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Distribution and Neurochemical Identification of Pancreatic Afferents in the Mouse

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Abstract

Dysfunction of primary afferents innervating the pancreas has been shown to contribute to the development of painful symptoms during acute and chronic pancreatitis. To investigate the distribution and neurochemical phenotype of pancreatic afferents, Alexa Fluor-conjugated cholera toxin B (CTB) was injected into the pancreatic head (CTB-488) and tail (CTB-555) of adult male mice to label neurons retrogradely in both the dorsal root ganglia (DRG) and nodose ganglia (NG). The NG and DRG (T5–T13) were processed for fluorescent immunohistochemistry and visualized by using confocal microscopy. Spinal pancreatic afferents were observed from T5 to T13, with the greatest contribution coming from T9–T12. The pancreatic afferents were equally distributed between right and left spinal ganglia; however, the innervation from the left NG was significantly greater than from the right. For both spinal and vagal afferents there was significantly greater innervation of the pancreatic head relative to the tail. The total number of retrogradely labeled afferents in the nodose was very similar to the total number of DRG afferents. The neurochemical phenotype of DRG neurons was dominated by transient receptor potential vanilloid 1 (TRPV1)-positive neurons (75%), GDNF family receptor alpha-3 (GFR α 3)-positive neurons (67%), and calcitonin gene-related peptide (CGRP)-positive neurons (65%) neurons. In the NG, TRPV1-, GFR α 3-, and CGRP-positive neurons constituted only 35%, 1%, and 15% of labeled afferents, respectively. The disparity in peptide and receptor expression between pancreatic afferents in the NG and DRG suggests that even though they contribute a similar number of primary afferents to the pancreas, these two populations may differ in regard to their nociceptive properties and growth factor dependency.

Keywords

TRPV1; CGRP; retrograde labeling; immunohistochemistry; dorsal root ganglion; nodose ganglia; visceral pain

Patients suffering from chronic pancreatitis (CP) often complain of debilitating abdominal pain, which dramatically affects their quality of life. Two main theories have arisen regarding the source of the intractable pain accompanying CP. The first suggests that the pain arises due to an increase in intraductal pressure, caused by strictures, pancreatic fibrosis, interstitial hypertension, and pancreatic ischemia (Friess et al., 2004; Di Sebastiano et al., 2004). The second theory maintains that various aspects of primary afferent dysfunction contribute to the pain (Bockman et al., 1988; Friess et al., 2002). This has been shown as an increase in the

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number and size of pancreatic axons, damage to the perineural sheaths allowing inflammatory infiltration, and increased neurotransmitter immunoreactivity (Di Sebastiano et al., 2004).

Changes in growth factor expression, which affect the functioning of primary afferents, have also been shown to occur in CP. Nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) are both members of the neurotrophin family and signal through a receptor complex consisting of the nonselective p75 receptor and a specific tyrosine kinase receptor, TrkA and TrkB, respectively. The expression levels of both NGF and BDNF are increased in pancreata from patients suffering from CP (Friess et al., 1999; Zhu et al., 2001). In both studies, a significant correlation was observed between the degree of NGF or BDNF immunostaining and the global pain scores of the patients (Friess et al., 1999; Zhu et al., 2001). A similar increase in neurotrophin expression has also been observed in animal models of CP, as well as an increase in pain responses (Toma et al., 2000, 2002; Winston et al., 2005).

Recently the role of another growth factor, artemin, has been explored in CP. Artemin, unlike NGF and BDNF, is a member of the glial cell line-derived neurotrophic factor (GDNF) family and signals through a receptor complex including the nonselective tyrosine kinase receptor, ret, and a selective GPI-linked co-receptor, GDNF family receptor alpha-3 (GFR α 3) (Baloh et al., 1998). Artemin is increased following inflammation and is also capable of potentiating transient receptor potential vanilloid 1 (TRPV1), the capsaicin receptor expressed by a large population of nociceptors (Malin et al., 2006). Artemin and its co-receptor, GFR α 3, were both increased in CP, and artemin expression levels were significantly correlated with the severity of pain reported by patients (Ceyhan et al., 2007).

Viscera are unique in that pain sensations arising from these structures result from integration of input from both spinal (dorsal root ganglia [DRG]) and the inferior vagal, or nodose, ganglia (NG). Visceral afferents in the DRG encode and process the physical, nociceptive aspects of visceral pain, whereas those in the NG contribute to the physiological, affective aspects of visceral pain (Grundy, 2002). Interestingly, recent reports have revealed different neurochemical phenotypes for DRG and NG neurons innervating visceral structures (Peeters et al., 2006; Zhong et al., 2007). These differences include the expression of TRPV1 and growth factor receptors, both of which are important in inflammation and pain arising from CP (Friess et al., 1999; Toma et al., 2000, 2002; Zhu et al., 2001; Winston et al., 2005; Wick et al., 2006; Ceyhan et al., 2007; Liddle, 2007).

Previous studies have examined the distribution and neurochemical expression of pancreatic afferents arising from the DRG (Won et al., 1998) and/or NG (Sharkey and Williams, 1983; Sharkey et al., 1984; Carobi, 1987), with an emphasis on the expression of neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP). However, the expression of TRPV1 and other molecules shown to be important in CP have not been investigated in pancreatic sensory neurons in any species. Here, we have employed retrograde tracing and immunohistochemistry to determine the distribution, neurochemical identity, and growth-factor responsiveness of individual mouse pancreatic sensory neurons in the NG and DRG, particularly with regard to their expression of TRPV1, CGRP, and GFR α 3. We have specifically chosen to examine the mouse pancreas because genetic manipulations and electrophysiological techniques make it possible to explore questions that are not amenable in any other species (Bielefeldt et al., 2006).

MATERIALS AND METHODS

Animals

Experiments were performed on 8-week old male C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME) housed in the Department of Laboratory Animal Resources at the University of

Pittsburgh Medical Center. All research performed conformed to NIH guidelines in accordance with the guidelines specified by the University of Pittsburgh Medical Center Animal Care and Use Protocols. All mice received water and food ad libitum.

