Chapter 2

Development of Human Pancreatic Innervation

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Abstract

Human pancreatic innervation is of particular interest due to its possible role in the pathogenesis of such diseases as diabetes mellitus, pancreatitis and pancreatic cancer. Despite the clinical importance, data concerning pancreatic innervation during human ontogeny and in various disorders are very limited. In this chapter, we present a review on human pancreatic autonomic innervation on the basis of the literature data and our previous results. Special attention is paid to the innervation of the endocrine pancreas. Gradual branching of neural network was seen during human pancreatic development. Innervation of the foetal pancreas is more abundant than in adults. In agreement with previous observations, we have revealed a close integration and similarity between endocrine cells and nervous elements in the developing human pancreas. Moreover, simultaneous interactions between the nervous system components, epithelial cells and endocrine cells were detected in the pancreas during prenatal human development. It has been suggested that pancreatic innervation plays an important role not only in regulation of endocrine and exocrine activity but also in normal islet morphogenesis.

Keywords: pancreatic innervation, islets of Langerhans, human development, sympathetic system, parasympathetic system

1. Introduction

The pancreas of most vertebrates is an organ that combines both endocrine and exocrine functions. Functions of the exocrine pancreas are the synthesis, accumulation and secretion of digestive enzymes (protease, amylase, lipase and nucleases) and preferment (elastase, procarboxypeptidase, trypsinogen, pepsinogen, deoxyribonuclease and ribonuclease). The main

function of the endocrine pancreas is regulation of carbohydrate metabolism. Specialised endocrine cells are grouped in units called pancreatic islets or islets of Langerhans. Islets of mammals (including humans) contain four major types of endocrine cells: beta cells secreting insulin, alpha cells secreting glucagon, delta cells secreting somatostatin and PP cells that synthesise pancreatic polypeptide [1]. Recently, another type of pancreatic endocrine cells was described—ghrelin-containing cells (epsilon cells) [2]. Pancreatic innervation is of interest due to its role in the pathogenesis of some diseases including chronic pancreatitis, pancreatic cancer and type 1 diabetes. Pain is the dominant clinical symptom in the majority of cases (73–93%) in patients with pancreatic cancer and pancreatitis. At the same time, the aetiology and pathogenesis of pain in chronic pancreatitis and pancreatic cancer are still unclear and are the subject of numerous studies [3].

In experiments on rodents (mice and rats) and cell cultures, it was indicated that nerve fibres and glial cells located in pancreatic islets may be the first target of autoimmune attack in type 1 diabetes [4–7]. Recently, there were reports of involvement of the peripheral nervous system in the pathogenesis of types 1 and 2 diabetes in humans [8, 9]. Moreover, the participation of the nervous system in the regulation of maturation, level of proliferation and number of insulin-producing beta cells, both in prenatal pancreatic development and in the postnatal period, was indicated in a number of experimental studies. Therefore, detailed information about the innervation of the endocrine pancreas is needed for understanding the mechanisms of beta cell pool renewal.

The pancreas is well innervated by the autonomic nervous system in various mammalian species [3, 10–15]. Rich innervation of the blood vessels and the exocrine part of the pancreas as well a more abundant innervation of the islets compared with the surrounding acinar part was detected already in the early studies [16, 17].

Connections between neurons are usually studied using anterograde and retrograde labelling of pathways. Pancreatic innervation was studied in various animal species using different tracing methods involving viruses, cholera toxin B, horseradish peroxidase, True Blue or DiI. It is believed that nerve fibres enter (and exit) in the pancreas as a part of neurovascular trunks. Within the pancreas, they also pass along the blood vessels and terminate (or, conversely, begin) near to the capillary wall and endocrine cells [18]. At the same time, they do not form classical synapses with target cells, but release neurotransmitters into the intercellular space, thus affecting more than one target simultaneously (i.e. they are enpassant synapses) [14]. Using retrograde labelling, the connection of pancreatic innervation with the central parasympathetic and sympathetic neurons in the brain stem, midbrain, hypothalamus and forebrain was shown [19–21]. Some of these brain centres are involved in monitoring of food intake or circadian rhythms, and it would be logical to assume that they send signals to the pancreas to adapt the digestive ferments and pancreatic hormone secretion to behavioural status. However, the central regulation of these processes has not yet been sufficiently studied [14].

In the pancreas, nerve endings were shown around blood vessels, as well as pancreatic acinar, ductal and endocrine cells, using immunohistochemistry and electron microscopy [17, 18]. Four types of plexuses (perivascular, periductal, periacinar and peri-insular) have been

identified in the mouse pancreas [18]. Similar data were obtained in studies on the pancreas of the rat and nutria [22, 23]. One of the most interesting features of the mammalian pancreas is that endocrine cells may form highly organised complexes with structures of the nervous system, so-called neuro-insular complexes (NICs). The structure of NIC in the human pancreas has not been studied in detail since their first description by van Campenhout [24] and Simard [25]. Fujita described two types of NIC, which he observed in the foetal and adult pancreas of the dog, cat and rabbit [26]. Some of the pancreatic ganglia contained endocrine cells forming NIC type I (NIC I). In NIC type II (NIC II), endocrine cells lie on the surface of, or even in the midst of, the nerve bundle. However, the distinction between these two types of complexes is conditional because there is an intermediate type of complex in which islets associate with nerve cells and nerve fibres simultaneously. Thus, in the pancreas, endocrine islets are closely associated with a dispersed neural network, which consists of autonomic nerves including sympathetic, parasympathetic and sensory nerves. Unfortunately, because of depth limitations in microscopy, this network cannot be easily portrayed by standard microtome-based two-dimensional (2D) histology. The systematic development of three-dimensional (3D) islet neurohistology has provided insight into neural-islet regulatory mechanisms and the role of neural tissue remodelling in the development of diabetes [27–29].

