at the beginning of tamoxifen administration. For prenatal analysis, 4-10 wild type CD1 embryos were analyzed per stage; for postnatal analysis, 5-8 wild type CD1 animals were analyzed per stage; for RIP-cmyc^{ER} islet regeneration analysis, 4-5 RIP-cmyc^{ER/+} experimental animals and 4 control animals were analyzed per stage. Males and females were represented in roughly equal proportions in all experimental groups. All experimental results were confirmed in duplicate or triplicate. The mice were housed in accordance with NIH guidelines and kept in a facility maintained at 22°C with an alternating 12h light/dark cycle. Mice were given standard mouse chow and water *ad libitum*. All procedures were approved by the Institutional Animal Care and Use Committee at the University of California, and were conducted in accordance with NIH and UCSF LARC guidelines.

Tissue preparation and immunofluorescent processing

Pancreata were isolated from wild type CD1 mice ranging from 12.5 days post coitus through 6 months in age and fixed 2-16 hours at 4°C in zinc-buffered formalin fixative (Anatech). Whole embryos ranging in age from e10.5-15.5 were fixed up to 16 hours at 4°C in zinc-buffered formalin fixative, after which they were either treated as whole mount tissues or pancreata were excised from the fixed embryos for cryoprotection. After fixation, tissues were cryoprotected 16 hours in 30% sucrose in 0.1M PBS at 4°C, then frozen in TissueTek (Sakura, Torrance, CA USA) and stored at -80°C. Tissues were cut on a cryostat at a thickness of 50µm. Serial sections were thaw mounted and affixed to glass slides before staining.

Sections were air dried at room temperature for 90 minutes to affix tissue to glass slides. Sections were incubated for 90 minutes in 0.3% Triton X-100 in 1× PBS to dissolve TissueTek, then blocked in 0.3% Triton X-100, 4% BSA, and 0.02% NaN₃ in 1× PBS at 4°C 16 hours. Primary antibodies were diluted in 0.3% Triton X-100, 4% BSA in 1× PBS and applied to sections (see Table 1 for antibodies and conditions employed). Sections were rinsed 4× quickly and washed 6×1 hour at room temperature in 0.3% Triton X-100 in 1× PBS, and blocked 16 hours in 0.3% Triton X-100, 4% BSA, and 0.02% NaN₃ in 1× PBS at 4°C. Secondary antibodies were diluted and applied in 0.3% Triton X-100, 4% BSA in 1× PBS and applied to sections (see Table 1), then rinsed 4× quickly and washed 6×1 hour at room temperature in 0.3% Triton X-100 in 1× PBS. Sections were rinsed 3×5 minutes in PBS, mounted with Vectashield + DAPI (Vector Labs) and imaged.

RIP-cmyc^{ER} Tamoxifen Protocol

The RIP-cmyc^{ER} line is a transgenic mouse line, in which the cell death gene *cmyc* is expressed under the control of the *rat insulin* promoter (RIP). Expression of the transgene in pancreatic β-cells is induced by the intraperitoneal administration of tamoxifen, and ceases when tamoxifen administration is stopped (Pelengaris et al. 2002). Tamoxifen (Sigma T5648) was dissolved 10 mg/mL in corn oil and 1mg was injected intraperitoneally into experimental mice once daily for 6 days. Control mice were injected with vehicle alone. Ascensia Elite blood glucose monitors and test strips (Bayer) were used to monitor non-fasting blood glucose. Non-fasting blood glucose was measured on days 3 and 7 of the tamoxifen regime, as well as at the time of sacrifice. Mice were considered hyperglycemic if blood glucose ≥ 200 mg/dL on day 7; blood glucose of all transgenic mice assayed was ≥350 mg/dL by day 7. Control animals had blood glucose levels below 200 mg/dL at day 7. Blood glucose measurements were taken

at days 3, 7, 9, 11, 13, 23, 30, 45, and 60 post initiation of tamoxifen administration. Pancreata were harvested on test dates, and tissues were fixed and prepared for cryosection as above. Pancreata were sectioned into 50 μ m sections and affixed to glass slides before staining. The immunofluorescence staining protocol was conducted as above.