Surgical procedures

All surgical procedures were performed under sterile conditions in a designated animal surgery area. Anesthesia was initiated by inhaled 4% isoflurane and maintained with inhaled 2% isoflurane. A laparotomy was performed to expose the abdominal viscera. Following exposure of the pancreatic head, three injections of Alexa Fluor 488-conjugated cholera toxin B (CTB; 2 mg/ml in sterile saline; Molecular Probes; Eugene, OR; cat. #C-22843) were made into the duodenal lobe pancreatic parenchyma (n = 8) by using a glass micropipette with a total volume of approximately 5 μ l. The area was swabbed to remove any excess of tracer, the wound was sutured shut, and the mice were allowed to recover for 4 days. In four of the eight mice, a second set of similar injections was made into the splenic portion of the pancreas by using Alexa Fluor 555-conjugated CTB (Molecular Probes, cat. #C-34775).

During development of this experimental protocol, we inadvertently injected the adipose tissue surrounding the gastropiploic artery. This resulted in visible leakage during the operation. These mice were used as experimental controls for dye leakage, and DRG and NG cell counts were performed similar to those for the above mice. In these mice, fewer than five total labeled cells could be found in any of the NG or DRG examined. Thus, we concluded that few, if any, of the labeled neurons observed following injection into pancreata were the result of leaked dye being taken up by surrounding tissues or organs.

Tissue preparation and immunohistochemistry

At 4 days postoperatively, animals were anesthetized by inhaled isoflurane, followed by intraperitoneal injection of 2.5% avertin (2,2,2-tribromoethanol and tert-amyl alcohol diluted in 0.9% saline; 20 μ l/g body weight) and transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. For mice that received CTB injections into both the head and tail of the pancreas, bilateral NG and T5–L1 DRG were dissected, and the left and right ganglia for each level were embedded separately into 10% gelatin. For those that received CTB only in the pancreatic head, paired NG and T5–L1 DRG were embedded together into 10% gelatin.

The embedded tissue was postfixed for 1 hour at 4°C and cryoprotected in 25% sucrose at 4°C overnight. Individual DRG and NG were sectioned at 35 μ m on a sliding microtome and stored at 4°C in 0.1 M PB. For sections containing both pancreatic head and tail afferents, every other section was mounted in 0.1% gelatin onto Superfrost Plus slides (Fisher, Chicago, IL). The stomach, duodenum, spleen, and pancreas were also removed en bloc, postfixed for 1 hour, and cryoprotected in 25% sucrose in 0.1 M PB overnight at 4°C. The tissue was sectioned at 20 μ m on a cryostat, and the sections were alternately mounted onto 10 consecutive Superfrost Plus slides.

The sections containing only pancreatic head afferents were stored in 0.1 M PB at 4°C and processed for immunohistochemistry. Briefly, sections were washed three times with 0.1 M PB and blocked for 1 hour in 0.1 M PB containing 5% normal horse serum and 0.25% Triton X-100. Sections were then incubated overnight at room temperature with primary antisera to TRPV1, GFR α 3, and CGRP, which were as follows:

1. The antiserum to TRPV1 (1:500; Calbiochem, San Diego, CA; cat. #PC420) was a purified rabbit polyclonal antibody developed against a synthetic peptide (EDA EVFKDS MVPGEK) corresponding to amino acids 824–838 of rat capsaicin receptor and conjugated to keyhole limpet hemocyanin (KLH). It recognizes both

mouse and rat TRPV1 protein and has been previously used to identify TRPV1-positive DRG neurons (Mizushima et al., 2005; Tamura et al., 2005). The antiserum recognizes a single band at ~100 kDa in a Western blot of lumbar DRG protein (Wang et al., 2006) or spinal cord dorsal horn (manufacturer's insert) in the mouse.

2. The GFR α 3 antiserum (1:40 in 0.01 mM CaCl₂ in 0.1 M PB; R&D Systems; Minneapolis, MN; cat. #AF2645) was produced in goats immunized with purified Sf21-derived rmGFR α -3 extracellular domain (corresponding to amino acids 33–379) and purified by affinity chromatography. Per the manufacturer, the antibody recognizes the recombinant protein using a Western blot and has been validated for immunohistochemistry and direct enzyme-linked immunosorbent assay (ELISA). By using direct ELISA, 10% cross-reactivity was observed with recombinant human GFR α 3 and less than 2% cross-reactivity with recombinant mouse GFR α 2 and GFR α 4. As the antiserum is a polyclonal antibody, epitope sequencing was not performed by the manufacturer. The GFR α 3 antiserum has previously been used to identify neurons responsive to the GDNF family member artemin (Elitt et al., 2006; Malin et al., 2006; Forrest and Keast, 2008).
3. The CGRP antiserum (1:1,000; Sigma, St. Louis, MO; cat. #C8198) was developed in rabbit against a synthetic whole rat α -CGRP conjugated to KLH as the immunogen. The antiserum was treated to remove lipoproteins. The CGRP antiserum is reactive with rat CGRP and human CGRP and with β -CGRP, when conjugated to bovine serum albumin (BSA), by using a dot blot immunoassay. However, the antiserum shows no cross-reactivity with SP, vasoactive intestinal peptide, neuropeptide Y, calcitonin, or somatostatin (per the manufacturer).
4. The isolectin *Griffonia simplicifolia* I-B₄ (IB₄) specifically binds to terminal α -galactose, the terminal sugar on galactose- α 1,3-galactose carbohydrate moieties present on glycoproteins and glycolipids (Goldstein and Winter, 1999) and has been commonly used to identify GDNF-responsive, nonpeptidergic DRG neurons (Molliver et al., 1997; Carlsten et al., 2001; Christianson et al., 2006b). IB₄ visualization was performed by incubating the sections overnight in Alexa Fluor 647-conjugated IB₄ (1:100 in 0.01 mM CaCl₂ in 0.1 M PB; Molecular Probes; cat. #I-32450).

Sections were washed three times with 0.1 M PB and incubated for 2 hours with secondary antibodies, donkey anti-rabbit, or goat CY2 and CY5 (1:300, Jackson ImmunoResearch, West Grove, PA). Sections were washed three times in 0.1 M PB and mounted in 0.1% gelatin onto Superfrost slides. Specificity of staining was established by testing TRPV1 and GFR α 3 antisera on DRG sections from respective knockout mice and preincubating CGRP antiserum at 4°C overnight with 10 μ M CGRP peptide (Sigma).