In addition, endocrine cells of pancreatic islets are similar to nervous cells in some biochemical and physiological characteristics. Some proteins expressed in endocrine cells of pancreatic islets are also specific to the nervous system: S100, GFAP (glial fibrillary acidic protein), GAD (glutamic acid decarboxylase), TH (tyrosine hydroxylase), NPY (neuropeptide Y), NSE (neuron-specific enolase) and others [6, 7, 30–32]. Moreover, a number of transcription factors that are characteristic of the nervous system, such as Ngn3 (neurogenin3), BETA2/NEUROD, etc., are expressed during the differentiation of pancreatic endocrine cells [33–35]. The cells of the endocrine pancreas are classified as cells of a dispersed (diffuse) endocrine epithelial system. The cells of the dispersed endocrine system are a part of the so-called APUD (amine precursor uptake and decarboxylation) system [36]. These cells have the combined ability to the capture and deposit amine precursors and synthesise biogenic amines. The obvious similarity between the pancreatic endocrine cells and nerve tissue leaves the issue of its causes open to discuss.

The precise innervation patterns of islets are unknown, particularly in humans [37]. Every year reviews are published, in which morphology and function of pancreatic innervation are discussed (see for review [10, 11, 14, 15, 38–40]). However, the nature and distribution of the nervous system structures in the pancreas were studied mainly in rodents. Interspecies differences in the structure and innervation of the pancreas between humans and experimental animals (mice and rats) are quite large. In humans, the pancreas is a compact organ, while in rodents it is treelike, distributed over the mesentery of the small intestine. Therefore, it is impossible to automatically transfer the data obtained on experimental animals to humans.

In addition, knowledge about the dynamics of innervation during ontogenesis and in various diseases of the pancreas is very limited. Single studies are devoted to the formation of innervation in prenatal human development (mainly in the last century, without the use of modern methods). Therefore, the fine details of pancreatic innervation (such as the distribution of

sympathetic and parasympathetic fibres and the formation of neuro-insular complexes) in human ontogenesis are insufficiently studied. This is mainly due to the inaccessibility of the material and to a number of technical difficulties, including the quality of pancreatic autopsy samples due to the activity of enzymes of the exocrine part [40].

However, over the past 10 years, different groups of researchers have made significant progress in the study of the peculiarities of innervation in rodents. The most attention was paid to the influence of the nervous system on the endocrine pancreas. It has been shown that both sympathetic and parasympathetic nervous systems affect postnatal development of the endocrine pancreas and its plasticity in adult animals [9, 41]. For example, after vagotomy there was a decrease in insulin-containing cell proliferation in mice and rats [42]. The important role of the sympathetic innervation for the formation of islet cytoarchitecture and their functional maturation during development was also shown [43].

Thanks to recent progresses in the field of islet research (including the study of isolated islets, in thick slices and in vivo), a number of issues concerning the structure and functions of pancreatic innervation have been clarified (see, e.g. [44–47]). In this chapter, we summarise the literature data and our previous results concerning the morphological organisation of autonomic innervation in the human foetal and adult pancreas. We also discuss the possible role of the close integration between the nervous system and epithelial and endocrine cells in the development of the endocrine pancreas.

2. Sources of pancreatic innervation

The pancreas is innervated by sympathetic and parasympathetic nerve fibres [11, 13]. The literature data indicate poor innervation of adult human pancreatic islets in comparison with rodents [44, 48–50]. At the end of the twentieth century, pancreatic innervation by postganglionic adrenergic and cholinergic fibres was intensively studied (for references, see [51]). Single nerve cells and nerve ganglia, both myelinated and unmyelinated nerve fibres of various diameters, have been detected in the human pancreas [23, 37, 48, 49]. In a simplified form, it can be considered that pancreatic sympathetic innervation is effected by the fibres of the ventral trunk and the parasympathetic innervation by the vagus nerve.

2.1. Efferent sympathetic fibres

Bodies of neurons, which form the efferent preganglionic sympathetic nerve fibres, are localised in the thoracic and upper lumbar segments of the spinal cord (T5–L1) [37, 52] or, according to some literature, in C8–L3 [21, 53]. Myelinated axons of these cells leave the ventral roots of the spinal cord and terminate on the bodies of neurons that lie in the ganglia of the paravertebral sympathetic chain, or pass through this chain via the n. splanchnicus to the celiac (*celiac*) and superior mesenteric (*mesenteric*) ganglia, and then terminate on neurons localised in these ganglia [54, 55]. The preganglionic fibres of the sympathetic system secrete acetylcholine (Ach). Postganglionic nerve fibres go to the pancreas, where they secrete norepinephrine, which binds to α and β adrenergic receptors and the neuropeptides galanin and NPY (neuropeptide Y) [10, 11, 53, 56].