Immunofluorescent confocal microscopy

Confocal images were taken using Leica DMRE SL and SP2 confocal microscopes and Leica confocal software. Images were obtained with 20x and 40x oil immersion lenses, and the microscopes were equipped with optical zoom capability. For 2-dimensional composite images, at least 1 confocal image was taken per micron of tissue thickness. Images were processed and contrast and brightness were adjusted using Adobe Photoshop.

Results

Innervation morphology of the adult pancreatic islet

Antibodies recognizing vesicular monoamine transporter 2 (VMAT2), calcitonin gene-related peptide (CGRP), and glial fibrillary acid protein (GFAP) were used to visualize postganglionic sympathetic neuronal processes, sensory neuronal fibers, and pancreatic astroglia, respectively. VMAT2⁺ staining was occasionally observed in adult β -cells as has previously been reported, though at a relatively low level and in a diffuse cytoplasmic pattern that was easily distinguishable from the bright vesicular reactivity observed in VMAT2⁺ neurons (Anlauf et al. 2003; Weihe and Eiden 2000). VMAT2⁺ staining was not observed in α -, δ -, or PP-cells in the endocrine pancreas; CGRP reactivity was observed only in CGRP⁺ neurons; and GFAP reactivity was specific to pancreatic astroglia.

In adult mice, VMAT2⁺ sympathetic and CGRP⁺ sensory fibers entered pancreatic islets at multiple points. Neural fibers were enriched at the islet periphery, although occasional detection of fibers within islet cores indicates that they are not necessarily excluded from the inside of the islets (Figure 1A, B, D and E). GFAP⁺ astroglia were highly associated with the islet periphery as they encapsulated pancreatic islets (Figure 1C, F).

Sympathetic (VMAT2⁺) neuronal fibers were quite distinct in the extent of their perivascular association in the adult mouse. Multiple VMAT2⁺ sympathetic tracts form an elaborate perivascular plexus around large and medium-sized pancreatic blood vessels as visualized with an antibody recognizing mouse pan-endothelial cell antigen (MECA32) (Figure 2A). Sympathetic capillary association was also occasionally detectable within islets (data not shown). Sensory fibers were no less elaborate than autonomic fibers, although CGRP⁺ tracts tended to align longitudinally with blood vessels, without forming plexuses (Figure 2B). None of the populations assessed were found to associate closely with pancreatic duct architecture (data not shown).

Sympathetic and sensory neurons enter the pancreatic bud during late embryogenesis (e10.5-e18.5)

Evagination of the mouse dorsal pancreatic bud is known to initiate at embryonic day 9.5 (e9.5) and pancreatic tissue continues to proliferate throughout embryonic development (Kim and Hebrok 2001). Neural crest cell migration is also known to begin in the early embryo (Kasemeier-Kulesa et al. 2005). To delineate the pattern of innervation during embryonic development, we began the analysis of tissue at e10.5 and continued to e18.5, just preceding birth. Staining of sagittal sections from e10.5 whole-mount embryos detected a VMAT2⁺ cell population present as a streak of cells along the length of the neural tube (Figure 3A). No VMAT2⁺ cell bodies were detected in the pancreatic bud at e10.5. The earliest presence of VMAT2⁺ cell bodies was detected at e12.5 in excised pancreatic tissue (Figure 3B).

VMAT2⁺ cell bodies were detected just inside the pancreatic periphery, indicating that e12.5 is probably the earliest point at which VMAT2⁺ cell bodies enter the pancreatic bud. VMAT2⁺ neuronal fibers were not detected in embryonic pancreatic tissues (Figure 3C).

CGRP was present at very low levels in individual sensory cell bodies along the neural tube at e10.5 (Figure 3D). By e12.5, a small number of single CGRP⁺ cell bodies were detected in excised pancreatic bud tissue, indicating that CGRP⁺ cells may first enter the pancreas at this stage in concert with VMAT2⁺ cells (Figure 3B, E). CGRP was detected more strongly after sensory precursors entered the pancreatic bud, and by e15.5 CGRP⁺ sensory fibers were highly elaborate (Figure 3F).

