Received: 25 December 2019 | Revised: 3 March 2020 | Accepted: 17 April 2020

DOI: 10.1111/nmo.13880

ORIGINAL ARTICLE

Neurogastroenterology & Motility NGM WILEY

Localisation analysis of nerves in the mouse pancreas reveals the sites of highest nerve density and nociceptive innervation

Ömer Cemil Saricaoglu[1] | Steffen Teller[1] | Xiaobo Wang[1] | Shenghan Wang[1] | Pavel Stupakov[1] | Tobias Heinrich[1] | Rouzanna Istvanffy[1] | Helmut Friess[1] | Güralp O. Ceyhan[1,2] | Ihsan Ekin Demir[1,2,3,4]

[1]Department of Surgery, Klinikum rechts der Isar, School of Medicine, Technical University of Munich, Munich, Germany

[2]Department of General Surgery, HPB-Unit, School of Medicine, Acibadem Mehmet Ali Aydinlar University, Istanbul, Turkey

[3]German Cancer Consortium (DKTK), Partner Site, Munich, Germany

[4]CRC 1321 Modelling and Targeting Pancreatic Cancer, Munich, Germany

Correspondence
Ihsan Ekin Demir, Department of Surgery, Klinikum rechts der Isar, Technical University of Munich, School of Medicine, Ismaninger Str. 22, D-81675 München, Germany.
Email: ekin.demir@tum.de

Funding information
IED was supported by a grant of the Deutsche Forschungsgemeinschaft/ DFG (DE 2428/3-1 and 3-2).

Abstract

Background: Neuropathy and neuro-inflammation drive the severe pain and disease progression in human chronic pancreatitis and pancreatic cancer. Mice, especially genetically induced-mouse models, have been increasingly utilized in mechanistic research on pancreatic neuropathy, but the normal "peripheral neurobiology" of the mouse pancreas has not yet been critically compared to human pancreas.

Methods: We introduced a standardized tissue-harvesting technique that preserves the anatomic orientation of the mouse pancreas and allows complete sectioning in an anterior to posterior fashion. We applied immunohistochemistry and quantitative colorimetry of all nerves from the whole organ for studying pancreatic neuro-anatomy.

Key Results: Nerves in the mouse pancreas appeared as "clusters" of nerve trunks in contrast to singly distributed nerve trunks in the human pancreas. Nerve trunks in the mouse pancreas were exclusively found around intrapancreatic *blood vessels*, and around *lymphoid structures*. The majority of nerve trunks were located in the pancreatic head (0.15 ± 0.08% of tissue area) and the anterior/front surface of the corpus/body (0.17 ± 0.27%), thus significantly more than in the tail (0.02 ± 0.02%, $P = .006$). Nerves in the tail included a higher proportion of nociceptive fibers, but the absolute majority, ie, ca. 70%, of all nociceptive fibers, were localized in the head. Mice heterozygous for *Bdnf* knockout allele ($Bdnf^{+/-}$) exhibited enrichment of nitrergic nerve fibers specifically in the head and corpus.

Conclusions & Inferences: Neuro-anatomy of the "mesenteric type" mouse pancreas is highly different from the "compact" human pancreas. Studies that aim at reproducing human pancreatic neuro-phenomena in mouse models should pay diligent attention to these anatomic differences.

KEYWORDS

BDNF, mouse, nerve trunks, nitrergic, pain, pancreas

1 | INTRODUCTION

Pancreatic cancer (PCa) and chronic pancreatitis (CP) are characterized by prominent alterations of intrapancreatic nerves like nerve hypertrophy, neuro-inflammation, and neural invasion, which are now to give rise to severe neuropathic abdominal pain.[1-3] Mouse models, particularly genetically induced mouse models, have been recently and increasingly introduced into the study of these neuropathic alterations.[4-6] However, these studies do not yet seem to take into account sufficiently the anatomic differences in the innervation of the mouse pancreas vs human pancreas. Indeed, human pancreas is a compact solid organ, which is localized in the retroperitoneum.[7] Conversely, mouse pancreas is a "mesenteric" type pancreas, which is not as compact and rather scattered in the adjacent small intestinal mesentery and is thus localized intraperitoneally.[7] For correct comparison and reporting of nerve alterations in mouse models and for purposes of simulating and studying human disease, it is imperative to know the localization and distribution of nerves in the mouse pancreas. Importantly, to date, despite some studies that compared the ganglia and the fine nerve fiber distribution,[8,9] there has been no study that investigated the differences in the nerve trunk anatomy between the mouse and the human pancreas.

In the present study, we performed a systematic morphological analysis of the nerves in correlation with their location in the pancreas. Here, we show that mouse pancreatic nerve trunks are solely located around peripancreatic lymphoid structures and around vascular complexes. Furthermore, we reveal a greater density of nerves in the head and anterior body (corpus) of the mouse pancreas, when compared to the remainder of the organ. Among pain-transmitting nerve fiber subtypes, we show that the distribution of nerves which contain pain-related neuropeptides like substance P (SP), CGRP, vasoactive intestinal peptide (VIP), or NOS, does not vary between different regions of the mouse pancreas and that they constitute around 10% percent of the total innervation. Overall, our study provides a reference for studying the mouse pancreatic innervation within the frame of morphological, anatomic, mechanistic, or neurochemical code studies.

Key Points

- Genetically engineered mice are increasingly used to study neuropathy and neural invasion due to pancreatic cancer. However, neuro-anatomy of mouse pancreas has not yet been critically compared to human pancreas.
- Here, we show that nerve trunks in the mouse pancreas were exclusively located around intrapancreatic lymphoid structure and vessels, and the density of nerve trunks and particularly of sensory nerves was highest in the pancreatic head.
- The present study will serve as a reference for mouse pancreatic nerve trunk anatomy.

2 | METHODS

2.1 | Systematic tissue harvesting and analysis

Methods of tissue harvesting can be deciding for the subsequent analysis of structures in the same tissue. Therefore, in this study, we applied a standardized harvesting method for complete embedding of the mouse pancreas with the adjacent organs. In 8-week-old C57BL/6J mice, we performed a median laparotomy and first grabbed the stomach and transected it distal to the pylorus. We then held the spleen and freed it from its retroperitoneal attachments. We then held the freed duodenum and the spleen, so that the pancreatic corpus was the only remaining part of the pancreas attached to mesentery of the small intestine. Here, the pancreas could be bluntly separated away from the mesentery under a stereomicroscope to definitely avoid collection of any mesentery structures. For the dorsal attachments, we also verified under a stereomicroscope the exclusion of any retropancreatic structures like lymph nodes.