Visualization and quantification of retrogradely labeled DRG and NG neurons

NG and DRG sections from mice receiving head and tail pancreatic CTB injections were viewed by using a Leica confocal microscope (Leica, Wetzlar, Germany). To visualize CTB-positive neurons, 16- μ m-thick optical sections were captured for every other tissue section throughout each ganglion. Each optical section was divided into four vertical slices, and the numbers of CTB-positive neurons from the first and last slice were counted and averaged together. Any CTB-positive neurons present in both slices were not counted, to ensure that large cells were not disproportionately represented, as recommended by the stereological technique contained in Pakkenberg and Gundersen (1988). The total numbers of CTB-positive neurons were calculated for the right and left ganglion at each level, and comparisons were made by using two-way analysis of variance (ANOVA) and Bonferroni's post-test for comparing multiple means (GraphPad Prism, GraphPad Software, San Diego, CA).

For sections processed for immunohistochemistry, both the left and right ganglia for a particular level were analyzed by using the same technique. The numbers of CTB-positive cells and the numbers of immunopositive CTB-positive cells were counted in the first and last slice and averaged together. Again, any CTB-positive neurons present in both the first and last slice were not counted. The average percentage of immunopositive CTB-labeled cells was calculated for each ganglion level and antibody. Comparisons were made by using two-way ANOVA and Bonferroni's post-test (GraphPad Prism). Adobe (San Jose, CA) Photoshop CS2 was used to adjust photomicrographs for brightness/contrast and to construct all figures.

RESULTS

Nodose ganglia (NG) and dorsal root ganglia (DRG) from T5–L1 were examined by using confocal fluorescent microscopy to visualize retrogradely labeled neurons 4 days after injection of Alexa Fluor 488- and 555-conjugated cholera toxin B (CTB) into the head and tail of the pancreas, respectively (Fig. 1). A previous study by Wang et al. (1998) demonstrated that CTB was more effective at retrogradely labeling bladder afferents than either wheat germ agglutinin or IB₄, most of which were small, unmyelinated afferents. CTB has also been used successfully in our lab to label colon, bladder, and/or stomach afferents in the same strain of mice and Sprague-Dawley rats (Christianson et al., 2006a,b,2007; Zhong et al., 2007). CTB labeling in the present study produced dense cytoplasmic staining mostly among small to medium-sized neurons (Fig. 1). CTB-positive pancreatic afferents were categorized as head-only if they contained only Alexa Fluor 488 (red), as tail-only if they contained only Alexa Fluor 555 (green), and as dually projecting if they contained both Alexa Fluor dyes (yellow, Fig. 1).

Quantification of pancreatic afferents

To determine possible leakage of CTB, the pancreas and surrounding organs including the stomach, duodenum, and spleen were dissected out of each mouse and examined by fluorescent microscopy. Alexa Fluor 555-CTB, which was injected into the pancreatic tail, was limited to the splenic lobe of the pancreas without leakage into the spleen or stomach (green, Fig. 1). Alexa Fluor 488-CTB (red, Fig. 1), which was injected into the pancreatic head, was faintly observed in the myenteric plexus of the proximal duodenum and gastric antrum. This was previously observed following FluoroGold injection into rat pancreata and was concluded to represent enteropancreatic innervation rather than diffusion of the injected tracer (Kirchgessner and Gershon, 1990).

CTB-positive pancreatic afferents were observed in both the NG and T5–13 DRG. T4 and L1 DRG were also examined but contained no CTB-positive cells. CTB-positive neurons were small in size, comparable to those observed in our previous publications of CTB-labeled colon and gastric afferents (Christianson et al., 2006a; Zhong et al., 2007). On average, 103.5 ± 11.5 CTB-positive NG neurons were observed per mouse when injections were made in both the head and tail of the pancreas. A similar number of CTB-positive DRG neurons were observed (127.0 ± 36.1) with most contained between T9 and T12 (Fig. 2). Within both populations, the predominant number of CTB-positive cells was from the pancreatic head only, comprising $84.8\% \pm 5.2$ of the NG and $66.3\% \pm 2.3$ of the DRG. In comparison, CTB-positive neurons innervating only the pancreatic tail comprised $6.6\% \pm 1.1$ and $17.1\% \pm 0.47$ of the NG and DRG, respectively ($P < 0.0001$, vs. head only), and those innervating both the head and tail portions of the pancreas comprised $8.6\% \pm 4.5$ of the NG and $16.6\% \pm 2.4$ of the DRG ($P < 0.0001$, vs. head only). This pattern was consistent throughout all spinal ganglia (Fig. 2).

The overall distribution of CTB-positive neurons (from combined head and tail injections) within the NG was asymmetrical, with most ($60.5\% \pm 0.7$) of the labeled neurons observed in the left NG compared with the right NG ($39.5\% \pm 0.7$; $P < 0.05$; Fig. 3). This pattern also held true for head-only afferents, with $63.7\% \pm 1.3$ being observed in the left NG and $36.3\% \pm 1.3$

in the right NG ($P < 0.0001$; Fig. 3). The opposite observation was made when examining tail-only afferents. On average, $27.3\% \pm 7.9$ were observed in the left NG and $72.7\% \pm 7.9$ within the right NG ($P > 0.05$; Fig. 3). However, this discrepancy has little impact on the overall distribution because tail-only afferents constitute only 6.6% of all NG pancreatic innervation. Likewise, those afferents that projected to both the head and tail region of the pancreas (dually projecting) were evenly split between the left and right NG ($54.9\% \pm 4.0$ and $45.1\% \pm 4.0$, respectively; $P > 0.05$) and did not dramatically impact on the overall distribution (Fig. 3). In contrast to the distribution patterns of the CTB-positive NG neurons, the DRG contained a symmetrical, bilateral distribution of CTB-positive afferents for all populations studied (Fig. 3). The only exception was a significantly higher percentage of tail-only afferents in the left T12 DRG ($2.2\% \pm 0.68$) compared with the right ($0.2\% \pm 0.15$; $P < 0.0001$; Fig. 3).

Neurochemical phenotype of pancreatic sensory afferents

For the following studies, sections of NG and DRG from mice receiving CTB injections only into the pancreatic head were processed for immunohistochemistry and lectin binding to determine their neurochemical phenotype and growth factor responsiveness. Single injections into the head of the pancreas were used in these studies because the great majority of pancreatic afferents innervated the pancreatic head (either as head-only or dually projecting afferents). In the following section, neurons labeled from these head injection will be referred to as pancreatic afferents.