Islet innervation and encapsulation take place coincident with postnatal islet maturation (p0-21)

Sympathetic innervation—The postnatal maturation of VMAT2⁺ sympathetic neural fibers can be described in terms of the changing nature of their contact with endocrine cells and blood vessels during development. At birth, endocrine cells may be found scattered or clustered throughout the pancreas, and VMAT2⁺ cell bodies are detected among scattered endocrine cells (Figure 4A). Unaffiliated endocrine cells migrate to join islet clusters until adulthood is reached. Adult islets have a characteristic arrangement of endocrine cells: β -cells are found at the islet core, surrounded by α -, δ - and PP cells (Kim and Hebrok 2001).

VMAT2⁺ fibers could be seen closely associated with endocrine cells during the first postnatal week, long before endocrine cell migration and islet formation have been completed (Figure 4B). During the second postnatal week, α - and β -cells continue their migration in the course of adopting the mature islet configuration. Sympathetic islet innervation remained largely concentrated at the periphery of the maturing islets, and closely resembled the adult configuration by p15 (Figure 4B, C).

At p4 VMAT2⁺ neural fibers were often observed in absence of associated blood vessels (Figure 5A). This appeared unique to the first postnatal week, as by p7 the incipient perivascular plexus began to be clearly visible on larger vessels (Figure 5B). Perivascular association of sympathetic fibers increased in complexity during the second postnatal week. The incipient plexus formation that was first visible at p7 reached a level of elaboration characteristic of the adult sympathetic perivascular plexus by p15 (Figure 5C).

Sensory Innervation—As in the case of sympathetic nerves, association of sensory fibers with endocrine cells takes place during the early processes of endocrine migration and islet formation. By p0, CGRP⁺ fibers were closely associated with endocrine cells in many nascent pancreatic islets (Figure 4D). CGRP⁺ fibers themselves did not change appreciably between p0 and p7; by the beginning of the second postnatal week, as endocrine cells coalesced into islets, CGRP⁺ fibers became clearly visible at the periphery of islets (Figure 4E). CGRP⁺ innervation remained largely concentrated at the periphery of islets throughout the maturation process. Thus sensory-endocrine association at p15 closely resembled that found in adult islets (Figure 4F).

Astroglial Encapsulation—GFAP is not detectable in astroglia before p6 (Figure 4G); this precludes its use to track the presence of astroglia in mouse embryos and neonates. Faintly GFAP-reactive cells were first visible at p6, after nascent pancreatic islets have begun to form but long before islet maturation is considered complete. At this stage, GFAP⁺ cells came into close contact with glucagon-producing cells at the islets' periphery. At p6, GFAP⁺ cells were also in contact with endocrine cells that had yet to become clearly affiliated with islets (data not shown). By p7, the beginning of the second postnatal week, rudimentary GFAP⁺ encapsulation of the maturing islets was evident (Figure 4H). The amount of GFAP⁺

encapsulation increased as pancreatic islets continued to mature, and by p15 astroglial encapsulation of islets closely resembled the adult morphology (Figure 4I).

Selective loss of β -cells differentially affects maintenance of islet innervation in RIP-cmyc^{ER} mice

We used the RIP-cmyc^{ER} regeneration model to determine the effect on existing neurons and neuroendocrine connections when the bulk of β -cells are ablated and replenished. As the β -cell population declines, mice quickly become hyperglycemic; they recover normal blood glucose levels as the islets are repopulated with β -cells over a two-month period. As such, this mouse line provides a β -cell regeneration model in which to study the maintenance of endocrine innervation during the course of β -cell loss and recovery (D. Cano and P. Heiser, personal communications).