In humans, the body and tail of the pancreas are innervated by nerve fibres originating from the ventral plexus and accompanying two arteries: the splenic artery and the transverse artery of the pancreas. The pancreatic head receives the largest number of nerve fibres [57, 58].

In the exocrine pancreas, sympathetic axons contact mostly with intrapancreatic ganglia, blood vessels and ducts. In mice, the innervation of the exocrine part is less pronounced than in humans. The major nerves run along the interlobular arteries and form the peri-insular plexus [18]. At the same time, in mice axons of sympathetic nerves contact alpha cells, while contact with beta cells is not found [44]. The axons of sympathetic nerves also innervate smooth muscle cells and pericytes of blood vessels and perivascular space, forming the so-called sympathetic neurovascular complex. In humans, sympathetic fibres innervate smooth muscle cells and pericytes and rarely contact directly with the endocrine cells. Apparently, the effects of the sympathetic innervation are likely mediated through indirect effects on local blood flow within the islet microcirculation [44, 59].

2.2. Efferent parasympathetic fibres

The bodies of the neurons forming the parasympathetic preganglionic nerve fibres lie in the dorsal motor nucleus of the n. vagus (X) [60–62] and, possibly, in the *nucleus ambiguus* [11–13]. Both of these nuclei are under the control of the hypothalamus. Preganglionic parasympathetic fibres are directed to the pancreas as a part of the vagus nerve branches. In the pancreas, parasympathetic fibres terminate on the bodies of parasympathetic neurons lying in intrapancreatic ganglia [38, 63]. These ganglia contain from 3 to 30 neurons and are usually located in intralobular connective tissue, within lobules or in close proximity to islets [13, 27, 29]. It is also important that these ganglia receive input not only from the parasympathetic nervous system but also from the sympathetic nervous system, as well as fibres from other intrapancreatic ganglia and also from the *myenteric plexus* [13]. Parasympathetic fibres are also involved in the formation of nerve plexuses around the arteries and mingle with sympathetic fibres.

Preganglionic parasympathetic fibres secrete acetylcholine (Ach), which binds to nicotine receptors on the membranes of neurons [53]. Short, unmyelinated postganglionic fibres terminate on the epithelial cells of acini and ducts, smooth muscle cells and islet cells. Postganglionic parasympathetic fibres release several neurotransmitters (Ach (acetylcholine) and NO (nitric oxide)) and neuropeptides (VIP (vasoactive intestinal peptide), GRP (gastrin-releasing peptide) and PACAP (pituitary-activating adenyl cyclase polypeptide)) [10, 11, 13, 56]. Postganglionic nerve fibres perform their functions mainly via Ach by binding to muscarinic receptors found, in particular, in the endocrine cells of the islets [12, 53]. In mice, postganglionic parasympathetic nerve fibres innervate all types of islets cells [10, 11, 44]. Recently, it was found that parasympathetic islet innervation in humans differs from that in mice: first, it was shown that only a small number of fibres penetrate inside the islets (most of the axons terminate in the exocrine part of the pancreas) [44], and, secondly, it was recently shown that stimulation with Ach mostly stimulates beta and delta cells, whereas alpha cells react to a lesser extent [64]. Interestingly, alpha cells themselves may be the primary source of Ach in human islets [45]. Apparently, in human islets, this classical neurotransmitter regulates the activity of other cell types in a paracrine manner. However, now, this concept is again under revision thanks recently to the work of Tang et al. [29].

2.3. The afferent fibres

In the pancreas, there are afferent (sensory) nerve fibres in addition to efferent sympathetic and parasympathetic innervation [10–12, 53, 54]. Bundles of sensory nerve fibres leave the pancreas and follow the sympathetic (*n. splanchnicus*) and vagus nerves. The bodies of sensory sympathetic neurons are localised in the ganglia of the dorsal roots in the spinal cord, mainly at the level of the lower thoracic segments (the so-called spinal afferents) projected on interneuron plates I and IV [52, 65]. For the parasympathetic system, the bodies of afferent neurons are localised in the ganglion nodosum, sending information to the nucleus of tractus solitarii [12, 54]. The neurotransmitters of the sensory nerve fibres are CGRP (calcitonin gene-related peptide) and SP (substance P). Most sympathetic and parasympathetic afferent nerves are sensitive to capsaicin [14]. Capsaicin (vanillin) receptors mainly transmit pain information [66]. In addition, Pacinian corpuscles were described in the pancreas of various mammalian species. The suggested function of this receptor is to transmit information about pressure and vibration stimuli. In the human pancreas, they were discovered in the early twentieth century [67]. Despite this fact being presented in many histology textbooks, in the modern literature, only three cases of these findings (all in pancreatic cancer) were described [67, 68]. In our research, we have studied pancreatic autopsies of 42 foetuses and neonates aged from the 10th to 40th week of gestation and of 65 adults, 18 of whom suffered from diabetes mellitus type 2. In total, more than 1000 sections were investigated. However, Pacinian corpuscles are a rare finding in the human pancreas: we were able to detect Pacinian corpuscles only in one pancreatic section of a newborn with diagnosed diabetic fetopathy. Thus, Pacinian corpuscles do not appear to play a significant role in the sensory innervation of the human pancreas.