The vast majority of pancreatic DRG afferents expressed TRPV1 ($74.9\% \pm 2.3$; Figs. 4, 7), a nonselective ion channel activated by capsaicin, low pH, and heat and shown to be expressed within a high percentage of visceral DRG afferents (Caterina et al., 1997; Davis et al., 2000; Robinson et al., 2004; Christianson et al., 2006a,b; Zhong et al., 2007). However, only $35.2\% \pm 5.5$ of pancreatic NG afferents expressed TRPV1 ($P < 0.01$, vs. DRG; Figs. 5, 7). No staining was observed when the TRPV1 antiserum was used on DRG sections from TRPV1 knockout mice (Fig. 6). Isolectin B₄ (IB₄) binds a subclass of primarily nonpeptidergic nociceptors that largely represents neurons responsive to growth factors in the glial cell line-derived neurotrophic factor (GDNF) family and is expressed by a very small percentage of visceral DRG afferents (Molliver et al., 1997; Wang et al., 1998; Robinson et al., 2004; Christianson et al., 2006a,b; Zhong et al., 2007). Not surprisingly, IB₄ was observed in only $2.5\% \pm 1.64$ of DRG and $10.6\% \pm 7.2$ of NG pancreatic afferents ($P < 0.05$; Figs. 4, 5, 7). A very small overlap in TRPV1 and IB₄ staining was observed in NG pancreatic afferents, constituting only $1.5\% \pm 1.3$ of all CTB-positive neurons (Figs. 5, 7). Approximately 3.2% of TRPV1-positive pancreatic NG afferents also bound IB₄, and 5.6% of IB₄-positive afferents expressed TRPV1 (Table 1). No overlap between TRPV1 and IB₄ was observed in pancreatic DRG afferents.

Artemin is a member of the GDNF family of neurotrophic factors and has been shown to be involved in the development of nociception following inflammation (Malin et al., 2006). Artemin signals through a two-receptor complex consisting of the tyrosine kinase receptor c-Ret and the GPI-linked receptor GFR α 3 (Baloh et al., 1998). GFR α 3 has been shown to be primarily expressed by TRPV1-positive afferents (Orozco et al., 2001; Elitt et al., 2006); therefore it was not surprising that a high percentage of pancreatic DRG afferents expressed GFR α 3 ($67.5\% \pm 3.0$; Figs. 4, 7). Only $1.0\% \pm 0.67$ of pancreatic NG afferents expressed GFR α 3 ($P < 0.01$; Figs. 5, 7). No staining was observed when the GFR α 3 antiserum was applied to DRG sections from GFR α 3 knockout mice (Fig. 6). The percentage of pancreatic DRG afferents that expressed both TRPV1 and GFR α 3 was $55.6\% \pm 7.2$ (Figs. 4, 7). Approximately 73.0% of TRPV1-positive pancreatic DRG neurons also expressed GFR α 3, whereas nearly all GFR α 3-positive afferents also expressed TRPV1 (96.1% ; Table 1). No detectable TRPV1/GFR α 3 double staining was observed in pancreatic NG ganglia (Figs. 5, 7).

Calcitonin gene-related peptide (CGRP) is a neuropeptide expressed by a subclass of nociceptive neurons responsive to nerve growth factor (NGF) and is also reportedly expressed by a majority of visceral afferents (Bennett et al., 1996; Wang et al., 1998; Robinson et al., 2004; Christianson et al., 2006a,b; Zhong et al., 2007). CGRP immunopositivity was observed in $64.5\% \pm 7.8$ of pancreatic DRG afferents but only $14.9\% \pm 7.2$ of pancreatic NG afferents ($P < 0.0001$; Figs. 4, 5, 7). CGRP immunoreactivity was abolished following preincubation with $10 \mu\text{M}$ CGRP peptide (Fig. 6). Armin and NGF have been shown to have coordinating and complimenting effects in increasing TRPV1 activation, thereby suggesting that they may support an overlapping population of sensory neurons (Malin et al., 2006). To determine whether this may occur within pancreatic afferents, we examined pancreatic afferents expressing both GFR α 3 and CGRP. Not surprisingly, $51.3\% \pm 8.6$ of pancreatic DRG afferents expressed both CGRP and GFR α 3, with 78.8% of CGRP-positive pancreatic DRG afferents also expressing GFR α 3 and 67.3% of GFR α 3-positive afferents also expressing CGRP (Fig. 7, Table 1). No CGRP/GFR α 3 double-labeled cells were observed within the pancreatic NG afferents (Figs. 5, 7).

DISCUSSION

Patients with chronic pancreatitis (CP) often suffer excruciating pain, which negatively affects their quality of life. Dysfunction of primary afferents and increases in pancreatic growth factor expression, specifically in NGF, BDNF and armin, have been shown to contribute to the pain (Bockman et al., 1988; Friess et al., 1999, 2002; Zhu et al., 2001; Di Sebastiano et al., 2004; Ceyhan et al., 2007). In order to determine the vagal and spinal distribution of pancreatic afferents and their potential to respond to pancreatic inflammation, we used Alexa Fluor-conjugated retrograde tracers to identify pancreatic afferents in the NG and DRG and antisera to TRPV1, CGRP, GFR α 3, as well as IB $_4$, to determine their neurochemical phenotype and growth factor responsiveness. GFR α 3 and CGRP were used to identify afferents that could be sensitized by armin and NGF, respectively, whereas TRPV1 expression was examined as it has been shown to be necessary for development of inflammatory pain in both visceral and somatic tissues.

Distribution of pancreatic afferents

Different conjugated forms of CTB were injected into the head and tail of mouse pancreata to determine the prevalence and distribution of pancreatic afferents in the NG and DRG. Roughly the same number of NG and DRG pancreatic neurons was observed, with most of the DRG afferents residing in T9–T12. The majority of all NG and DRG CTB-positive afferents projected only to the pancreatic head. Approximately the same number of afferents projected to the pancreatic tail or to both the head and tail regions, but each comprised a significantly smaller population than the head-only afferents. This coincides with a previous study showing that the highest density of sensory innervation was observed in the head region for both myelinated and CGRP-positive afferents, with significantly diminished innervation in the pancreatic tail (Lindsay et al., 2006). Similarly, a previous study by Won et al. (1998) reported a larger number of DRG neurons retrogradely labeled from the head of the rat pancreas versus the tail. Another study reported no difference in the number of labeled DRG neurons following injection of True Blue into the duodenal or splenic lobe of the rat pancreas (Sharkey et al., 1984); however, differences in retrograde tracer, injection technique, or species could account for this discrepancy.

