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ORIGINAL PAPER

Dichotomizing Axons in Spinal and Vagal Afferents of the Mouse Stomach

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Abstract

Visceral sensory input is typically poorly localized. We hypothesized that gastric sensory neurons frequently dichotomize, innervating more than one anatomically distinct region and contributing to the poor spatial discrimination.

Methods The neurochemical phenotype and projections of gastro-duodenal sensory neurons were determined in adult mice. Choleratoxin B (CTB) coupled to different fluorophors was injected into fundus, corpus, antrum, and/or distal duodenum. Immunoreactivity for TRPV1, neurofilament (N52), calcitonin gene-related peptide (CGRP), presence of isolectin B4 (IB4) and labeling for retrograde labels was determined.

Results Depending on the distance between injection sites, staining for two retrograde tracers was seen in 6–48% of neurons. Most dorsal root ganglion (DRG) neurons showed immunoreactivity for TRPV1 and CGRP. In contrast, about half of the gastric nodose ganglion (NG) neurons had TRPV1 immunoreactivity or showed IB4 labeling with only 10% CGRP-positive neurons. N52 immunoreactivity was present in one-fourth of gastroduodenal DRG and NG neurons.

Conclusion Visceral sensory neurons have neurochemical properties and may project to more than one anatomically distinct area. Neurons with such dichotomizing axons may contribute to the poor ability to localize or discriminate visceral stimuli.

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Introduction

Dyspeptic symptoms affect about 25% of the adults in the United States of America and are responsible for up to 15% of patients' visits to primary care physicians and up to one-third of the consultative visits to gastroenterologists [1]. Sensitization of peripheral and/or central nociceptive afferents can alter nociceptive processing, thereby inducing the visceral hyperalgesia that underlies the pain or discomfort experienced by these patients [2, 3]. Visceral stimuli, such as gastric distension, typically trigger vague, poorly localized symptoms, with affected patients displaying lower sensory thresholds than controls, consistent with the presence of hypersensitivity [4].

The stomach receives a dual sensory innervation, consisting of vagal and spinal afferents projecting from the nodose and dorsal root ganglia, respectively. Essentially, all mechanosensitive vagal afferents are activated by low-intensity stimuli [5–7]. In contrast, spinal afferents can be differentiated into low- and high-threshold fibers, with about 20% of the spinal afferents requiring distension pressures exceeding 20 mmHg [8, 9]. Recent studies of visceral afferents suggest that the low-threshold fibers are not homogeneous but can be further differentiated into mucosal or muscular afferents, based on responses to mucosal stimulation and stretch [10–13]. Such physiological studies also demonstrated that visceral afferents may have several receptive fields [14, 15]. Consistent with these results are those of Christianson et al., who showed that between 20% and 30% of colon sensory neurons are dually

labeled by choleratoxin B injected into two distinct areas of the distal colon, separated by 1 cm [16]. Experiments with different labels injected into neighboring organs, such as bladder and distal colon, further suggest the presence of dichotomizing axons with a single neuron projecting to two viscera [17].

On the basis of these findings, we decided to perform a detailed anatomical and neurochemical characterization of the extrinsic innervation of the mouse stomach and duodenum. We separately labeled vagal and spinal neurons projecting to different areas of the stomach or duodenum using Alexa Fluor-conjugated choleratoxin B (CTB). In addition, we performed immunohistochemical investigations, using antibodies against the cationic ion channel TRPV1 (capsaicin receptor) and the neuropeptide calcitonin gene-related peptide (CGRP), which typically co-localizes with the high-affinity receptor for nerve growth factor (trkA) and is often used to identify potential peptidergic nociceptiors [16, 18, 19]. To determine the fraction of possible non-peptidergic nociceptors, we examined binding of the plant isolectin IB4 [20]. Finally, we investigated the presence of neurons with myelinated axons by immunohistochemical staining for the neurofilament heavy chain [21]. These neurochemical markers are frequently used to classify sensory neurons, with TRPV1, CGRP, and IB4 identifying subgroups thought to play a role in the sensation of pain.

Methods

Experiments were performed on C57 black six male mice (The Jackson Laboratory, Bar Harbor, ME, USA), weighing 20–25 g. Animal handling adhered to the *Guide for the Care and Use of Laboratory Animals* (National Research Council); all procedures were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh. Mice were housed under a 12 h light and dark cycle and had free access to food and water.