2.4. Enteric nervous system

In some studies on pancreatic innervation, it is assumed that the pancreas is innervated not only by extrinsic efferent and afferent nerves but also by intrinsic enteric neurons of the so-called enteric nervous system (ENS) [12, 69]. The ENS controls the motor, secretion and other functions of the gastrointestinal tract and is closely related with the diffuse endocrine system [70]. Enteric ganglia have some morphological and functional differences from sympathetic and parasympathetic ganglia:

1. The ENS performs complex integrative functions independently of higher nerve centres.
2. In the ENS, a large number of various neurotransmitters, many of which are characteristic of the central nervous system, are produce.
3. Unlike other autonomous ganglia, enteric ganglia do not contain connective tissue and blood vessels. Enteric ganglia are demarcated from the surrounding tissue of the so-called blood-ganglionic barrier, similar to the blood–brain barrier. It is insufficiently studied, and not all researchers agree with its existence.
4. Glial cells of enteric ganglia are similar in morphology, cell markers and functions with astrocytes of the CNS.

The complex structure of the enteric nervous system, containing a variety of morphological and functional types of neurons and their neurotransmitters, allows the ENS to perform complex reflex acts, some of which are implemented autonomously and some in interaction with the central nervous system and other parts of the autonomous nervous system. Intrapancreatic ganglia are connected with autonomous ganglia in the intestinal nerve plexus [71–73]. Neurotransmitters for neurons of these ganglia are, among others, serotonin and nitric oxide (NO) [73]. However, according the dominant viewpoint, intramural pancreatic neurons belong to the parasympathetic system.

3. Functional role of pancreatic innervation

As was mentioned earlier, the pancreas combines exo- and endocrine functions, secreting digestive enzymes and hormones, which regulate glucose homeostasis. The nervous system regulates the activity of both the endocrine and exocrine pancreas. However, it is problematic to separate the innervation of the pancreatic endocrine part from the innervation of the exocrine, since the tracing method used for this purpose belongs to the pancreas as a whole. In addition, the activity of both endocrine and exocrine parts of the pancreas depends on food intake. Therefore, it is not surprising that the cephalic phase has been described for both pancreatic parts. Although the stimulation of the ventromedial hypothalamus and efferent sympathetic and parasympathetic neurons affects the secretion of islet hormones (see below), it is unknown whether this stimulation is direct through axons innervating the islet or indirect by activating other organs, which affect insulin and glucagon secretion [14]. Moreover, it is very difficult to separate the nervous system effects from other (e.g. humoral) influences.

So, in the laboratory of I.P. Pavlov, in 1895, I.L. Dolinsky conducted an experiment in which he established that acid injection into the duodenum causes a release of pancreatic juice [74]. In 1901, British physiologists William Baileys and Ernest Starling concluded that there is some substance released by the duodenum that stimulates secretion by the pancreas. In the following year, 1902, this substance was discovered and named secretin. Secretin was the first such "chemical messenger" identified. This type of substance is now called a hormone.

At the same time, in the classic studies of I. P. Pavlov with M. A. Afanasiev, the nervous mechanism of pancreatic secretion was found. In the work "On secretory nerves of the pancreas" (1877), they showed that vagus nerve stimulation causes pancreatic secretion. Moreover, I. P. Pavlov with his colleagues detected that imaginary feeding in animals with chronic pancreatic fistula causes an abundant release of pancreatic juice. Later, this was confirmed by the studies of K. M. Bykov and G. M. Davydov in patients with pancreatic fistula. An abundant pancreatic juice released by this patient occurred while talking about delicious food [74]. However, pancreatic juice obtained after vagus nerve stimulation is released in a small quantity and is rich in proteins and enzymes, whereas after the secretin injection, it contains little proteins and enzymes and is released in large quantities [74]. It should be noted that both these factors (nervous and humoral) act simultaneously and synergistically.

Currently, it is considered that efferent sympathetic nerve fibres indirectly inhibit the release of enzymes of the exocrine pancreas by suppressing the stimulating effects of ganglia and constriction of vessels (vasoconstriction), thereby reducing blood flow [13, 59]. The stimulation of short, unmyelinated postganglionic parasympathetic fibres increases release from secretory cells of the exocrine pancreas and ducts causing vasodilation [13, 57].

The autonomous nervous system also regulates hormone release in the endocrine pancreas, thereby affecting glucose metabolism [10, 11, 14, 53]. Many various chemical factors affect insulin and glucagon expression. Auto-, juxta-, para- and endocrine ways potentially regulate secretion of islet hormones. Since the classical studies of Claude Bernard, which showed that injection into the floor of the fourth ventricle causes hyperglycemia, the involvement of the nervous system in the regulation of pancreatic endocrine function and metabolic control has been shown in many studies. It is, therefore, rather difficult to separate one effect from the other [14, 53].