The report by Sharkey et al. (1984), however, did agree with our finding that most of the pancreatic head afferents were observed in the left NG, whereas most of the pancreatic tail afferents were observed in the right NG. This observation has also been described by other reports (Sharkey and Williams, 1983; Carobi, 1987; Sternini et al., 1992). In contrast to the

NG neurons, no significant difference was observed between the number of CTB-positive neurons in the left and right DRG for any of the injection-specific populations, with the single exception of there being significantly more tail-only afferents in the left T12 DRG than the right. Interestingly, the previous report by Won et al. (1998) showed a higher number of pancreatic head afferents in the right DRG, as well as a higher number of pancreatic tail afferents in the left DRG.

Anatomical (Christianson et al., 2006b, 2007; Zhong et al., 2007) and electrophysiological (Bahns et al., 1987; Berkley et al., 1990; Malykhina et al., 2006; Ustinova et al., 2006) studies have shown that single afferents are capable of innervating more than one region within the same organ or of sending projections to two separate organs. Similarly, here we observed a small population of pancreatic afferents that appear to innervate both the head and tail portions of the organ. These large projections may contribute to the diffuse characteristic of pancreatic pain.

Neurochemical phenotype of pancreatic afferents and their potential susceptibility to inflammation

NG and DRG neurons have long been thought to convey different types of sensory information from the viscera. DRG neurons respond to physiological levels of stimulation and are capable of encoding noxious input. Therefore, they are believed to mediate painful sensations, and their close association with the vasculature also suggests an efferent/cytoprotective role in response to injury or inflammation (Grundy, 2002). NG neurons have low thresholds for activation, and previous studies have suggested that these afferents do not code into the noxious range (Sengupta, 2000). Therefore it was believed that these afferents could only be involved in the physiological regulation of visceral stimulation, including emotional and behavioral aspects. However, recent studies of intact vagal afferents innervating the stomach and esophagus have shown that whereas they do generally have low thresholds, they are capable of coding noxious stimuli (Blackshaw et al., 2000; Bielefeld et al., 2006; Bielefeld and Davis, 2008). Whether these responses from vagal afferent directly contribute to pain sensation or to negative emotional aspects of visceral pain (e.g., nausea, fullness, depression) is unclear.

Whereas both DRG and NG afferents can respond to a wide range of stimulus intensities, differences in their neurochemical profiles suggest that they may diverge in their ability to respond to inflammation. Data have been accumulating over the past decade showing that persistent inflammatory pain requires the expression of TRPV1 in primary afferents. TRPV1 was expressed in both DRG and NG pancreatic neurons, but at a significantly lower level in the NG (79% for DRG vs. 35% for NG). A similar discrepancy in neurochemical phenotype was observed in a study of mouse gastric neurons (Zhong et al., 2007). Not only did they report a lower expression of TRPV1 and CGRP in the NG, they also reported a higher expression of IB₄ among gastric NG afferents, compared with DRG afferents, similar to what was observed here. Visceral DRG neurons have also been shown to have higher mRNA expression levels of TRPV1, as well as TRPA1 and SP, than NG neurons (Peeters et al., 2006).

Local release of growth factors and cytokines in response to an inflammatory insult is capable of potentiating and increasing the responsiveness of the TRPV1 channel, thereby contributing to the production of inflammatory hyperalgesia (Ma and Quirion, 2007). Most pancreatic DRG neurons in this study expressed CGRP and/or GFR α 3. CGRP has long been used as a marker for NGF-responsive neurons (Averill et al., 1995) and has been shown previously to be expressed by the vast majority (88%) of rat pancreatic DRG neurons (Won et al., 1998). GFR α 3 is the co-receptor for artemin (Baloh et al., 1998), and the expression of these markers indicates that these neurons may be particularly susceptible to increases in either NGF or artemin in the event of CP (Friess et al., 1999; Ceyhan et al., 2007). These neurons also expressed TRPV1, which has been shown to be potentiated by NGF and/or artemin in calcium

imaging studies of DRG neurons (Malin et al., 2006). A high prevalence of TRPV1 expression among visceral afferents has been shown by a number of publications in recent years (Robinson et al., 2004; Brierley et al., 2005; Zhong et al., 2007; Christianson et al., 2006a,b), and TRPV1 has also been shown to contribute to observed visceral hyperalgesia in animal models of CP. Ablation of TRPV1-positive fibers via application of resiniferatoxin to celiac ganglia significantly reduced SP release and inflammation in a rat model of pancreatitis (Noble et al., 2006). Similarly, TRPV1 mRNA and protein expression were both increased in pancreatic DRG neurons following induction of CP in rats, and treatment with a TRPV1 antagonist reduced both visceral and referred somatic pain behaviors (Xu et al., 2007).

In conclusion, the present study has demonstrated that, in terms of density, the mouse pancreas receives comparable sensory innervation from both spinal and vagal ganglia. Despite their relatively similar contributions, these two populations have markedly different neurochemical phenotypes and presumed growth factor-response profiles. Pancreatic DRG neurons are largely TRPV1 positive and putatively responsive to NGF and/or artemin. In contrast, a much smaller percentage of pancreatic NG neurons express TRPV1, and few, if any, express molecules indicative of NGF and/or artemin responsiveness. The next step will be to determine how inflammation, particularly that which occurs in acute and chronic pancreatitis, affects these two separate populations and whether they contribute to persistent visceral hypersensitivity.