RIP-cmyc^{ER} mice were treated with a 6-day course of intraperitoneal tamoxifen injection, monitored for changes in blood glucose, and sacrificed for tissue preparation as described above. Mice were clearly hyperglycemic, with a mean non-fasted blood glucose levels exceeding 400 dl/ml at day 3 of cmyc induction, compared to under 200 dl/ml in untreated controls; animals began to recover normoglycemia shortly after cessation of cmyc induction, and blood glucose levels returned to normal range by 60 days after cmyc induction. Mice were observed to have similar blood glucose levels at similar points along the recovery trajectory, regardless of sex. No sexual dimorphism was observed with regard to the expression patterns detailed below.

Loss of β -cells is sufficient to cause pancreas-wide delocalization of a monoamine transporter in post-ganglionic sympathetic nerves—Pancreata harvested 7 days post-initiation (dpi) of cmyc activation were found to lack VMAT2⁺ reactivity, a marker of sympathetic innervation (Figure 6A). The loss of sympathetic VMAT2 was homogeneous throughout the pancreas. By 13dpi (1 week after cmyc activation has been discontinued), VMAT2 had begun to reappear in perivascular sympathetic fibers. Low levels of VMAT2 were detectable in a pattern characteristic of the sympathetic perivascular plexus, but sympathetic VMAT2 reactivity remained absent around pancreatic islets (Figure 6B). At roughly 30dpi, VMAT2 was observed in islet-associated sympathetic fibers, and VMAT2 reactivity in perivascular fibers closely resembled that of wild type animals. As the methods used were not quantitative, no conclusions could be reached about relative levels of VMAT2 expression during recovery of normoglycemia. However, subcellular VMAT2 localization appeared to be largely restored in islet-associated fibers by 60dpi (Figure 6C). Overall, the recovery of VMAT2 reactivity in pancreatic sympathetic nerve fibers coincided temporally with the repopulation of islets with β -cells, and with recovery of normoglycemia as measured by blood glucose tests (Figure 6A–C).

To more closely determine the extent of disruption in postganglionic sympathetic neurons, RIP-cmyc^{ER} specimens were also stained with antibodies to detect the presence of neuropeptide Y (NPY), a modulator of glucagon secretion that is also present in postganglionic sympathetic neurons (Adeghate 2002). NPY was present throughout the loss and resurgence of β -cells, indicating that although VMAT2 localization was disrupted, the architecture of postganglionic sympathetic neurons was intact (Figure 7). Thus, we conclude that large-scale β -cell loss alone is sufficient to disrupt VMAT2 localization, but insufficient to disrupt the structure of sympathetic neurons in the pancreas.

Large-scale β -cell loss has a delayed and transient effect on astroglial-endocrine association—The effect of large-scale β -cell depletion on pancreatic astroglia was both subtle and delayed relative to its effects on the sympathetic nerve population.

Astroglial association with islets visualized at 7dpi and 9dpi did not appear decreased (Figure 6D) relative to that in wild type animals (Figure 1C). However, astroglial encapsulation of pancreatic islets was visibly lessened by 13dpi (Figure 6E). Thus we surmise that the decrease in astroglial-islet association occurred after, rather than during, the initial loss of β -cells. This decrease appeared to be transient, as islet encapsulation at 60 days post cmc activation looked very similar in extent to encapsulation in wild type animals (Figure 6F). It should be noted that, in contrast to the case of sympathetic neural fibers, the observed decrease in astroglial islet encapsulation was not characterized by a loss in astroglial GFAP reactivity. Remaining GFAP⁺ cells were still clearly associated with islets, and appeared to be healthy.

Islet sensory innervation appears unaffected in RIP-cmyc^{ER} mice—In contrast to the demonstrated effects of large-scale β -cell depletion on sympathetic neurons, no readily apparent interruption of sensory innervation of pancreatic islets was revealed in RIP-cmyc^{ER} mice. CGRP reactivity was detected and resembled wild type reactivity at 7dpi and 9dpi; the same was true of tissues from animals isolated at 60dpi (Figure 6G, H). No morphological changes were detected in sensory neurons at islets throughout stages of β -cell repopulation.