Surgical procedures

Mice (n = 13) were anesthetized with an intraperitoneal injection of a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg), and a midline laparotomy was performed under sterile conditions. Stomach and duodenum were exposed, and 3 µl Alexa Fluor 488-conjugated CTB (2%) was injected beneath the serosal layer along the anterior wall of the fundus with a Hamilton microsyringe (33-gauge needle). The needle was left in place for 30 s to avoid dye leakage. After withdrawal of the needle, the area was swabbed to remove any excess tracer. A second injection was placed on the corresponding area of the dorsal wall. Alexa Fluor 555-conjugated CTB (2%) was then injected into the corpus, pyloric antrum or distal duodenum, by the same technique. Injection sites were separated by 1 cm, 2 cm, and more than 3 cm, respectively. Considering the course of vagal fibers within the gastric wall [22], we placed the injections close to the greater curvature to minimize the labeling of passing fibers. For immunohistochemical experiments, only Alexa Fluor 488-conjugated CTB (2%) was injected into the corpus or distal duodenum as described above (n = 17). To compare the neurochemical properties of gastroduodenal with those of muscular and cutaneous afferents, we injected 3 µl Alexa-Fluor 488-conjugated CTB (single injection) into the lateral aspect of the intercostal muscle (T10; n = 4) or the overlying skin (n = 4). For these control experiments, neurons were sampled only from T10.

Tissue preparation and processing

Five to 7 days after surgery, the animals were anesthetized and transcardially perfused with ice-cold paraformaldehyde (4% in 0.1 M phosphate buffer). Both nodose ganglia and paired dorsal root ganglia (T4–L2) were dissected, embedded in 10% gelatin. The tissue was transferred into 0.1 M phosphate buffer (PB) with 25% sucrose and stored at 4°C for at least 18 h, prior to our cutting sections (35 µm) on a sliding microtome. Sections were washed three times in 0.1 M PB, placed in phosphate-buffered solution containing 5% normal goat serum and 0.25% Triton X-100 (PBT) and then incubated overnight at room temperature with antibodies against TRPV1 (raised in rabbits; 1:500; Calbiochem, San Diego, CA, USA; cat. # PC420), CGRP (raised in rabbits; 1:2000; Chemicon, Temecula, CA, USA; cat. # AB1971) and/or the neurofilament heavy chain (generated from mouse ascites fluid; 1:400; N52, Sigma, St. Louis, MO, USA; cat. # N0142) and/or Alexa Fluor 647-conjugated isolectin Griffonia simplifolica IB4 (1:100; IB4, Molecular Probes, Eugene, OR, USA; cat. # I-32450). After being washed three times in 0.1 M PB, sections were incubated for 2 h at room temperature with secondary antibodies, donkey anti-rabbit Cy3 or anti-mouse Cy5 (1:150; Jackson Immunoresearch, West Grove, PA, USA). The sections were then washed three times in 0.1 M PBT and mounted in 0.1% gelatin.

Labeling was analyzed using a Leica confocal microscope (Leica Microsystems; Wetzlar, Germany) and sequential scanning to prevent bleed-through of the different fluorophors. To visualize CTB-positive neurons, we captured 16 µm-thick optical sections for every other tissue section throughout each ganglion. Each optical section was divided into four sections, and the number of CTB-positive neurons from the first and last section was counted and

averaged together. Any CTB-positive neurons present in both sections were not counted, to ensure that large cells were not disproportionately represented, as recommended by the stereological technique contained in Pakkenberg and Gundersen [23]. We calculated the total number of CTB-positive neurons for each ganglion by multiplying the number of CTB-positive cells counted in each section by 2. For sections processed for immunohistochemistry, both the left and right ganglia from each spinal level were analyzed. A similar optical section was captured and divided into four sections. The number of CTB-positive cells and the number of CTB-positive cells with immunoreactivity for the marker of interest were counted in the first and last section and averaged together. Again, any CTB-positive neurons present in both the first and last sections were not counted. The average percentage of immunopositive CTB-labeled cells was calculated for each spinal level and antibody. The diameter of gastric sensory neurons was determined with imaging processing software (Image J 1.29, Wayne Rasband, National Institutes of Health). Only cells with visible nuclei were used for this analysis. Adobe Photoshop 7.0 was used to adjust photomicrographs for brightness/contrast and to construct the figure.

Statistical analysis

All data are given as mean ± standard error. Results were analyzed with *t*-test and analysis of variance (ANOVA), using SigmaStat software (Jandel Scientific; San Rafael, CA, USA). A value of $P < 0.05$ was considered statistically significant.

Results

Distribution of gastroduodenal sensory neurons

Subserosal injection of Alexa Fluor-conjugated CTB in the fundus, corpus, and pylorus of the stomach and the duodenum resulted in dense cytoplasmic staining of DRG and nodose neurons. The CTB-positive neurons were uniformly distributed within each individual DRG and nodose ganglion. The majority of the CTB-positive DRG neurons were medium sized with an average diameter of 32 ± 1 μm. The total number of CTB-positive neurons did not differ between the gastric-specific populations (fundus, corpus, and pylorus); however, significantly fewer neurons were labeled by CTB injection into the duodenum than into the gastric-specific afferents ($P < 0.05$; Fig. 1). This was true for both vagal (Kruskal–Wallis ANOVA on ranks, $H = 8.4$; $P < 0.05$) and spinal neurons (Kruskal–Wallis ANOVA on ranks, $H = 12.5$; $P < 0.01$). Within the fundus-specific population, significantly more CTB-positive afferents were observed in the DRG than in nodose ($P < 0.05$; Fig. 1). A similar discrepancy was observed within the corpus- and pylorus-specific populations; however, the differences were not significant ($P > 0.05$). Within the spinal ganglia, gastroduodenal neurons were found from T4 through L2, with a peak around T10 (Fig. 1). No differences were observed in the distribution along the rostro-caudal axis between the different target-specific populations.