The resected mouse pancreas-duodenum-spleen bloc was immediately fixed in 4% paraformaldehyde followed by paraffin embedding, as described previously.[1] The paraffin embedding of the pancreas was performed by strictly adhering to its normal anatomic location (Figure 1A-C). Thus, we were able to preserve the anatomic orientation and subsequently performed a complete front to back, that is, anterior to posterior sectioning of the organ. This way, we generated around 400 slides from a single pancreas, where every 5th slide was immunostained with pan-neuronal markers such as S100 or PGP 9.5 (Figure 1A-C). Hematoxylin was used as counterstain. Depending on the size of the nerve trunks, to ensure best visualization and quantification, the photomicrographs were taken at 10×, 20×, or 40× magnification. Scale bars were integrated on all images during image acquisition. The human normal pancreas sections were obtained from healthy organ donors (six male, four female) whose pancreas was not allocated, as reported previously.[10,11]

2.2 | Immunohistochemistry (IHC) & quantification of neuro-immunoreactivity

Consecutive 3 µm sections from the paraffin-embedded mouse duodenum-pancreas-spleen blocs were analyzed for the immunoreactivity of each nerve for the pan-neural markers protein-gene-product 9.5 (PGP9.5) and S100, and for SP, calcitonin-gene-related-peptide (CGRP), VIP, and neuronal nitric oxide synthase (nNOS). Each nerve on every immunostained section was photomicrographed with the Keyence BioRevo BZ-9000 system (Neu-Isenburg) and measured for the percent proportion of the immunostained nerve area by using the "Threshold function" of the ImageJ software on 8-bit images

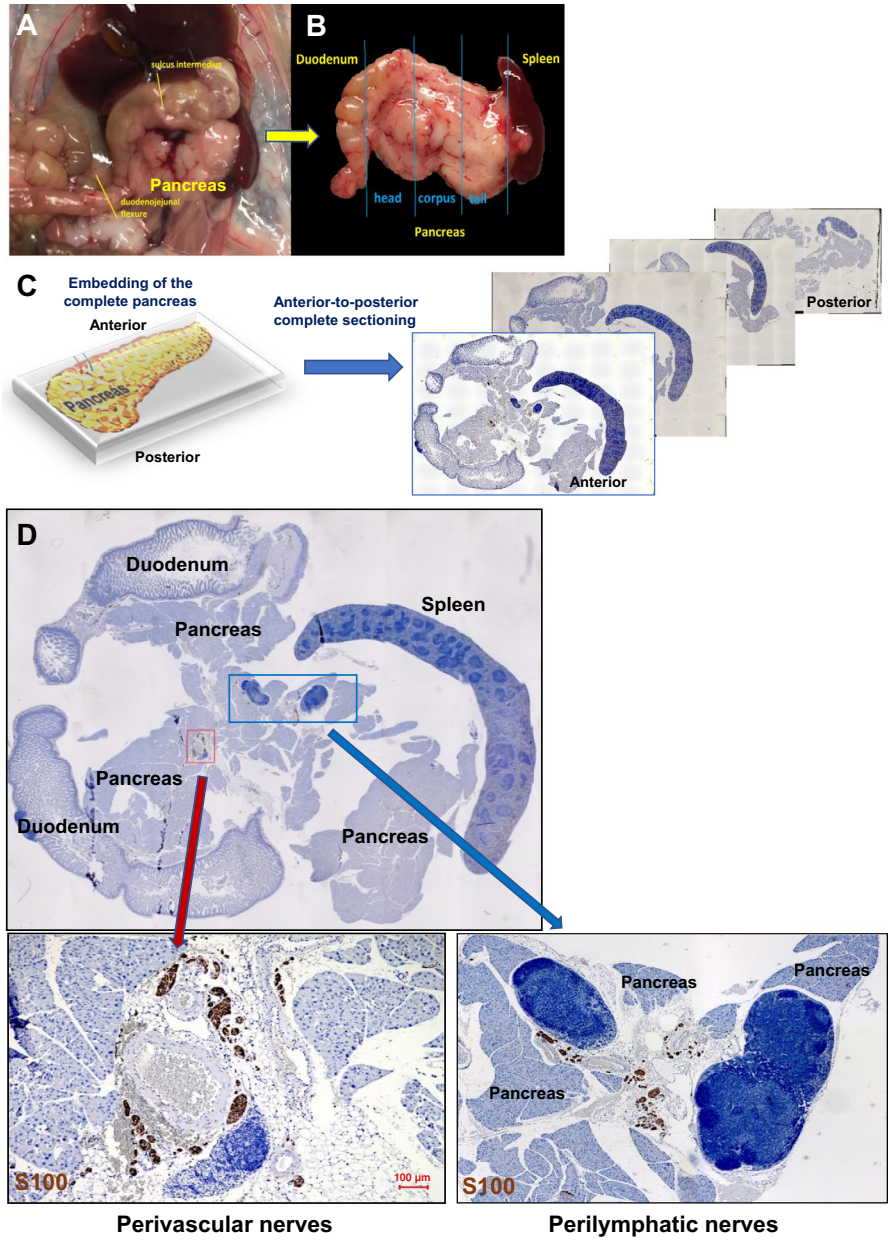

FIGURE 1 Systematic harvesting and whole-tissue embedding of the mouse pancreas. A,B, To ensure analysis of the complete organ and to preserve the anatomic orientation of the mouse pancreas (n = 5), we explanted the mouse pancreas *en bloc* together with the adjacent duodenum and spleen, and strictly avoided collection of the neighbouring mesentery. C, The paraffin-embedded whole duodenum-pancreas-spleen bloc was completely sectioned in an antero-posterior fashion. D, Mouse intrapancreatic nerves were nearly solely found in two niches, ie (a) the perivascular regions, and (b) the perilymphoid areas. The remaining regions, including the intrapancreatic septae, did not include nerves, which is in contrast with human pancreas

(Wayne Rasband; NIH, version 1.44), as described previously.[11] The average percent stained area of all nerves on all sections was termed "%immunoreactivity per nerve". The applied antibodies are shown on Table 1.

2.3 | Wild-type and knockout mice

Male C57BL/6J and B6.129S4-Bdnftm1Jae/J mouse strain was also purchased from the Jackson Laboratory (herein termed "Bdnf ± mice"). The mice were sacrificed at the age of 8 weeks for histological analysis. All animals were housed for at least 1 week prior to experimental use in micro isolators under specific pathogen-free conditions, according to Federation of Laboratory Animal Science Associations and institutional recommendations.

2.4 | Study approval

The study was approved by the ethics committee of the Technical University of Munich, Germany (Approval-Nr: 550/16s). The breeding of the animals was approved by the Government of Upper Bavaria (Approval Nr 55.2-1-54-2532-223-2015).

2.5 | Statistics

Results are expressed as mean ± standard deviation (SD). Only two-group analyses were performed, which were carried out using the unpaired *t* test. All tests were two-sided, and a *P* value of <.05 was considered to indicate statistical significance.

TABLE 1 Primary antibodies

Antibody	Species	Type	Dilution	Source
PGP9.5	Mouse	Monoclonal	1:2000	DAKO, Hamburg, Germany
S100	Mouse	Monoclonal	1:150	Merck Millipore, Darmstadt, Germany
CGRP	Rabbit	Polyclonal	1:200 (IHC)	Merck Millipore, Darmstadt, Germany
Substance P	Mouse	Monoclonal	1:150 (IHC)	Santa Cruz, Dallas, TX, USA
VIP	Rabbit	Polyclonal	1:500 (IHC)	Sigma-Aldrich, St. Louis, MO, USA
nNOS	Rabbit	Polyclonal	1:500 (IHC)	Cell Signaling, Cambridge, UK

Abbreviation: IHC, immunohistochemistry.