Discussion

Innervation and encapsulation of islets occur in tandem with islet maturation

VMAT2⁺ sympathetic and CGRP⁺ sensory cell bodies migrate into the pancreatic bud from the neural tube, where they are detected at e10.5. Both are first detected in the pancreatic bud at e12.5. While CGRP is detected in sensory fibers soon after at e15.5, VMAT2 does not clearly mark sympathetic neural fibers until shortly after birth.

The development of islet innervation by sympathetic and sensory neurons, and islet encapsulation by GFAP⁺ astroglia, are closely integrated with postnatal islet maturation. All three of these neural crest derived populations are detected in close apposition to endocrine cells at or within a few days after birth. They then appear to coalesce with endocrine cells to form nascent islets during the first postnatal week, and adopt their adult position within islets by the second postnatal week. Meanwhile, the association of sympathetic tracts with blood vessels in the maturing postnatal pancreas is a unique developmental hallmark of postganglionic sympathetic neurons. At p7, the beginning of the second postnatal week, there is increasing complexity of early postnatal sympathetic association with the vasculature. By the beginning of the third postnatal week at p15, elaboration of the perivascular plexus closely resembles adult perivascular morphology. In comparison, sensory perivascular association does not appear to become more elaborate during the course of pancreatic maturation.

The adult morphology of pancreatic innervation by the sympathetic and sensory nerve populations is similar in that observed neural fibers are enriched around the islet periphery compared to the islet core. By contrast, the nerve populations are distinct in their extents of islet innervation and in their modes of association with pancreatic blood vessels. Sympathetic islet innervation is greatly enriched in comparison to sensory islet innervation. VMAT2⁺ sympathetic processes tend to fasciculate into tracts of multiple processes that surround pancreatic blood vessels in a perivascular plexus. CGRP⁺ sensory processes are less fasciculated by comparison, and single processes tend to appear separate from particular blood vessels. The neuronal and astroglial antibodies that we found suitable to our purpose had all been raised in rabbit hosts, and therefore could not be used simultaneously to compare the cell populations.

The presence of VMAT2 provides clues to the status of autonomic function in developmental and disease states of the pancreas

Intracellular localization of VMAT2 to small synaptic vesicles (SSVs) and dense core vesicles (DCVs) has been reported in catecholaminergic neurons in the CNS (Nirenberg et al. 1995, 1996, 1997a, 1997b). Additionally, there is a wealth of evidence suggesting that peripheral sympathetic neurons modulate visceral responses through the slow, nonsynaptic release of monoamines from DCVs in axon terminals (Nirenberg et al. 1995; Vizi 1991; Beaudet and Descarries 1978). Monoamines can also be released from dendrites, occasionally by exocytosis from DCVs (Li et al. 2005; Nirenberg et al. 1995; Morris and Pow 1991; Geffen et al. 1976). The ultrastructural studies that would further define the intracellular localization of VMAT2 in postganglionic sympathetic pancreatic neurons have yet to be done. However, the subcellular VMAT2⁺ localization in these neurons during postnatal maturation implies that postganglionic sympathetic neurons may be physiologically able to accumulate and secrete noradrenaline during maturation of the endocrine pancreas. Pancreatic neurons do not form true synapses with islet cells (Fisher and Bourque 2001; Fujita and Kobayashi 1979; Serizawa et al. 1979). While they contact target cells, to date there is no evidence to suggest the presence of postsynaptic densities that define the canonical synapse. Therefore, since early contacts between neurons and endocrine cells resemble the adult associations, the morphological evidence supports the possibility that neuroendocrine signaling takes place before pancreatic islets are fully mature, although we provide no physiological evidence for this type of signaling at this stage of development. Whether such signaling occurs, and concomitantly, whether that signaling has a role in directing the extent of pancreatic innervation during development, are open questions.