The cellular architecture of islets affects paracrine regulation and synchronises the release of insulin [75]. All pancreatic islets secrete hormones consistently, with an approximately 5-min interval [76]. In order to create this secretion pattern, the activity of insulin-containing beta cells must be consistent both within the individual islet and between the islets [14]. At the same time, the secretory activity of other islets endocrine cells, such as glucagon-secreting alpha cells that have opposite effects on glucose homeostasis, should be consistent with the activity of beta cells. Thanks to this interaction, endocrine cells can simultaneously send signals regulating the effective delivery of islet hormones into the circulatory system and, ultimately, to the liver, regulating the maintenance of glucose homeostasis [76].

However, the islets of Langerhans are a part of a complex coherent system. They are also exposed to humoral factors such as circulating plasma hormones (e.g. epinephrine). The brain also regulates the secretion of islet hormones via the autonomic nervous system [14]. Thus, in works by Akmaev et al. [19], it was shown that the hypothalamus is able to stimulate insulin secretion from beta cells of pancreatic islets along the nerve pathway, which was named "paraventricular-vagal." This pathway starts from small neurons of the paraventricular nucleus (PVN) of the hypothalamus, synaptically switches in the medulla oblongata to neurons of the dorsal nucleus of the vagus nerve and reaches the pancreatic islets in the composition of the vagus nerve. In this pathway, beta cells receive stimulating signals. Inhibitory signals come from neurons by a humoral way: PVN neurons secrete corticotropin-releasing hormone, which stimulates the secretion of adrenocorticotropic hormone in the pituitary gland that induces the secretion of glucocorticoids in the adrenal cortex. Glucocorticoids inhibit insulin release from beta cells. This kind of double control, according to the authors, is typical for the regulation of endocrine functions. Recently, there has been data that significantly complements this concept: various areas of the hypothalamus have different effects on the secretion of insulin and/or glucagon [77]. So, a detailed study of this system is needed to further identify both neurons and functionally related projections of the central nervous system regulating islet functions.

For most species studied, it is characteristic that nerve fibres are localised mainly at the periphery of the pancreatic islets, forming a peri-insular nervous network [17]. Only single

nerve fibres are detected within islets. The bodies of ganglion neurons are also rarely localised in the pancreatic islets and may be in direct contact with endocrine cells [17, 27, 29, 78, 79].

It is believed that autonomic innervations indirectly affect the release of insulin in the cephalic phase during food intake and also take part in the increase of glucagon and decrease of insulin release by sympathetic stimulation [10, 80]. Stimulation of the splanchnic nerve increases the release of glucagon and reduces the release of insulin and somatostatin from endocrine cells of the pancreas [12, 14, 15]. Sympathetic nerves are also believed to be involved in islet response for hypoglycemia, which includes increased glucagon secretion and inhibition of insulin secretion. The general sympathetic effect is expressed by reducing the insulin concentration in plasma (by increasing the concentration of catecholamines that inhibit insulin secretion) [10, 11].

Parasympathetic nerves are responsible for the early phase of insulin secretion, including the cephalic phase (i.e. insulin secretion, which occurs during anticipation of eating). In general, parasympathetic stimulation is believed to increase the release of insulin, glucagon, somatostatin and pancreatic polypeptide in many different species (for review, see [10, 11, 14, 15]).

Sensory nerves are also involved in the regulation of hormone secretion by endocrine cells [11]. Following chemical destruction of sensory nerves (capsaicin treatment) in mice, there is an increase in insulin secretion in response to glucose compared to control [81].

In conclusion, it should be added that pancreatic innervation is insufficiently studied, especially in humans [40, 44]. Interestingly, the innervation of the islets is very plastic: it has been shown that islets transplanted into the portal vein of diabetic rats were reinnervated by the nerves of the liver [82]. This makes it necessary to further study the role of innervation in the regulation of glucose homeostasis and plasticity of the endocrine part of the pancreas.

4. Pancreatic innervation during prenatal development

Despite the clinical importance, data concerning pancreatic innervation during human ontogeny and in diseases are very limited [37]. Such studies have been performed on rodents and mostly concern the sympathetic innervation [43, 55, 83]. The embryonic sources of neural elements are fibres of the vagus (*n. vagus*) and splanchnic nerves (*n. splanchnicus*) growing into the developing pancreas and neurons that differentiate from the neural crest cells migrating to the pancreas. Sympathetic fibres innervate the developing mouse pancreas starting from the 15th day of embryonic development (E14.5) [43]. Consequently, the degree of sympathetic innervation increases until 20 days of postnatal development (P20) [55]. The development of the pancreatic sympathetic innervation depends on nerve growth factor (NGF) [43].

The human pancreas receives extensive innervation, showing peculiar growth dynamics during gestation [37]. Ingrowths of nerves in the human pancreas start at 6 weeks of development. Further morphogenesis of pancreatic innervation is characterised by the increase of sources of innervation and degree of nervous element differentiation [84, 85]. Large bundles of nerve fibres and groups of poorly differentiated neurons are found in the human pancreas

starting from the 8th week of development. At the end of the 9th week, the pancreas is innervated from almost all sources, characteristic of adults (celiac plexus, superior mesenteric plexus and posterior vagal trunk) [85]. In 1940, it was shown that pancreatic nerve cells migrate from the solar plexus and from ganglia located in the wall of the duodenum and along the branches of the vagus nerve (mainly right). At the same time, neuroblasts were detected in the pancreas of 20-week-old foetuses. Moreover, even in newborns pancreatic nerve cells were neuroblastic [86].