3 | RESULTS

3.1 | Nerve trunks in the mouse pancreas are exclusively localized in perilymphoid and perivascular niches

Due to the widespread application of mouse models in the study of pancreatic diseases, it is becoming increasingly important to correctly understand the anatomy of the mouse pancreas. For this purpose, we systematically investigated the mouse intrapancreatic nerve anatomy and first performed a complete harvesting and paraffin embedding of the pancreas, strictly adhering to its normal anatomic location (Figure 1A-C). This way, we were able to preserve the anatomic orientation and subsequently performed a complete front to back, that is, anterior to posterior sectioning of the organ. This way, we generated around 400 slides from a single pancreas, where every 5th slide was immunostained with panneuronal markers such as S100 or PGP 9.5 (Figure 1A-C). Here, it is important to consider that PGP9.5 antibodies tend to stain pancreatic islets as well, which need to be excluded from quantitative analyses (Figure S1).

As we were able to preserve the natural location of the pancreas between the duodenum and the spleen (Figure 1). We first looked at the localization of nerves in the mouse pancreas. Here, we noticed that intrapancreatic nerve trunks in the mouse pancreas were almost exclusively localized in two major locations: First, numerous small to large diameter nerve trunks were found around intrapancreatic blood vessels (Figure 1D). Second, in addition to these paravascular nerves, nerves were unequivocally encountered around intrapancreatic round lymphoid, lymph-node like structures (Figure 1D). This second class of nerves exhibited obvious proximity to the capsule of these lymphoid clusters, whereas there was no visible penetration of these nerves into these lymphoid structures. Beyond these two specialized locations, we hardly identified nerve trunks in the interlobular connecting tissue bridges (septae), which is the most common location of nerves in the human pancreas (Human: 82.7 ± 18.0% of all nerves localized in interlobular septae, without adjacent vessels, vs. mouse: 5.9 ± 7.8%, $P < .0001$, Figure 2A,B).

When looking closely at the morphology of these nerves, there was also a major difference between mouse and human pancreatic nerves. Mouse intrapancreatic nerves appeared in clusters composed out of 5 to 10 small-to-large nerve trunks (Figures 1D and 2A), which, as mentioned, appeared around these specialized locations. This is in contrast with human intrapancreatic nerves, which normally appear as singular, unclustered, nerve trunks that are readily present in the normal parenchyma, between acinar cells (Figure 2A).

3.2 | The majority of nerve trunks are in the head and anterior corpus region of the mouse pancreas

With the help of the anatomic embedding and sectioning method we applied, we first looked at the quantitative innervation of the mouse pancreas (Figure 2C,D). Here, we detected a prominently greater nerve area (0.15 ± 0.08% of the tissue area) and nerve density (2.02 ± 0.77 nerves per tissue area) in the head of the pancreas when compared to the tail (area: 0.02 ± 0.02%, density: 0.17 ± 0.08, Figure 2C,D). The corpus exhibited an intermediate nerve area and density (area: 0.11 ± 0.14%, density: 1.34 ± 1.46, Figure 2C,D). We then compared the total nerve area in the anterior vs posterior regions of the pancreas. Here, we found a significantly greater nerve area in the anterior (0.14 ± 0.08%) and posterior head (0.16 ± 0.09%), and anterior corpus (0.17 ± 0.27%), when compared to posterior corpus (0.07 ± 0.04%), anterior tail (0.01 ± 0.01%), or posterior tail (0.02 ± 0.01%, Figure 2E). Thus, these findings suggested that the majority of nerves were located toward the anterior parts of the *right-sided* pancreas, which has many implications for comparative studies of innervation in mouse models of pancreatic disease.