Sympathetic neurons, sensory neurons, and astroglia are differentially affected by large-scale fluctuations in the β -cell population

The RIP-cmyc^{ER} mouse line provides a disease model in which to explore the physiology of various adult pancreatic cell populations, both in response to a controlled large-scale depletion of β -cells and the resulting hyperglycemia that mimic type I diabetes, and during β -cell repopulation of the islets of Langerhans and recovery of normoglycemia. Observation of impaired glucose tolerance during high-stringency glucose tolerance tests after these animals were otherwise considered fully recovered (P. Heiser, personal communication) led us to hypothesize that autonomic islet innervation is affected, and that the recovery of some physiological aspect of autonomic innervation in these animals may not be complete after 60 days of recovery. Remodeling of pancreatic sensory neurons has been associated with pain in the context of pancreatic cancer (Lindsay et al. 2005). In contrast, pancreatic pain has not been reported in association with diabetic phenotypes. Hence the lack of detectable sensory remodeling in the RIP-cmyc^{ER} line is perhaps expected. Finally, changes in pancreatic astroglia have been previously reported in non-obese diabetic (NOD) mice, and we believed that our system would allow us to view astroglial remodeling in closer detail (Persson-Sjogren et al. 2005; Homo-Delarche 2004; Winer et al. 2003; Persson-Sjogren 2001).

As noted previously, the availability of antibodies suitable for pancreatic analysis necessarily limited our findings. Thus, the markers utilized in this study cannot provide an exhaustive description of sympathetic, sensory, and astroglial innervation. In addition, although the dimensional information contained in 50 μ m sections is far superior to that of thinner (5–10 μ m) sections, 50 μ m sections were not thick enough to provide a full 3-dimensional rendering of labeled islets and neurons. Sections over 50 μ m thick yielded no more information, as 50 μ m was found to be the limit at which antibodies were fully able to penetrate sections. This technical limitation made it impossible to ascertain all the projections coming from a single neural or astroglial cell body, or to connect more distal projections with specific cell bodies. Similarly, in this study we were unable to ascertain whether projections from single neurons

were sent to multiple islets, or to trace projections from specific intrapancreatic or extra-pancreatic ganglia to particular parts of the pancreas. Particularly in the context of β -cell regeneration, it is very possible that other changes that could be taking place in the neurons and astroglia but were missed in our analysis simply because we lacked markers adequate to observe and document them.

Response of sympathetic innervation to loss and replenishment of the β -cell population, and correlation of neural recovery with physiological recovery of the endocrine pancreas—Loss and recovery of VMAT2 reactivity in postganglionic sympathetic neurons temporally coincides with the incidence of hyperglycemia and the recovery of normoglycemia in RIP-cmyc^{ER} mice following tamoxifen insult. At 60dpi, we observe recovery of normal blood glucose levels coincident with the restoration of subcellular VMAT2 localization. The VMAT2 transporter molecule has a recognized role in the storage and transport of catecholamines, particularly in the modulation of monoamine neurotransmission in dopamine-producing cells in the central nervous system (CNS) (Nirenberg 1997a, b). This transporter may have a similar function in pancreatic sympathetic neurons. Since the pancreatic sympathetic neurons remain while VMAT2 immunoreactivity is lost, we conclude that the ability of postganglionic sympathetic neurons to accumulate noradrenaline may be compromised by the large-scale loss of β -cells in RIP-cmyc^{ER} animals. However, the persistence of NPY localization indicates that the architecture of sympathetic neurons is maintained throughout changes in the β -cell population.

Loss of the β -cell population does not affect the subcellular localization of CGRP, a molecule that is central to nociception—Changes in CGRP⁺ pancreatic sensory innervation have been implicated in pancreatic cancer-associated pain (Lindsay et al. 2005). Pancreatic pain has not been associated with β -cell loss itself-- as occurs in type 1 diabetes and in synthetic diabetic mouse models such as non-obese diabetic or streptozotocin mice. In the RIP-cmyc^{ER} model, CGRP-reactivity is retained in sensory fibers observed at islets. Additionally, no architectural changes are apparent during the loss and recovery of β -cells in RIP-cmyc^{ER} mice. These results underscore that the effects of β -cell loss on islet-associated neurons are specific to cell type, and not general effects of pancreatic ill health or islet deterioration.