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LITERATURE CITED

- Averill S, McMahon SB, Clary DO, Reichardt LF, Priestley JV. Immunocytochemical localization of trkA receptors in chemically identified subgroups of adult rat sensory neurons. *Eur J Neurosci* 1995;7:1484–1494. [PubMed: 7551174]
- Bahns E, Halsband U, Janig W. Responses of sacral visceral afferents from the lower urinary tract, colon and anus to mechanical stimulation. *Pflugers Arch* 1987;410:296–303. [PubMed: 3684516]
- Baloh RH, Tansey MG, Lampe PA, Fahrner TJ, Enomoto H, Simburger KS, Leitner ML, Araki T, Johnson EM Jr, Milbrandt J. Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. *Neuron* 1998;21:1291–1302. [PubMed: 9883723]
- Bennett DL, Dmtrieva N, Priestley JV, Clary D, McMahon SB. trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. *Neurosci Lett* 1996;206:33–36. [PubMed: 8848275]
- Berkley KJ, Hotta H, Robbins A, Sato Y. Functional properties of afferent fibers supplying reproductive and other pelvic organs in pelvic nerve of female rat. *J Neurophysiol* 1990;63:256–272. [PubMed: 2313344]
- Bielefeldt K, Davis B. Differential effects of ASIC3 and TRPV1 deletion on gastroesophageal sensation in mice. *Am J Physiol Gastrointest Liver Physiol* 2008;294:G103–G108.
- Bielefeldt K, Zhong F, Koerber HR, Davis BM. Phenotypic characterization of gastric sensory neurons in mice. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G987–997.
- Blackshaw LA, Page AJ, Partosoedarso ER. Acute effects of capsaicin on gastrointestinal vagal afferents. *Neuroscience* 2000;96:407–416. [PubMed: 10683581]
- Bockman DE, Buchler M, Malfertheiner P, Beger HG. Analysis of nerves in chronic pancreatitis. *Gastroenterology* 1988;94:1459–1469. [PubMed: 3360267]
- Brierley SM, Carter R, Jones W 3rd, Xu L, Robinson DR, Hicks GA, Gebhart GF, Blackshaw LA. Differential chemosensory function and receptor expression of splanchnic and pelvic colonic afferents in mice. *J Physiol* 2005;567:267–281. [PubMed: 15946967]

- Carlsten JA, Kothary R, Wright DE. Glial cell line-derived neurotrophic factor-responsive and neurotrophin-3-responsive neurons require the cytoskeletal linker protein dystonin for postnatal survival. *J Comp Neurol* 2001;432:155–168. [PubMed: 11241383]
- Carobi C. Capsaicin-sensitive vagal afferent neurons innervating the rat pancreas. *Neurosci Lett* 1987;77:5–9. [PubMed: 2439955]
- Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, Julius D. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 1997;389:816–824. [PubMed: 9349813]
- Ceyhan GO, Bergmann F, Kadihasanoglu M, Erkan M, Park W, Hinz U, Giese T, Muller MW, Buchler MW, Giese NA, Friess H. The neurotrophic factor artemin influences the extent of neural damage and growth in chronic pancreatitis. *Gut* 2007;56:534–544. [PubMed: 17047099]
- Christianson JA, McIlwrath SL, Koerber HR, Davis BM. Transient receptor potential vanilloid 1-immunopositive neurons in the mouse are more prevalent within colon afferents compared to skin and muscle afferents. *Neuroscience* 2006a;140:247–257. [PubMed: 16564640]
- Christianson JA, Traub RJ, Davis BM. Differences in spinal distribution and neurochemical phenotype of colonic afferents in mouse and rat. *J Comp Neurol* 2006b;494:246–259. [PubMed: 16320237]
- Christianson JA, Liang R, Ustinova EE, Davis BM, Fraser MO, Pezzone MA. Convergence of bladder and colon sensory innervation occurs at the primary afferent level. *Pain* 2007;128:235–243. [PubMed: 17070995]
- Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, Hughes SA, Rance K, Grau E, Harper AJ, Pugh PL, Rogers DC, Bingham S, Randall A, Sheardown SA. Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* 2000;405:183–187. [PubMed: 10821274]
- Di Sebastiano P, di Mola FF, Buchler MW, Friess H. Pathogenesis of pain in chronic pancreatitis. *Dig Dis* 2004;22:267–272. [PubMed: 15753609]
- Elitt CM, McIlwrath SL, Lawson JJ, Malin SA, Molliver DC, Cornuet PK, Koerber HR, Davis BM, Albers KM. Artemin overexpression in skin enhances expression of TRPV1 and TRPA1 in cutaneous sensory neurons and leads to behavioral sensitivity to heat and cold. *J Neurosci* 2006;26:8578–8587. [PubMed: 16914684]
- Forrest SL, Keast JR. Expression of receptors for glial cell line-derived neurotrophic factor family ligands in sacral spinal cord reveals separate targets of pelvic afferent fibers. *J Comp Neurol* 2008;506:989–1002. [PubMed: 18085594]
- Friess H, Zhu ZW, di Mola FF, Kulli C, Graber HU, Andren-Sandberg A, Zimmermann A, Korc M, Reinshagen M, Buchler MW. Nerve growth factor and its high-affinity receptor in chronic pancreatitis. *Ann Surg* 1999;230:615–624. [PubMed: 10561084]
- Friess H, Shrikhande S, Shrikhande M, Martignoni M, Kulli C, Zimmermann A, Kappeler A, Ramesh H, Buchler M. Neural alterations in surgical stage chronic pancreatitis are independent of the underlying aetiology. *Gut* 2002;50:682–686. [PubMed: 11950816]
- Goldstein IJ, Winter HG. The *Griffonia simplicifolia* I-B4 isolectin. A probe for alpha-D-galactosyl end groups. *Subcell Biochem* 1999;32:127–141. [PubMed: 10391994]
- Grundy D. Neuroanatomy of visceral nociception: vagal and splanchnic afferent. *Gut* 2002;51(suppl 1):i2–5. [PubMed: 12077054]
- Kirchgessner AL, Gershon MD. Innervation of the pancreas by neurons in the gut. *J Neurosci* 1990;10:1626–1642. [PubMed: 2159059]
- Liddle RA. The role of transient receptor potential vanilloid 1 (TRPV1) channels in pancreatitis. *Biochim Biophys Acta* 2007;1772:869–878. [PubMed: 17428642]
- Lindsay TH, Halvorson KG, Peters CM, Ghilardi JR, Kuskowski MA, Wong GY, Mantyh PW. A quantitative analysis of the sensory and sympathetic innervation of the mouse pancreas. *Neuroscience* 2006;137:1417–1426. [PubMed: 16388907]
- Ma W, Quirion R. Inflammatory mediators modulating the transient receptor potential vanilloid 1 receptor: therapeutic targets to treat inflammatory and neuropathic pain. *Expert Opin Ther Targets* 2007;11:307–320. [PubMed: 17298290]
- Malin SA, Molliver DC, Koerber HR, Cornuet P, Frye R, Albers KM, Davis BM. Glial cell line-derived neurotrophic factor family members sensitize nociceptors in vitro and produce thermal hyperalgesia in vivo. *J Neurosci* 2006;26:8588–8599. [PubMed: 16914685]