The gradual branching of the vascular and neural networks is observed in the human pancreatic development. Primitive free nerve endings are detected starting from the 12th week of development. In an immunohistochemical study of pancreatic innervation development in human foetuses, two peaks of increase in the number of structures of the nervous system in the head of the gland were revealed at the 14th and 22th weeks. In the pancreatic body and tail, the number of nerve structures increases from the 20th week [37]. By 30–32 weeks of development, the density of nerve endings is reduced compared to previous periods [85]. The innervation of pancreatic islets in humans is formed from the 14 to 15th weeks of the development. It differs from experimental mammals (rodents): the development of pancreatic islet innervation in rodents (mouse, Mongolian gerbil and golden hamster) is observed in the first weeks after birth [83, 87, 88].

Our study was performed on a collection of pancreatic autopsies, which allows us to explore the features of intrapancreatic innervation directly in humans using a variety of methods: classical histology; immunohistochemistry; light, fluorescent and confocal microscopy; morpho- and stereometry; statistical analysis; 3D histology; and computer reconstruction. The study was performed on 50 pancreatic autopsies of foetuses from the 10th to 40th gestational week (g.w.). Foetal pancreatic autopsies were divided into four groups according to the classification of the foetal period: pre-foetal period (10–12 g.w.), early foetal period (13–20 g.w.), middle foetal period (21–28 g.w.) and late foetal period (29–40 g.w.). A panel of antibodies for nervous system proteins (chromogranin A, neuron-specific enolase (NSE), neural cell adhesion molecule (NCAM), synaptosomal-associated protein of 25 kDa (SNAP-25, peripherin, S100 protein and neuron-specific class III β-tubulin), endocrine cell hormones (insulin, glucagon and somatostatin) and epithelial cells (cytokeratin 19 (CK19)) were used in this work [89, 90]. We generated new data concerning the spatio-temporal distribution of the innervation in the human pancreas during prenatal development.

In the pre-foetal period (10–12 g.w.), large weakly branched bundles of nerve fibres and nerve ganglia were detected already at the 10th week of gestational development using antibodies to NSE, NCAM and neuron-specific β-III tubulin (**Table 1**). The largest bundles of nerve fibres were detected in the dense peri-pancreatic mesenchyme, and the group of neurons and bundles of nerve fibres of smaller diameter were located in the loose mesenchyme between pancreatic ducts (**Figure 1a**). A network of fine nerve fibres was not developed. In some cases, bundles of nerve fibres were found near large vessels. Nerve ganglia in the pancreas of 10–12 week foetuses were small groups of cells.

Starting from 12 weeks, cells immunopositive for antibodies to S100 protein were found in nervous system structures. Localisation of neuromarkers was different. In the nerves, NSE-positive

Markers	NSE	NCAM	Neuron-specific β-III tubulin	S100 protein	Chromogranin A	SNAP-25	Peripherin
Nerve fibres and ganglions	10 weeks	10 weeks	10 weeks	12 weeks	14 weeks (weak staining)	14 weeks	14 weeks
Endocrine cells	12 weeks	14 weeks	14 weeks	15–16 weeks (some islets cells)	12 weeks	16 weeks	—

Table 1. Appearance of immunopositive reactions to neural proteins in the developing human pancreas.

fibres formed the core, while small S100-positive cells surrounded them. The ganglionic cells were NSE-positive, and the small cells surrounding them S100-positive. The bodies of ganglion neurons were immunonegative to S100, that is, the positive reaction to S100 protein was observed in satellite cells of intrapancreatic ganglia and in Schwann cells of nerve fibre bundles, while NSE was detected in neuronal bodies and processes. In addition, NSE- and chromogranin A-positive endocrine cells were first found in 12-week foetuses (**Table 1**).

The formation of the human pancreatic islets starts only at 12 weeks of development. In the pre-foetal period, only contacts between single endocrine cells or small groups and fine nerve fibres were detected, and classical NIC I and NIC II were not found. At gestational week 10 (postconception week 8), thickening of the ductal epithelial layer was found, in which endocrine cells were concentrated forming "buds" on pancreatic ducts. As development proceeds, buds containing different types of endocrine cells separate from the ducts forming small clusters or mantle-type islets. In our studies, contacts between the structures of the nervous system and epithelial cells of primitive ducts were detected in the foetal pancreas at early stages of development (10–13 weeks) before the formation of islets.

The formation of the pancreatic lobules begins in the early foetal period, from 13 weeks. At the same time, active formation of the islets of Langerhans and innervation of the endocrine part starts (**Figure 1b**). Nervous system of the pancreas of 14–15 week foetuses becomes more branched in comparison with 10–12 weeks of development. Large bundles of nerve fibres are localised in the connective tissue of gland's capsule. Smaller nerves pass into the interlobular connective tissue separately or along the blood vessels. Nerve fibres and ganglia are first found within the lobules. At the 16th week of development, the nervous apparatus of the pancreas is presented by bundles of nerve fibres of different diameters and nerve ganglia, which are located in the interlobular connective tissue and within the lobules. The nerve fibres connecting two nerve ganglia were found in 14–15 week foetuses, i.e. the first clearly detected integration of the nervous system structures was shown.