Astroglial islet encapsulation is responsive to changes in islet state—Pancreatic astroglia appear to be affected by the large-scale loss and replenishment of β -cells, but the effect is distinct from observed effects on sympathetic fibers in that astroglial changes occur later in the blood glucose recovery curve than do changes in the sympathetic nerves. While the astroglial population diminishes in response to β -cell loss, astroglial contact with islets as detected by GFAP reactivity is observed throughout the loss and replenishment of the β -cell population.

This transient decrease in islet encapsulation by astroglia appears to be a function of glial remodeling in response to changes in islet state; recovery of encapsulation also appears to be swift, indicating that islet state and level of astroglial contact may be linked. This possibility would be consistent with the hypothesis that astroglia are performing a support role at the pancreatic islets.

It should be noted that islet encapsulation by astroglia appears to increase above wild type levels roughly 1–1.5 weeks after initiation of cmyc activation. We believe this is a visual artifact brought about by the loss of islet volume and surface area such that the ratio of astroglia to islet area has increased, rather than a true representation of astroglial increase. The exact function of pancreatic astroglia is unknown at this time. It is possible that there is a transient increase in encapsulation by astroglia as part of a response to physiological stress. This could

then be followed by a transient astroglial decrease, either in response to a decrease in stress as recovery of the β -cell population begins, or in response to decreased islet size, and another increase in islet encapsulation as the islet is repopulated with β -cells and accordingly grows in size and nutritive need. A quantitative analysis of this phenomenon in three dimensions would be informative in this respect, as would genetic manipulation of possible molecules involved. The presence of the different protein markers assayed does provide some clues to the possibility of peripheral nervous system (PNS) function in both the developmental and islet recovery assays, but does not provide conclusive functional information about the cells visualized, nor can it conclusively link pancreatic neural function with pancreatic function. Further morphological and functional analyses would have to be undertaken to conclusively define a role for PNS function during both pancreatic development and adult islet recovery.

There are a number of species-specific variations in the enteric nervous system (ENS), notably between large and small mammals (Faussone-Pellegrini 2006; Furness et al. 2006; reviewed in Hansen 2003a). These include differences in anatomical organization of the ENS as well as neurophysiology. Hence a direct analogy between these findings and the human context cannot automatically be assumed. Yet this study does contribute to a greater understanding of how physiological systems develop together and coordinate their functions, which can be highly instructive in terms of elucidating how perturbation of one organ system through stress or disease can specifically affect parallel systems in an organism. Such an understanding may also have some interesting applications to islet transplantation biology. This analysis of endocrine innervation in the context of islet development and perturbation begins to clarify the relationship between endocrine pancreas development, physiology and innervation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Pancreatic neurons and astroglia are intimately associated with the islets of Langerhans. Sympathetic and sensory neurons, as well as astroglia (green), all populate pancreatic islets. Sympathetic and sensory neural fibers appear concentrated at the islet periphery defined by glucagon-producing α -cells (red, A–C), relative to the islet core defined by insulin-producing β -cells (red, D–F). N=8. Scale bar=50 μ m

Figure 2.

Adult pancreatic neural processes have distinct modes of perivascular association. Sympathetic fibers (green, A) are highly fasciculated relative to sensory fibers, and form an elaborate perivascular plexus that surrounds large and medium-sized blood vessels (blue, A). Sensory fibers (green, B) are somewhat less fasciculated, and tend to align with the vasculature (blue, B). N=8. Scale bar=10µm

Figure 3.

Sympathetic and sensory cell bodies enter the pancreatic bud during organogenesis. At embryonic day 10.5 (e10.5), the sympathetic VMAT2⁺ cell population is visible along the neural tube of a sagittally sectioned whole mount (green, A; somites are outlined in yellow). No VMAT2⁺ cell bodies are seen in the pancreatic bud at this time (N=10). Sensory CGRP⁺ cell bodies are also detected along the neural tube at e10.5 (green, D), but are morphologically distinct from VMAT2⁺ cells (N=8). VMAT2⁺ and CGRP⁺ cells are first detected in the excised pancreas at e12.5; CGRP⁺ cells are detected in much lower numbers than VMAT2⁺ cells in e12.5 (N=4) pancreas (green, B and E). In the e15.5 pancreas (N=5), VMAT2⁺ is still confined to sympathetic cell bodies, while CGRP is detected in elaborate neural fibers (C, F). Tangential edges of cell bodies are occasionally depicted (white arrowheads, C and F). Scale bar=50µm

Figure 4.