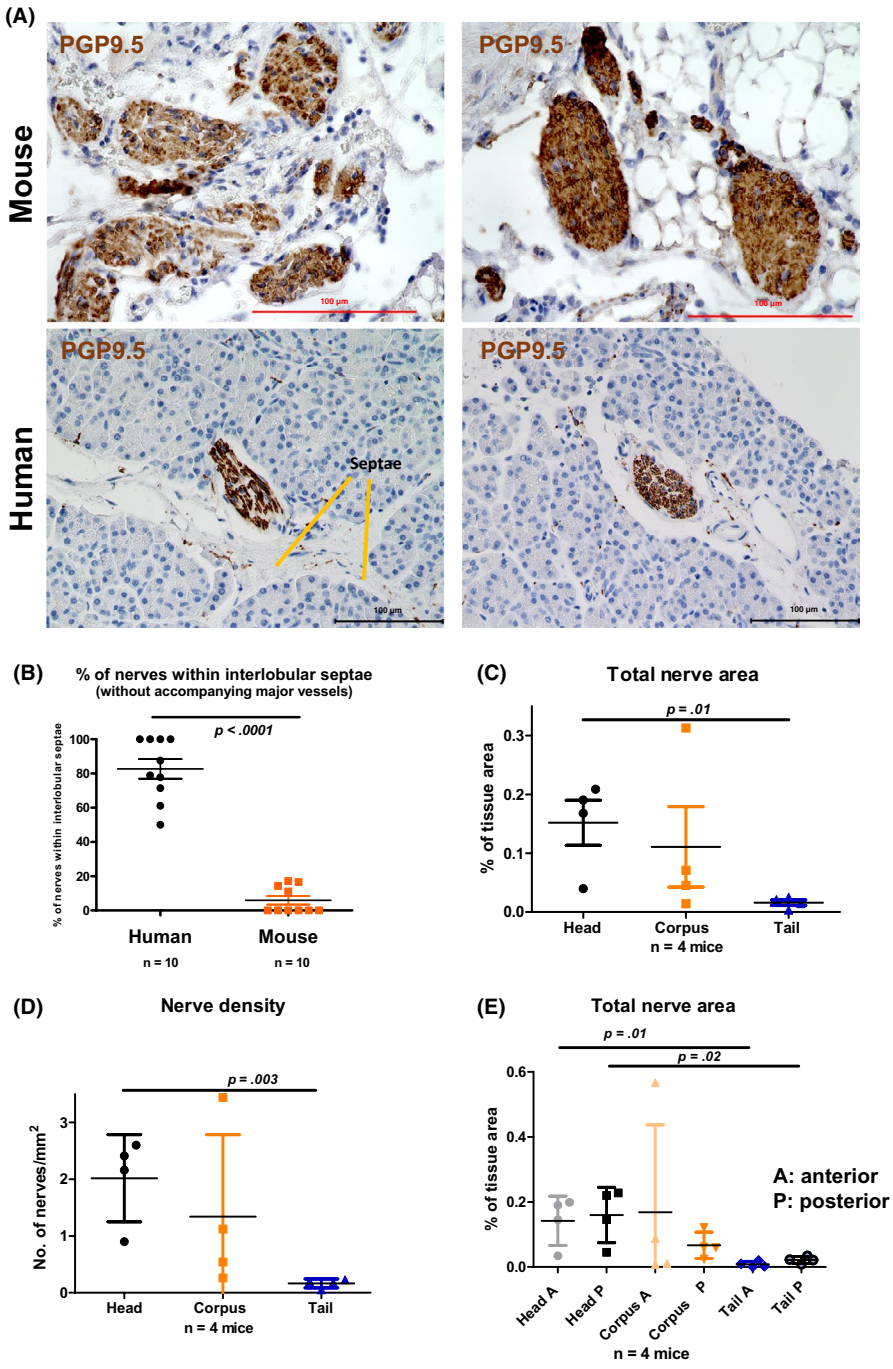

FIGURE 2 Distribution of nerve trunks in the mouse pancreas. A-B, PGP9.5 or S100 were used as pan-neural markers. Intrapancreatic nerve trunks in the mouse pancreas appear as small-to-large caliber clusters of several nerves at one of the above mentioned two particular locations. In contrast, human pancreas contains several singular nerve trunks within the normal parenchyma, between the acinar cells. C-D, Comparison of the nerve area and nerve density in the head, corpus, and tail of the mouse pancreas. E, Analysis of the differences in the nerve area at the anterior (A) vs posterior (P) surfaces of the head, corpus, and tail of the mouse pancreas. Unpaired *t* test

3.3 | Most nociceptive fibers are in the head of the mouse pancreas

In the next step, we placed our focus on the distribution of the nociceptive nerve fibers, since pain represents the cardinal and most severe symptom of exocrine pancreatic diseases like PCa and CP. Here, we first looked at the amount of SP+ or CGRP+ nerve fibers within nerve trunks in the head, corpus, and tail of the mouse pancreas (Figure 3A). Here, we found a remarkably higher proportion of SP+ (68.9 ± 19.4%) or CGRP+ (71.2 ± 21.2%) nerve fibers in nerve trunks in the pancreatic head when compared to the corpus (SP: 20.2 ± 12.7%, CGRP: 23.2 ± 21.1%) or tail (SP: 11.0 ± 10.0%, CGRP: 5.6 ± 4.8%) regions of the pancreas (Figure 3B). This observation held true for the total proportion of SP+ or CGRP+ fibers within the total nerve area (Figure 3B). However, when we looked at the average proportion of such nerve fibers per individual nerve trunk, we again detected a greater proportion SP+ fibers in the head when compared to corpus (SP-head: 9.1 ± 2.6%, SP-corpus: 7.8 ± 1.6%, SP-tail: 9.6 ± 6.1%, Figure 3C). Interestingly, the amount of CGRP+ nerve fibers per nerve also tended to be greater in the pancreatic tail (CGRP-head: 8.5 ± 2.4%, CGRP-corpus: 9.6 ± 3.6%, CGRP-tail: 10.8 ± 5.9%, Figure 3C). These observations suggested on the one

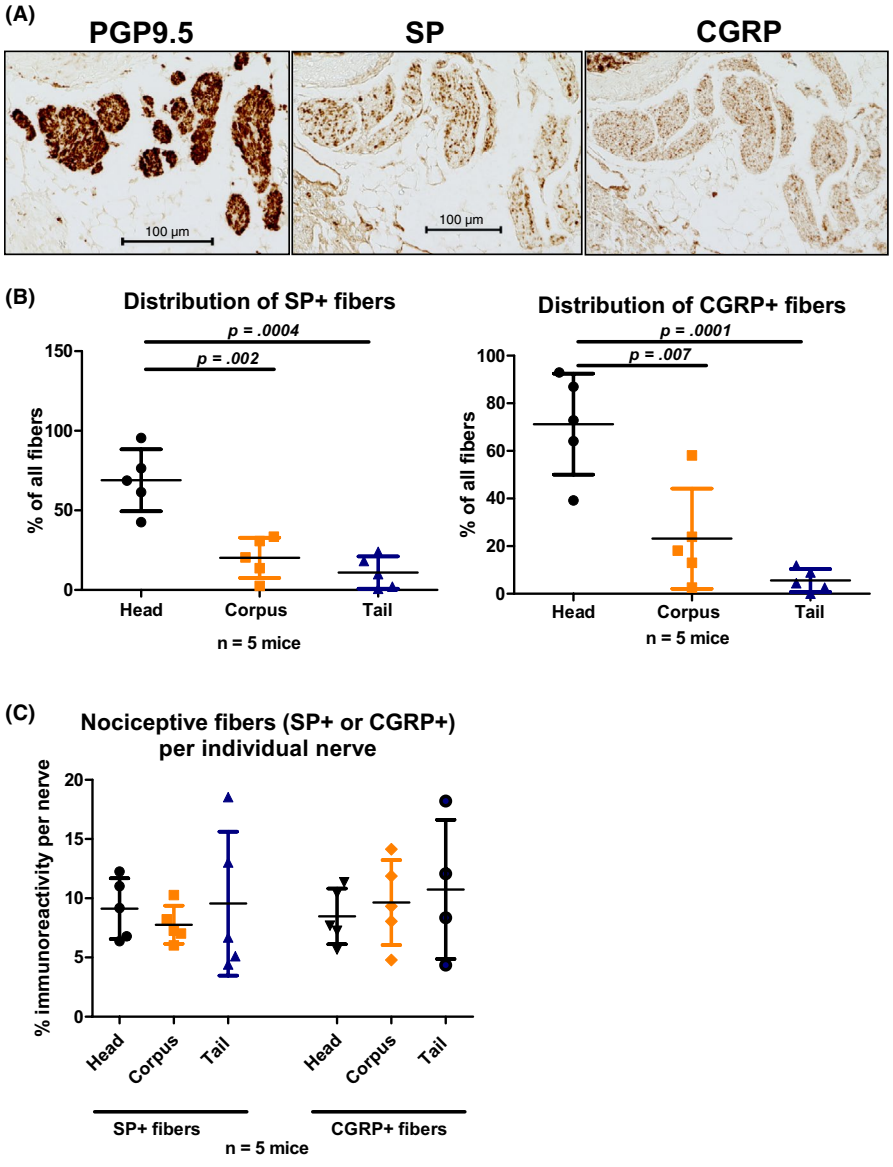

FIGURE 3 Analysis of the nociceptive (substance P [SP] and calcitonin-gene-related-peptide [CGRP]-containing) nerve fiber distribution in the mouse pancreas. A, All nerve trunks were identified with the help of a consecutive, PGP9.5-immunostained section. The proportion of SP- or CGRP-immunostained area in each nerve was proportioned to the total area of each nerve. B, Comparison of the total area of the SP+ or CGRP+ nerve fibers in the head, corpus, and tail of the mouse pancreas. C, Comparison of average portion of SP+ or CGRP+ nerve fibers per nerve in the head, corpus, and tail of the mouse pancreas. Unpaired *t* test

hand that the proportion of SP+ or CGRP+ nerve fibers varies between ca. 6%-10% of all nerve fibers per nerve. Moreover, although the majority of nociceptive nerve fibers were in the mouse pancreatic head, the ones in the pancreatic tail tended to have on average a higher proportion of CGRP+ nerve fibers.