Localisation of antigens in the structures of the nervous system was also similar with the pre-foetal period. In addition, the immunopositive cells for chromogranin A, SNAP-25 and peripherin were detected in the nerve fibres and ganglia starting from 14 to 15 weeks of the development (**Table 1**). SNAP-25, NCAM, NSE, peripherin and neuron-specific β-III tubulin

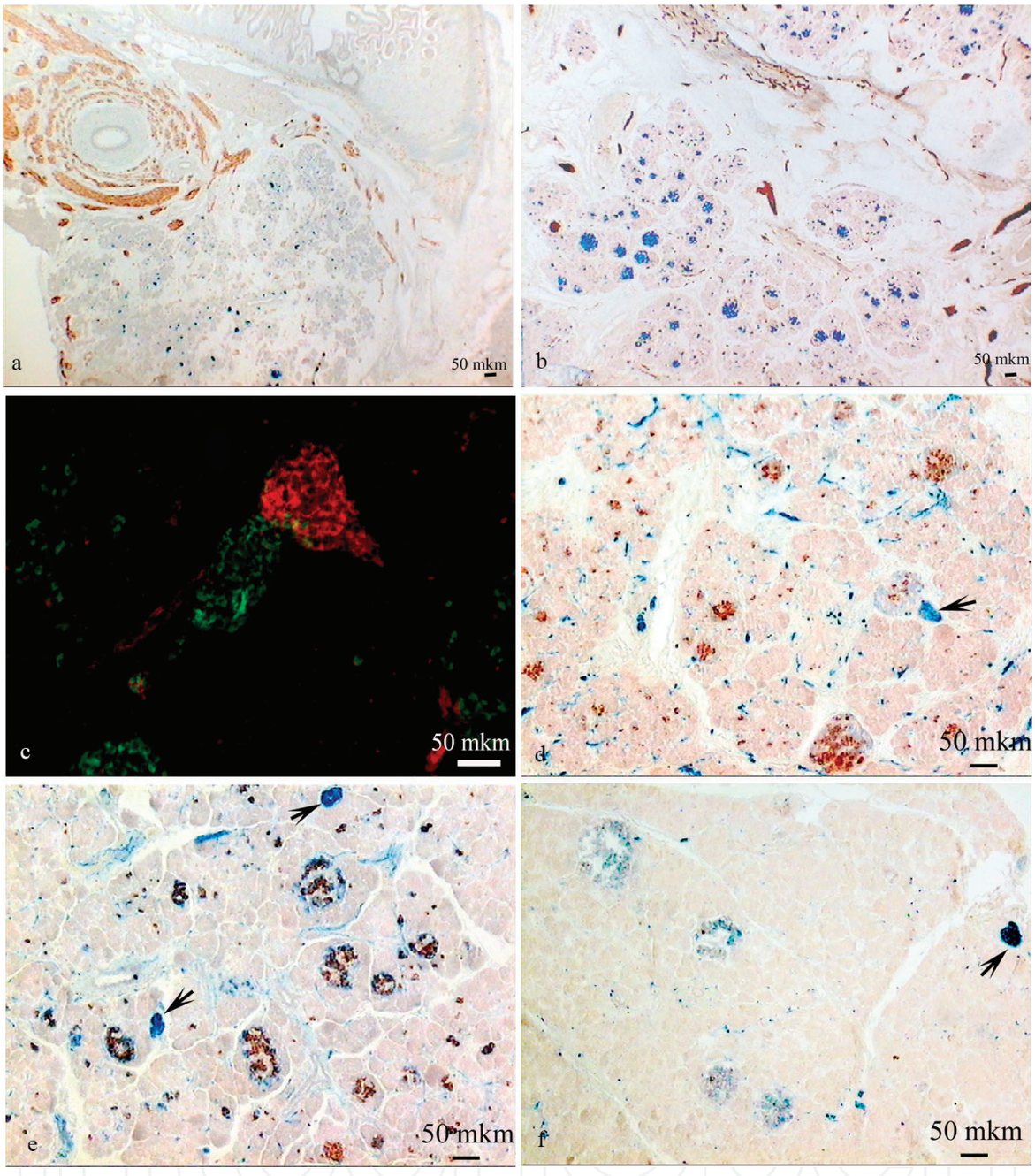

Figure 1. Spatio-temporal distribution of the nervous system structures in the human pancreas during ontogenesis. (a, b, d–f) double immunohistochemistry on the pancreatic slices of foetuses ((a) 12 g.w., (b) 16 g.w., (d) 28 g.w.), child ((e) 3 months) and adult ((f) 88 years): (a, b) insulin (blue) + S100 (red), (d, e) insulin (red)+ NSE (blue) and (f) glucagon (red) + NSE (blue). Arrows indicate some ganglia. (c) Stack of serial immunofluorescence images of NIC in the foetal pancreas (20 g.w.) (sum thickness of slices 90 mkm): Glucagon (green) + S100 (red).

were detected in bundles of nerve fibres of different diameters and the bodies of neurons in human foetuses. However, there were fine nerve fibres located in the acinar parenchyma that were immunonegative for peripherin but reacted with other markers in all investigated cases. This suggests that nerve fibres of the human pancreas differ according to the set of expressed proteins. In addition, positive immunostaining for NCAM and neuron-specific β-III tubulin was observed in endocrine cells starting from 14 weeks of development,

while SNAP-25-positive endocrine cells were detected only from 16 weeks of development. Immunopositivity to antibodies against S100 protein was found only in some islet cells starting from 15 to 16 weeks of development (**Table 1**).