Nerve growth and islet innervation during postnatal pancreatic maturation, p0-p15.

Sympathetic cell bodies (green, white arrowheads) are interspersed with endocrine cells (red) at birth (A; N=5), as endocrine cells are still migrating to form nascent islets; VMAT2⁺ fibers become more elaborate as islets begin to adopt their mature configuration (B; N=5), but tend to remain concentrated at the periphery of the maturing islets (C; N=4). Sensory fibers become associated with glucagon-producing α -cells as early as p0 (D; N=5); as with sympathetic fibers, sensory nerves follow islet capillaries (E), but remain enriched at the islet periphery throughout islet maturation (E, F; N=5 for each). GFAP is not a viable marker at birth (G; N=7), but astroglial encapsulation of nascent islets is under way by p7 (H; N=4), and increases as islets mature (I; N=4). Scale bar=50 μ m

Figure 5. Growth of the sympathetic perivascular plexus during postnatal pancreas maturation. VMAT2⁺ fibers (green) associate with blood vessels (blue) by p4, but occasionally depart from the vasculature (A; N=4); initial perivascular plexus formation is detected at p7 (B; N=5); vascular association resembles the adult perivascular plexus by p15 (C; N=4). Scale bar=25μm

Figure 6.

Differential effects of β -cell loss on sympathetic and sensory neurons and astroglia. VMAT2-reactivity in sympathetic fibers (green, A–D) was lost by 7dpi (B) and restored during the next 7 weeks (C, D) of β -cell recovery in RIP-cmycER mice. Fluctuations in the β -cell population resulted in a transient decrease in GFAP⁺ islet-associated astroglia (green, E–H). Although overall GFAP-reactivity did not seem to be affected, the degree of astroglial surround of islets had declined markedly by 2 weeks following initiation of cmyc activation in β -cells (G). Islet encapsulation increased over the following 6 weeks to become indistinguishable from wild type (H). CGRP-reactivity in sensory fibers (green, I–K) was retained in islets during the same time frame. Control RIP-cmyc^{ER} mice were not tamoxifen-treated. Control N=4; 7dpi N=4; 13dpi N=5; 60dpi N=5. Scale bar=20 μ m

Figure 7.

The structure of sympathetic fibers is intact throughout β -cell loss and resurgence. NPY-reactivity in sympathetic fibers (green, A–H) persists at perivascular plexuses along blood vessels (blue, A–D), and in close apposition to glucagon-producing α -cells (red, E–H). Control RIP-cmyc^{ER} mice were not tamoxifen-treated. Control N=4; 7dpi N=4; 13dpi N=5; 60dpi N=5. Scale bar=20 μ m

Table 1**Antibodies Used**

Antibody	Source	Conditions
Rabbit anti rat VMAT2	Phoenix	1:400 16 hours 4°C
Rabbit anti CGRP	Sigma	1:700 16 hours 4°C
Rabbit anti GFAP	Signet	1:50 16 hours 4°C
Rabbit anti Neuropeptide Y	Chemicon	1:400 16 hours 4°C
Guinea pig anti Insulin	Linco	1:400 16 hours 4°C
Guinea pig anti Glucagon	Linco	1:400 16 hours 4°C
Guinea pig anti Pancreatic Polypeptide	Linco	1:1000 16 hours 4°C
Rat anti Somatostatin	Chemicon	1:200 16 hours 4°C
Rat anti MECA32	BD Pharmingen	1:200 16 hours 4°C
Donkey anti Rabbit Alexa Fluor 488	Molecular Probes	1:500 16 hours 4°C
Goat anti Guinea pig Alexa Fluor 633	Molecular Probes	1:500 16 hours 4°C
Goat anti Rat Cy3	Jackson	1:200 16 hours 4°C