- Malykhina AP, Qin C, Greenwood-van Meerveld B, Foreman RD, Lupu F, Akbarali HI. Hyperexcitability of convergent colon and bladder dorsal root ganglion neurons after colonic inflammation: mechanism for pelvic organ cross-talk. *Neurogastroenterol Motil* 2006;18:936–948. [PubMed: 16961697]
- Mizushima T, Obata K, Yamanaka H, Dai Y, Fukuoka T, Tokunaga A, Mashimo T, Noguchi K. Activation of p38 MAPK in primary afferent neurons by noxious stimulation and its involvement in the development of thermal hyperalgesia. *Pain* 2005;113:51–60. [PubMed: 15621364]
- Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD. IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* 1997;19:849–861. [PubMed: 9354331]
- Noble MD, Romac J, Wang Y, Hsu J, Humphrey JE, Liddle RA. Local disruption of the celiac ganglion inhibits substance P release and ameliorates caerulein-induced pancreatitis in rats. *Am J Physiol Gastrointest Liver Physiol* 2006;291:G128–134. [PubMed: 16769810]
- Orozco OE, Walsh L, Sah DW, Pepinsky RB, Sanicola M. GFR α 3 is expressed predominantly in nociceptive sensory neurons. *Eur S Neurosci* 2001;13:2177–2182.
- Pakkenberg B, Gundersen HJ. Total number of neurons and glial cells in human brain nuclei estimated by the disector and the fractionator. *J Microsc* 1988;150:1–20. [PubMed: 3043005]
- Peeters PJ, Aerssens J, de Hoogt R, Stanisz A, Gohlmann HW, Hillsley K, Meulemans A, Grundy D, Stead RH, Coulie B. Molecular profiling of murine sensory neurons in the nodose and dorsal root ganglia labeled from the peritoneal cavity. *Physiol Genomics* 2006;24:252–263. [PubMed: 16303873]
- Robinson DR, McNaughton PA, Evans ML, Hicks GA. Characterization of the primary spinal afferent innervation of the mouse colon using retrograde labelling. *Neurogastroenterol Motil* 2004;16:113–124. [PubMed: 14764211]
- Sengupta JN. An overview of esophageal sensory receptors. *Am J Med* 2000;108(suppl 4a):87S–89S. [PubMed: 10718458]
- Sharkey KA, Williams RG. Extrinsic innervation of the rat pancreas: demonstration of vagal sensory neurones in the rat by retrograde tracing. *Neurosci Lett* 1983;42:131–135. [PubMed: 6664624]
- Sharkey KA, Williams RG, Dockray GJ. Sensory substance P innervation of the stomach and pancreas. Demonstration of capsaicin-sensitive sensory neurons in the rat by combined immunohistochemistry and retrograde tracing. *Gastroenterology* 1984;87:914–921. [PubMed: 6205934]
- Sternini C, De Giorgio R, Furness JB. Calcitonin gene-related peptide neurons innervating the canine digestive system. *Regul Pept* 1992;42:15–26. [PubMed: 1475404]
- Tamura S, Morikawa Y, Senba E. TRPV2, a capsaicin receptor homologue, is expressed predominantly in the neurotrophin-3-dependent subpopulation of primary sensory neurons. *Neuroscience* 2005;130:223–228. [PubMed: 15561438]
- Toma H, Winston J, Micci MA, Shenoy M, Pasricha PJ. Nerve growth factor expression is up-regulated in the rat model of L-arginine-induced acute pancreatitis. *Gastroenterology* 2000;119:1373–1381. [PubMed: 11054396]
- Toma H, Winston JH, Micci MA, Li H, Hellmich HL, Pasricha PJ. Characterization of the neurotrophic response to acute pancreatitis. *Pancreas* 2002;25:31–38. [PubMed: 12131768]
- Ustinova EE, Fraser MO, Pezzone MA. Colonic irritation in the rat sensitizes urinary bladder afferents to mechanical and chemical stimuli: an afferent origin of pelvic organ cross-sensitization. *Am J Physiol Renal Physiol* 2006;290:F1478–1487. [PubMed: 16403832]
- Wang HF, Shortland P, Park MJ, Grant G. Retrograde and transganglionic transport of horseradish peroxidase-conjugated cholera toxin B subunit, wheatgerm agglutinin and isolectin B4 from *Griffonia simplicifolia* I in primary afferent neurons innervating the rat urinary bladder. *Neuroscience* 1998;87:275–288. [PubMed: 9722157]
- Wang S, Davis BM, Zwick M, Waxman SG, Albers KM. Reduced thermal sensitivity and Nav1.8 and TRPV1 channel expression in sensory neurons of aged mice. *Neurobiology of aging* 2006;27:895–903. [PubMed: 15979214]
- Wick EC, Hoge SG, Grahn SW, Kim E, Divino LA, Grady EF, Bunnett NW, Kirkwood KS. Transient receptor potential vanilloid 1, calcitonin gene-related peptide, and substance P mediate nociception in acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2006;290:G959–969. [PubMed: 16399878]

- Winston JH, He ZJ, Shenoy M, Xiao SY, Pasricha PJ. Molecular and behavioral changes in nociception in a novel rat model of chronic pancreatitis for the study of pain. *Pain* 2005;117:214–222. [PubMed: 16098667]
- Won MH, Park HS, Jeong YG, Park HJ. Afferent innervation of the rat pancreas: retrograde tracing and immunohistochemistry in the dorsal root ganglia. *Pancreas* 1998;16:80–87. [PubMed: 9436867]
- Xu GY, Winston JH, Shenoy M, Yin H, Pendyala S, Pasricha PJ. Transient receptor potential vanilloid 1 mediates hyperalgesia and is up-regulated in rats with chronic pancreatitis. *Gastroenterology* 2007;133:1282–1292. [PubMed: 17698068]
- Zhong F, Christianson JA, Davis BM, Bielefeldt K. Dichotomizing axons in spinal and vagal afferents of the mouse stomach. *Dig Dis Sci* 2008;53:194–203. [PubMed: 17510799]
- Zhu ZW, Friess H, Wang L, Zimmermann A, Buchler MW. Brain-derived neurotrophic factor (BDNF) is upregulated and associated with pain in chronic pancreatitis. *Dig Dis Sci* 2001;46:1633–1639. [PubMed: 11508661]

Fig. 1.