3.4 | Loss of *Bdnf* alters the nitrergic, but not the VIPergic, innervation of the mouse pancreatic head and corpus

In the final part, we looked at the amount of VIP+ or nitrergic, that is, nNOS-containing nerve fibers in the normal mouse pancreas (Figure 4A) and compared these amounts to mice that were heterozygously knocked out for brain-derived neurotrophic factor (BDNF, here termed the *Bdnf*$^{+/-}$ mice), since homozygous *Bdnf*$^{-/-}$ mice are hardly alive until the adult age.[12] We recently reported that the amount of nitrergic fibers in the mouse pancreas is higher in the *Bdnf*$^{+/-}$ mice.[10] Furthermore, BDNF was previously reported to control the expression of the neuropeptides SP,[13,14] CGRP,[15] VIP,[16] and nNOS[17,18] in various neuronal subclasses. In the present study, we found that the total amount of the VIPergic nerve fibers did not change in the nerves of *Bdnf*$^{+/-}$ pancreas when compared to wildtype (WT) mice (8.6 ± 0.9% vs 7.8 ± 1.0%, Figure 4B). When we had a closer look at the anatomic localization of these VIPergic fibers, we found that they were rather equally present in the nerves of the pancreatic head, corpus and tail (VIP-head: 8.5 ± 1.1%, VIP-corpus: 8.4 ± 0.8%, VIP-tail: 7.5 ± 2.9%, Figure 4C). In the *Bdnf*$^{+/-}$ mice, the VIP content of pancreatic nerves was also homogeneous between the three different regions of the pancreas (VIP-head: 8.2 ± 2.0%, VIP-corpus: 8.7 ± 1.5%, VIP-tail: 7.8 ± 1.9%, Figure 4C).

When we analyzed the distribution of nitrergic fibers depending on the location in the pancreas, we found a strong tendency to enrichment of such fibers in the head and corpus, but not in the tail, of the *Bdnf*$^{+/-}$ pancreas (nNOS-head: 7.5 ± 3.3%, nNOS-corpus: 7.3 ± 3.3%, nNOS-tail: 5.5 ± 3.0%, Figure 4D), when compared to WT

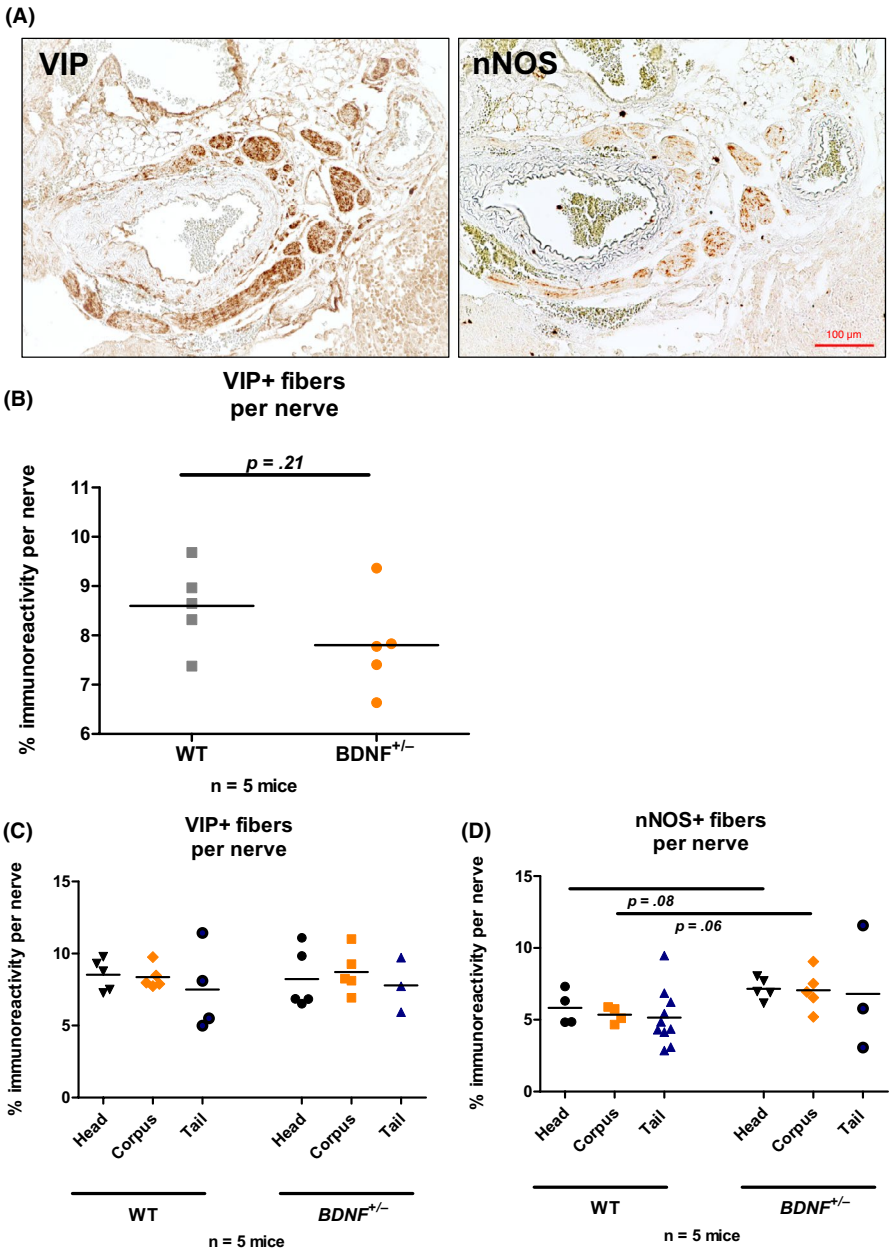

FIGURE 4 Analysis of the VIPergic and nNOS-containing/nitrergic nerve fiber distribution in the mouse pancreas. A, All nerve trunks were identified with the help of a consecutive, PGP9.5-immunostained section. The proportion of VIP- or BDNF-immunostained area in each nerve was proportioned to the total area of each nerve. B, Comparison of average portion of VIP+ nerve fibers per nerve in the head, corpus, and tail of the wildtype (WT) or Bdnf+/−mouse pancreas. C-D, Comparison of the total area of the VIP+ or nNOS+ nerve fibers in the head, corpus, and tail of the pancreas in WT vs *Bdnf*$^{+/-}$ mice (n = 5 each). Unpaired *t* test

mouse pancreas (nNOS-head: 5.2 ± 2.7%, nNOS-corpus: 5.5 ± 3.0%, nNOS-tail: 5.2 ± 2.0%, Figure 4D). These results suggested that the increase of the nitrergic innervation in *Bdnf*$^{+/-}$ pancreas that we previously reported is encountered in the head and corpus regions.[10]