The contacts of nerves fibres with endocrine cells were detected starting from 12 weeks of development. Already in the early foetal period, it was possible to identify NIC I (single insulin- or glucagon-containing cells in ganglia (Supplementary Video 1) or ganglia associated with the islets) and NIC II (single endocrine cells in the nerve (Supplementary Video 2), nerve endings associated with single endocrine cells or with the islets) and make their 3D reconstruction. The analysis of three-dimensional reconstructions allowed us to show ganglia associated with two islets at once, islets associated simultaneously with two ganglia, and NIC of mixed (intermediate) type [91]. Moreover, in the foetal pancreas, starting from 13 weeks, we showed simultaneously neuro-insular complexes and contacts between the structures of nervous system and epithelial cells located in ducts as well as in cell clusters that were often connected with the ducts. Based on these findings, we suggested that the development of neuro-insular complexes may be due to integration between the structures of the nervous system and epithelial progenitors at the initial stages of islet formation. Furthermore, endocrine cells are supposed to migrate along nerve fibres from the ducts, small clusters of endocrine cells and islets to the other islets, which are located a distance from pancreatic ducts, due to exocrine pancreatic growth, thus increasing their pool of endocrine cells. We suppose that the mechanism of pancreatic islet formation is similar to the formation of some peripheral analysers.

The pattern of immunoreactivity of neural markers during the middle (21–28 g.w.) and late foetal periods is similar to those in the early foetal period. In the middle of the foetal period, the density of pancreatic innervation is higher than in the early foetal period (**Figure 1c**, **d**). Despite increasing the size of pancreatic lobules and more sparse distribution of large and medium bundles of nerve fibres, the network of fine nerve fibres gradually branch and become denser. However, during late foetal and neonatal development, this network is much sparser (**Figure 1e**). This is due to the increase in the size of lobules. However, at all stages of human prenatal development, density of distribution of the nervous system structures is higher than in adults (**Figure 1f**). The density of NIC distribution also gradually decreases at birth. Our quantitative data indicate that the largest number of NIC I was observed in the early and middle foetal periods, during the active morphogenesis of pancreatic islets, whereas at birth (in the late foetal period) and in the adult, NIC II became more prevalent [91]. During the middle and late foetal periods, the nervous system components also contact epithelial cells located in ducts or in clusters outside the ductal epithelium and form complexes with separate epithelial cells. We observed CK19-positive cells inside the ganglia and nerve bundles, which were located separately or integrated within the islets [90].

In this study, our previous data were confirmed and refined [89] that the formation of the nervous system in the development of human pancreas can be divided into three stages. In the pre-foetal period, the nervous apparatus of the pancreas is represented by slightly branched bundles of nerve fibres and nerve ganglia. However, the structures of the nervous system differ from the late foetuses and adults by antigenic composition. Expression of various neural proteins does not begin simultaneously in the foetal pancreas.

The second stage of development of the nervous apparatus of the pancreas (during the early and middle foetal periods) is characterised by gradual branching of the neural network and formation of connections between the structures of the nervous system and exocrine and endocrine parts. In the early foetal period, nerve fibres gradually branch, nerve fibres and nerve ganglia appear localised between the acini, and a network of fine nerve fibres starts to form. In the later stages of development, the distribution of neural structures (nerve fibres, nerve ganglia and parenchymal network of fine nerve fibres) become sparser with increase in the size of the pancreas. Thus, innervation of the pancreas at this stage of development gradually becomes similar to the distribution structures of the nervous system in the adult pancreas.

In our studies, we demonstrated close integration between the structures of the nervous system and endocrine cells in the human pancreas, which were more frequently observed during prenatal development. Thus, a dense network is formed in the developing human pancreas, in which the structures of the nervous system are associated with the islets of Langerhans. The close relationship between developing islets and structures of the nervous system suggests that neuroendocrine interactions can influence not only the secretion of hormones but also to participate in the morphogenesis of the islets, presumably due to the participation in migration of endocrine cells from ducts to islets. Understanding the role of NICs in islet formation can lead to new approaches to understanding the mechanisms and treatment of diabetes.

5. Conclusions

Thus, our knowledge about the peripheral nervous system in the human pancreas is limited. Importantly, human islet development has not been examined for the presence of classical markers of the parasympathetic and sympathetic nervous systems. Furthermore, the exact location where neuronal axons terminate within the human islets in adults was not shown until recently.

However, the human pancreas is abundantly innervated during the gestational period. The value of such an abundant innervation of the pancreas and pancreatic islets, in particular, in human development is not clear. The observed differences between the nervous apparatus of foetuses and adults may have functional significance for pancreatic morphogenesis. Interestingly, some authors have described similar dynamics of innervation development in other internal human organs. The close relationship between the nervous and endocrine systems makes it necessary to further study the role of innervation in the plasticity of the endocrine pancreas both during formation of endocrine function and disorders of carbohydrate metabolism.

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Conflict of interest

The authors declare no competing interests.

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