Pancreatic neurons in the mouse were retrogradely labeled from the head and/or tail portions of the pancreas by using Alexa Fluor-conjugated cholera toxin B (CTB). CTB labeling produced dense cytoplasmic staining among mostly small to medium-sized neurons. Neurons projecting exclusively to the pancreatic head contained only CTB-488 (green somata, **A,D,G**), those projecting exclusively to the pancreatic tail contained only CTB-555 (red somata, **B,E,H**) and those projecting to both areas of the pancreas contained both CTB-488 and 555 (yellow somata, **C,F,I**). **J**: Reconstructed montage of a mouse pancreas injected with CTB-488 in the pancreatic head (H) and CTB-555 in the tail (T). The stomach (S), pylorus (P), and duodenum (D) are also present. Scale bar = 20 μ m in I (applies to A–I); 100 μ m in J.

Fig. 2.

Approximately the same number (**A**) and percentage (**B**) of CTB-positive neurons was observed in the nodose (NG) and dorsal root ganglia (DRG). The greatest number of spinal pancreatic neurons was observed from T9 to T12. The majority of all pancreatic afferents projected only to the pancreatic head (black bars), with similar, significantly smaller proportions projecting to the pancreatic tail (white bars) or to both the tail and head (gray bars). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ vs. head only.

Fig. 3.

A: The left nodose ganglia (NG) contained a significantly higher percentage of all pancreatic neurons than did the right NG; however, no differences were observed between the left and right dorsal root ganglia (DRG), regardless of spinal level. **B:** The same pattern held true for neurons innervating only the pancreatic head. **C:** The opposite was observed for neurons retrogradely labeled from the pancreatic tail, with a significantly higher percentage being observed in the right NG. There was also a significantly higher percentage of pancreatic tail neurons in the left T12 DRG than the right. **D:** No differences in dually projecting neurons were observed between left and right ganglia for either NG or DRG.

Fig. 4.

Immunohistochemistry using antisera to TRPV1, CGRP, and GFR α 3, along with IB₄ binding, was performed on nodose ganglia to identify neurochemically cholera toxin B (CTB)-positive afferents innervating the pancreas. A small percentage of pancreatic vagal neurons expressed TRPV1 (arrows; **A–H**) or bound IB₄ (open arrows; **A–D**); however, very few (<2%, not shown) showed reactivity with both markers. CGRP was also expressed by a very small percentage of pancreatic vagal neurons (arrows, **I–L**); however, GFR α 3 was uniformly not expressed among pancreatic vagal neurons (**E–L**). Scale bar = 20 μ m in **L** (applies to **A–L**).

Fig. 5.

Immunohistochemistry using antisera to TRPV1, CGRP, and GFR α 3, along with IB₄ binding, was performed on dorsal root ganglia to identify neurochemically cholera toxin B (CTB)-positive afferents innervating the pancreas. The vast majority of pancreatic spinal neurons expressed TRPV1 (arrows and arrowheads; **A–H**); however, very few bound IB₄ (not shown, **C**), and none were found to both express TRPV1 and bind IB₄ (**D**). CGRP was also expressed by the majority of pancreatic spinal neurons (arrows and arrowheads, **I–L**), as was GFR α 3 (arrowheads, **E–L**). GFR α 3 was expressed by nearly all TRPV1-positive (arrowheads, **H**) and CGRP-positive neurons (arrowheads, **L**). Scale bar = 20 μ m in **L** (applies to **A–L**).

Fig. 6. Specificity of staining was tested for each antiserum. The TRPV1 antiserum labels primarily small and medium-sized DRG neurons in wild-type mice (**A**) but did not react with tissue sections taken from TRPV1 knockout mouse DRG (**B**). GFR α 3 antiserum also labels many small and medium-sized DRG neurons in wild-type mice (**C**); however, staining was absent in DRG sections taken from GFR α 3 knockout mice (**D**). CGRP staining is observed in many DRG neurons in wild-type mice (**E**), and preabsorption with 10 μ M CGRP peptide abolished all antibody staining (**F**). Scale bar = 20 μ m in B (applies to A,B), D (applies to C,D), and F (applies to E,F).

Fig. 7.

The neurochemical phenotype of cholera toxin B (CTB)-positive neurons innervating the pancreatic head was evaluated in nodose (NG) and dorsal root ganglia (DRG). **A,B:** TRPV1 was expressed by a significantly larger percentage of DRG neurons than NG. A small percentage of NG neurons bound IB₄, and less than 2% expressed both TRPV1 and bound IB₄ (A). Only 2.5% of pancreatic DRG neurons bound IB₄, and none of these expressed TRPV1 (A). **C:** CGRP had a similar significantly higher expression among pancreatic DRG neurons, compared with NG. GFRα3 was also expressed by the majority of pancreatic DRG neurons and was practically absent among NG neurons (<1%, B,C). Most pancreatic DRG neurons that expressed TRPV1 or CGRP also expressed GFRα3; however, this neurochemical phenotype was not observed among pancreatic NG neurons (B,C). ND, not detected. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$ vs. nodose.

TABLE 1
Percent of Pancreatic Afferents Expressing Two Immunohistochemical Markers¹

	TRPV1/IB ₄	IB ₄ /TRPV1	TRPV1/GFRα 3	GFRα 3/TRPV1	CGRP/GFRα 3	GFRα 3/CGRP
Nodose	3.2 ± 2.7	5.6 ± 4.8	0	0	0	0
DRG	0	0	73.0 ± 4.4	96.1 ± 3.9	78.8 ± 7.5	67.3 ± 9.8

¹ Percentages are shown of pancreatic afferents expressing the first marker that were also positively labeled by the second. Data are means ± SEM. Abbreviations: CGRP, calcitonin gene-related peptide; DRG, dorsal root ganglia; GDNF, family receptor alpha 3 (GFRα3); IB₄, isolectin B₄; TRPV1, transient receptor potential vanilloid 1.