4 | DISCUSSION

Pancreatic diseases like PCa and CP exhibit remarkable neuroplastic changes that are closely linked to disease progression and pain status.[3,19,20] Understanding of the mechanism behind these prognostically relevant nerve alterations is highly dependent on the application and choice of the correct models. Mouse models, particularly genetically engineered ones, have recently been increasingly applied for deciphering the mechanisms behind nerve-cancer-inflammation interactions in the pancreas.[5,21-25] The present study aimed at providing a systematic analysis of the anatomic distribution of nerve trunks and selected nociceptive nerve fiber classes in the mouse pancreas. Here, we report a significantly stronger innervation of the mouse pancreatic head and corpus when compared to the tail, and a significant enrichment of nociceptive, pain-transmitting fibers particularly in the pancreatic head. Furthermore, there seem to be differences in the amount of nerves and nerve fibers depending on the anterior vs posterior surfaces of the mouse pancreas. These observations therefore have deciding implications for all the neuroanatomic studies that analyze nerves in the mouse pancreas under different disease conditions.[5,21-25]

In the human pancreas, the sympathetic efferent fibers are known to travel through splanchnic nerves to form synapses within the prevertebral sympathetic ganglia and the

TABLE 2 The differences and similarities in the nerve trunk distribution and content of mouse vs. human pancreas as detected by the current study

Features of nerve trunks	Mouse pancreas	Human pancreas
Localization of nerve trunks	1. Perivascular 2. Perilymphoid 3. Not in the intralobular septae	Rather arbitrary distribution, including intralobular septae
Highest density of nerve trunks	In the head and anterior corpus	In the head[29,31]
Highest total amount of SP+ or CGRP+ fibers in nerve trunks	In the head	Not known
Highest proportion of SP+ nerve trunks	In the head and tail	Not known
Highest proportion of CGRP+ nerve trunks	In the tail	Not known
Highest proportion of VIP+ nerve trunks	Homogeneous	Not known

intrapancreatic sympathetic ganglia.[26,27] These fibers project from the prevertebral ganglia and enter the pancreas either within mixed autonomic nerves or directly.[28] Furthermore, in humans, the body and the tail of the pancreas are known to be innervated from nerves fibers that arise from the celiac plexus and enter the pancreas along the branches of the splenic archery and the transverse pancreatic artery.[26] On the other hand, the pancreatic head receives the majority of the nerve fibers from the nerve plexus along the hepatic artery, the portal vein, and the inferior pancreaticoduodenal artery.[29-31] Thus, the entrance of nerves along neurovascular stalks is a well-known phenomenon from the human pancreas. The only other study that analyzed the anatomy of mouse pancreas previously reported that the distribution of sympathetic nerve fibers in the mouse pancreas is more homogenous between the three different parts of the pancreas when compared to human pancreas.[32] However, our study clearly showed that there is a concentration of nerves in the pancreatic head and corpus, when compared to the tail. Considering the additional difference between the anterior and posterior surface of the pancreas particularly in the corpus region, we underline that any analysis of pancreatic innervation in mouse models should pay strong attention anatomic region of tissue collection. We propose that due to lack of any significant difference between the anterior and posterior part, the choice of the pancreatic head for analysis may be more accurate for the purpose of comparison.

Another key finding of our study is the specific localization of intrapancreatic nerves around two major sites in the mouse pancreas. The first site; that is, the perivascular area, probably corresponds to the intrapancreatic continuation of the neurovascular stalks that are derived from the extrapancreatic, retroperitoneal regions. The second localization, that is, the vicinity of lymph-node like modular structures, is a novel finding that deserves attention. Lymphoid cells and immune cells are known to be regulated in their activity and differentiation by neural signals.[33,34] The close anatomic relationship that we hereby report for the first time maybe an indicator of such in your writing me on a regulation in the pancreas. When one considers the extremely high frequency of neuritis as neuro-inflammation in human CP and PCa,[1,35] it is imaginable that the proximity of nerves to lymphoid cell conglomerates may be an anatomic factor that predisposes to intrapancreatic neuro-inflammation.

It should also be underlined that the lymphoid structures that we detected in the present study are strictly intrapancreatic structures, as, due to the complete serial sectioning of the pancreas in a defined direction, we were able to exclude any structures that were not surrounded by pancreatic parenchyma. All the lymphoid structures that were encircled by intrapancreatic nerves were also localized within the pancreas. A possible explanation for this phenomenon, but also for all the differences in the innervation of the mouse pancreas when compared to the human pancreas, lies in the differences in their intra-abdominal location. Indeed, human pancreas is a “compact, dense type” pancreas that is located in the retroperitoneum. Conversely, the mouse pancreas is a so-called “mesenteric type” pancreas, which is quite diffusely distributed and embedded in the attached small intestine mesentery and that has an intraperitoneal localization.[7] Due to this basic difference in the anatomy of human vs mouse pancreas, it is estimated the mouse pancreas has a similar lymph-node drainage as the small intestine, which carries multiple lymph nodes in its mesentery.

The differences in the anatomic localization of nerves between the different regions of the pancreas are more prominent for nociceptive nerve fibers. So far, a definitive or deciding role for classical nociceptive neuropeptides like SP and CGRP, for example, CP-associated pain has not yet been shown; in fact, we could recently provide evidence for the lack of a role for these neuropeptides in the promotion of human CP-associated pain.[10] Still, the levels of the SP receptors neurokinin-1 and neurokinin-2 receptor,[36] and CGRP levels in the intrathecal space have been found to associate with pain in human and rat CP.[37] Based on our results, it seems that the majority of SP- and CGRP-containing nerve fibers are located in the pancreatic head. From human studies, it is known that the highest density of nerves is detectable in the pancreatic head, which is assumed to represent one of the reasons for the effectiveness of pancreatic head resection for relieving CP- or PCa associated pain.[3,38] Thus, analyzing and targeting the pancreatic head in mouse models may yield similarly relevant clues

for pancreas-associated pain generation in human disease. In our view, researchers should increasingly evaluate the use of animals other than mice for the study of neuropathy and innervation in pancreatitis and cancer. Indeed, a similarly "compact" type pancreas is, for example, encountered in pigs, and porcine genetic models of pancreatic disease are currently developed with the aim of improved translation.[39]

Anatomic considerations do seem to be of strong importance for studying pain in pancreatic diseases. In human CP of Middle European patients, there is a prominently higher prevalence of inflammatory tumor formation in the pancreatic head, when compared to American patients who exhibit a rather diffuse disease in the whole gland.[40-42] This difference in the anatomic location of the disease not only impacts the symptoms (eg, pain), but also the surgical treatment strategy. Furthermore, obstruction of the pancreatic duct by, for example, stones or an inflammatory mass in the pancreatic head seems to be the maintaining factor with regard to the severe pain of CP patients.[43] Animal models with similar anatomic-mechanic drivers of disease, such as the duct-ligation model of CP,[44] or similarly unilocular, rather than multilocular, disease (as seen in human PCa and as recently also reproduced in murine "resectable" genetic PCa[45]) may thus provide further clues with potentially higher relevance for human disease.

Our present study implies a quite homogeneous distribution of VIPergic nerve fibers in the three main pancreatic regions. Furthermore, it seems that the loss of VIPergic fibers in the pancreas is accompanied by an increase of nitrergic, that is, nNOS-containing nerve fibers in the pancreatic head and corpus. Considering the recently discovered, potentially key role of nitrergic fibers in CP-associated pain,[10] we hereby underline the importance of the anatomic region of the pancreas when analyzing the nitrergic innervation in the mouse pancreas.

The current study certainly harbors also some limitations. Importantly, the deduced conclusions relate to our observations on nerve "trunks", thereby possibly omitting the distribution of small fiber networks, intrinsic, peri-islet neurons, and intrapancreatic ganglia that are inherently present in the human and mouse pancreas.[9,46,47] Second, we currently have no quantitative information related to differences in the 3D structure of nerve trunks and how they transverse into and through the pancreas. Therefore, future studies should increasingly apply 3D reconstruction and imaging technologies[8,9] for comparative analyses on human and mouse pancreas. Third, we limited our study to the analysis of neuropeptides like VIP, SP, CGRP, and nNOS with regard to sensation and pain, yet these analyses can certainly be expanded to include further neuronal subgroups, including TRPV1-, PACAP, 5-HT, TRPA1- or TRPV4-containing fiber subclasses.[48,49]

In conclusion, the present study provided a detailed quantitative illustration of the innervation and nociceptive fiber distribution in the mouse pancreas. Importantly, intrapancreatic nerves in mice appear as clusters of numerous small-to-large nerve trunks around two special niches, that is, the "perivascular" and "intrapancreatic perilymphoid" areas. Therefore, mouse intrapancreatic nerves exhibit major morphological differences when compared to human intrapancreatic nerves and are very difficult to analyze within efforts of murine modelling of human pancreatic disease (Table 2). However, mouse models will certainly continue to be of major benefit for understanding the molecular, genetic, and cellular repertoire of pancreatic diseases during their development and progression. Still, a one-to-one transfer of conclusions from anatomic-histological observations in the mouse model to human disease should be avoided. In addition to calling attention to these major differences, we also hope that these observations will serve as a guide for researchers who study the role of innervation in pancreatic disease generation and progression.

CONFLICT OF INTEREST

None.

AUTHOR CONTRIBUTIONS

IED, GOC, and HF designed and supervised the study. ÖCS, ST, XW, and SW performed the experiments. RI provided major intellectual input and supervised the study. All authors critically read the manuscript and agreed on its final version.

ETHICAL APPROVAL

The study was approved by the ethics committee of the Technical University of Munich, Germany (Approval-Nr: 550/16s). The breedings of the animals were approved by the Government of Upper Bavaria (Approval Nr 55.2-1-54-2532-223-2015).

DATA AVAILABILITY STATEMENT

All data in the manuscript are available on request from the Corresponding Author.

ORCID

Ihsan Ekin Demir https://orcid.org/0000-0001-8241-1871

REFERENCES

1. Ceyhan GO, Bergmann F, Kadihasanoglu M, et al. Pancreatic neuropathy and neuropathic pain–a comprehensive pathomorphological study of 546 cases. *Gastroenterology*. 2009;136(1):177-186 e171.
2. Demir IE, Friess H, Ceyhan GO. Nerve-cancer interactions in the stromal biology of pancreatic cancer. *Front Physiol*. 2012;3:97.
3. Demir IE, Friess H, Ceyhan GO. Neural plasticity in pancreatitis and pancreatic cancer. *Nat Rev Gastroenterol Hepatol*. 2015;12(11):649-659.
4. Stopczynski RE, Normolle DP, Hartman DJ, et al. Neuroplastic changes occur early in the development of pancreatic ductal adenocarcinoma. *Cancer Res*. 2014;74(6):1718-1727.
5. Renz BW, Takahashi R, Tanaka T, et al. beta2 adrenergic-neurotrophin feedforward loop promotes pancreatic cancer. *Cancer Cell*. 2018;33(1):75-90 e77.
6. Renz BW, Tanaka T, Sunagawa M, et al. Cholinergic signaling via muscarinic receptors directly and indirectly suppresses pancreatic tumorigenesis and cancer stemness. *Cancer Discov*. 2018;8(11):1458-1473.
7. Tsuchitani M, Sato J, Kokoshima H. A comparison of the anatomical structure of the pancreas in experimental animals. *J Toxicol Pathol*. 2016;29(3):147-154.
8. Chien HJ, Chiang TC, Peng SJ, et al. Human pancreatic afferent and efferent nerves: mapping and 3-D illustration of exocrine,

endocrine, and adipose innervation. *Am J Physiol Gastrointest Liver Physiol*. 2019;317(5):G694-G706.
9. Tang SC, Baeyens L, Shen CN, et al. Human pancreatic neuro-insular network in health and fatty infiltration. *Diabetologia*. 2018;61(1):168-181.
10. Demir IE, Heinrich T, Carty DG, et al. Targeting nNOS ameliorates the severe neuropathic pain due to chronic pancreatitis. *EBioMedicine*. 2019;46:431-443.
11. Ceyhan GO, Demir IE, Rauch U, et al. Pancreatic neuropathy results in "neural remodeling" and altered pancreatic innervation in chronic pancreatitis and pancreatic cancer. *Am J Gastroenterol*. 2009;104(10):2555-2565.
12. Ward NL, Hagg T. BDNF is needed for postnatal maturation of basal forebrain and neostriatum cholinergic neurons in vivo. *Exp Neurol*. 2000;162(2):297-310.
13. Wang P, Du C, Chen FX, et al. BDNF contributes to IBS-like colonic hypersensitivity via activating the enteroglia-nerve unit. *Sci Rep*. 2016;6:20320.
14. Cantarella G, Lempereur L, Presta M, et al. Nerve growth factor-endothelial cell interaction leads to angiogenesis in vitro and in vivo. *FASEB J*. 2002;16(10):1307-1309.
15. Fukuoka T, Miki K, Yoshiya I, Noguchi K. Expression of beta-calcitonin gene-related peptide in axotomized rubrospinal neurons and the effect of brain derived neurotrophic factor. *Brain Res*. 1997;767(2):250-258.
16. Cellerino A, Arango-Gonzalez B, Pinzon-Duarte G, Kohler K. Brain-derived neurotrophic factor regulates expression of vasoactive intestinal polypeptide in retinal amacrine cells. *J Comp Neurol*. 2003;467(1):97-104.
17. Cheng A, Wang S, Cai J, Rao MS, Mattson MP. Nitric oxide acts in a positive feedback loop with BDNF to regulate neural progenitor cell proliferation and differentiation in the mammalian brain. *Dev Biol*. 2003;258(2):319-333.
18. Wu W, Li L, Yick LW, et al. GDNF and BDNF alter the expression of neuronal NOS, c-Jun, and p75 and prevent motoneuron death following spinal root avulsion in adult rats. *J Neurotrauma*. 2003;20(6):603-612.
19. Demir IE, Schafer KH, Tieftrunk E, Friess H, Ceyhan GO. Neural plasticity in the gastrointestinal tract: chronic inflammation, neurotrophic signals, and hypersensitivity. *Acta Neuropathol*. 2013;125(4):491-509.
20. Demir IE, Wang K, Tieftrunk E, et al. Neuronal plasticity in chronic pancreatitis is mediated via the neurturin/GFRalpha2 axis. *Am J Physiol Gastrointest Liver Physiol*. 2012;303(9):G1017-G1028.
21. Demir IE, Boldis A, Pfitzinger PL, et al. Investigation of Schwann cells at neoplastic cell sites before the onset of cancer invasion. *J Natl Cancer Inst*. 2014;106(8). pii: dju184. https://www.ncbi.nlm.nih.gov/pubmed/?term=25106646
22. Demir IE, Kujundzic K, Pfitzinger PL, et al. Early pancreatic cancer lesions suppress pain through CXCL12-mediated chemoattraction of Schwann cells. *Proc Natl Acad Sci USA*. 2017;114(1):E85-E94.
23. Demir IE, Tieftrunk E, Schorn S, et al. Activated Schwann cells in pancreatic cancer are linked to analgesia via suppression of spinal astroglia and microglia. *Gut*. 2016;65(6):1001-1014.
24. Gil Z, Cavel O, Kelly K, et al. Paracrine regulation of pancreatic cancer cell invasion by peripheral nerves. *J Natl Cancer Inst*. 2010;102(2):107-118.
25. Saloman JL, Albers KM, Li D, et al. Ablation of sensory neurons in a genetic model of pancreatic ductal adenocarcinoma slows initiation and progression of cancer. *Proc Natl Acad Sci USA*. 2016;113(11):3078-3083.
26. Dolensek J, Rupnik MS, Stozer A. Structural similarities and differences between the human and the mouse pancreas. *Islets*. 2015;7(1):e1024405.
27. Gilon P, Henquin JC. Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. *Endocr Rev*. 2001;22(5):565-604.
28. Ahren B, Taborsky GJ Jr. The mechanism of vagal nerve stimulation of glucagon and insulin secretion in the dog. *Endocrinology*. 1986;118(4):1551-1557.
29. Tiscornia OM. The neural control of exocrine and endocrine pancreas. *Am J Gastroenterol*. 1977;67(6):541-560.
30. Tiscornia OM, Martinez JL, Sarles H. Some aspects of human and canine macroscopic pancreas innervation. *Am J Gastroenterol*. 1976;66(4):353-361.
31. Yi SQ, Miwa K, Ohta T, et al. Innervation of the pancreas from the perspective of perineural invasion of pancreatic cancer. *Pancreas*. 2003;27(3):225-229.
32. Lindsay TH, Halvorson KG, Peters CM, et al. A quantitative analysis of the sensory and sympathetic innervation of the mouse pancreas. *Neuroscience*. 2006;137(4):1417-1426.
33. Wulfing C, Gunther HS. Dendritic cells and macrophages neurally hard-wired in the lymph node. *Sci Rep*. 2015;5:16866.
34. Panuncio AL, De La Pena S, Gualco G, Reissenweber N. Adrenergic innervation in reactive human lymph nodes. *J Anat*. 1999;194(Pt 1):143-146.
35. Demir IE, Schorn S, Schremmer-Danninger E, et al. Perineural mast cells are specifically enriched in pancreatic neuritis and neuropathic pain in pancreatic cancer and chronic pancreatitis. *PLoS ONE*. 2013;8(3):e60529.
36. Michalski CW, Shi X, Reiser C, et al. Neurokinin-2 receptor levels correlate with intensity, frequency, and duration of pain in chronic pancreatitis. *Ann Surg*. 2007;246(5):786-793.
37. Liu L, Shenoy M, Pasricha PJ. Substance P and calcitonin gene related peptide mediate pain in chronic pancreatitis and their expression is driven by nerve growth factor. *JOP*. 2011;12(4):389-394.
38. Friess H, Shrikhande S, Shrikhande M, et al. Neural alterations in surgical stage chronic pancreatitis are independent of the underlying aetiology. *Gut*. 2002;50(5):682-686.
39. Li S, Edlinger M, Saalfrank A, et al. Viable pigs with a conditionally-activated oncogenic KRAS mutation. *Transgenic Res*. 2015;24(3):509-517.
40. Keck T, Marjanovic G, Fernandez-del Castillo C, et al. The inflammatory pancreatic head mass: significant differences in the anatomic pathology of German and American patients with chronic pancreatitis determine very different surgical strategies. *Ann Surg*. 2009;249(1):105-110.
41. Sendler M, van den Brandt C, Glaubitz J, et al. NLRP3 Inflammasome regulates development of systemic inflammatory response and compensatory anti-inflammatory response syndromes in mice with acute pancreatitis. *Gastroenterology*. 2020;158(1): 253-269 e214.
42. Aghdassi AA, Mayerle J, Christochowitz S, Weiss FU, Sendler M, Lerch MM. Animal models for investigating chronic pancreatitis. *Fibrogenesis Tissue Repair*. 2011;4(1):26.
43. Issa Y, Kempeneers MA, Bruno MJ, et al. Effect of early surgery vs endoscopy-first approach on pain in patients with chronic pancreatitis: the ESCAPE randomized clinical trial. *JAMA*. 2020;323(3):237-247.
44. Sendler M, Beyer G, Mahajan UM, et al. Complement component 5 mediates development of fibrosis, via activation of stellate cells, in 2 mouse models of chronic pancreatitis. *Gastroenterology*. 2015;149(3): 765-776 e710.
45. Gurlevik E, Fleischmann-Mundt B, Brooks J, et al. Administration of gemcitabine after pancreatic tumor resection in mice induces an antitumor immune response mediated by natural killer cells. *Gastroenterology*. 2016;151(2):338-350 e337.
46. Kirchgessner AL, Gershon MD. Innervation of the pancreas by neurons in the gut. *J Neurosci*. 1990;10(5):1626-1642.
47. Kirchgessner AL, Gershon MD. Innervation and regulation of the pancreas by neurons in the gut. *Z Gastroenterol Verh*. 1991;26:230-233.
48. Schwartz ES, La JH, Scheff NN, Davis BM, Albers KM, Gebhart GF. TRPV1 and TRPA1 antagonists prevent the transition of acute to

chronic inflammation and pain in chronic pancreatitis. *J Neurosci*. 2013;33(13):5603-5611.
49. Zhang LP, Kline RH, Deevska G, Ma F, Nikolova-Karakashian M, Westlund KN. Alcohol and high fat induced chronic pancreatitis: TRPV4 antagonist reduces hypersensitivity. *Neuroscience*. 2015;311:166-179.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Saricaoglu ÖC, Teller S, Wang X, et al. Localisation analysis of nerves in the mouse pancreas reveals the sites of highest nerve density and nociceptive innervation. *Neurogastroenterol Motil*. 2020;00:e13880. https://doi.org/10.1111/nmo.13880