Injections of differently conjugated forms of CTB into the fundus and either the corpus or pylorus or into the duodenum were performed to determine the percentage of dually-projecting gastroduodenal afferents in the DRG and nodose ganglia. Injection of CTB into the fundus and corpus of the stomach (injection distance 1 cm) resulted in 210 ± 23 and 111 ± 24 CTB-positive neurons in the DRG and nodose, respectively (Fig. 2). Of these CTB-positive neurons, double labeling was observed in 48.3 ± 3.3% and 22.6 ± 4.0% of DRG and nodose neurons, respectively. Similar to the results seen following singular CTB injections, the percentage of double-labeled neurons was higher in the DRG than in the nodose ganglia ($t = 5.6$; $P < 0.01$; Fig. 3). Whole mounts of the stomach revealed that the injected CTB had spread from the two injection sites to produce an approximately 20% overlap between the two CTB conjugates (data not shown). Injection of CTB into the fundus and pyloric antrum of the stomach (injection distance 2 cm) resulted in 294 ± 59 and 211 ± 32 CTB-positive neurons in the DRG and nodose, respectively. Within this population, double labeling was observed in 36.2 ± 10.5% and 19.7 ± 6.5% of DRG and nodose neurons, respectively (Fig. 3). No significant difference was observed between the percentage of dually labeled DRG and nodose neurons ($t = 1.2$; $P > 0.05$; Fig. 3). Whole mounts of the stomach revealed that the spread of the CTB injected at each site did not overlap (data not shown). Injection of CTB into the fundus of the stomach and the distal duodenum resulted in 128 ± 24 and 127 ± 34 neurons in the DRG and nodose ganglia, respectively. Within this population, double labeling was observed in 7.4 ± 4.6% and 13.9 ± 7.5% of DRG and nodose neurons, respectively (Fig. 3). Again, no significant difference was observed between the percentage of DRG and nodose neurons that were double labeled ($t = –0.05$; $P > 0.05$). No significant difference was observed between the percentage of double-labeled afferents that projected to fundus and corpus or to fundus and pylorus, regardless of the increased distance between the two injection sites (1 cm vs 2 cm; DRG: $t = 1.27$, $P > 0.05$; nodose ganglion: $t = –0.004$, $P > 0.05$). However, the percentage of double-labeled DRG neurons innervating both the fundus and duodenum was significantly lower than that within the gastric-specific

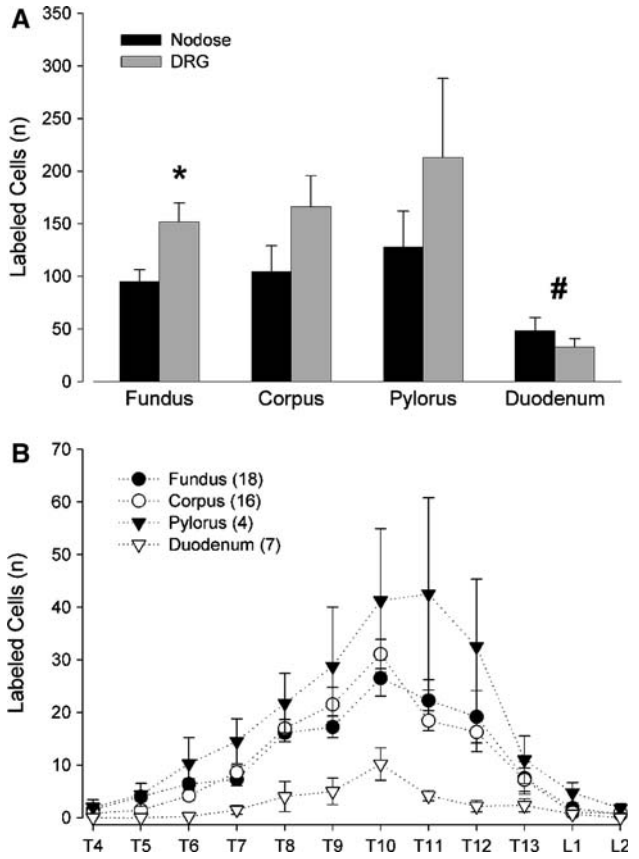

Fig. 1 Number and distribution of gastroduodenal sensory neurons. *The bar graph* (**A**) shows that more vagal (*black boxes*) and spinal (*gray boxes*) innervate the stomach than the duodenum ($^{\#}P < 0.05$ for duodenal compared to gastric neurons). Only in the fundus were differences between the number of vagal and spinal afferents significant ($^{*}P < 0.01$). The distribution of gastroduodenal afferents (**B**) shows labeled neurons from T4 to L2, with a peak around T10

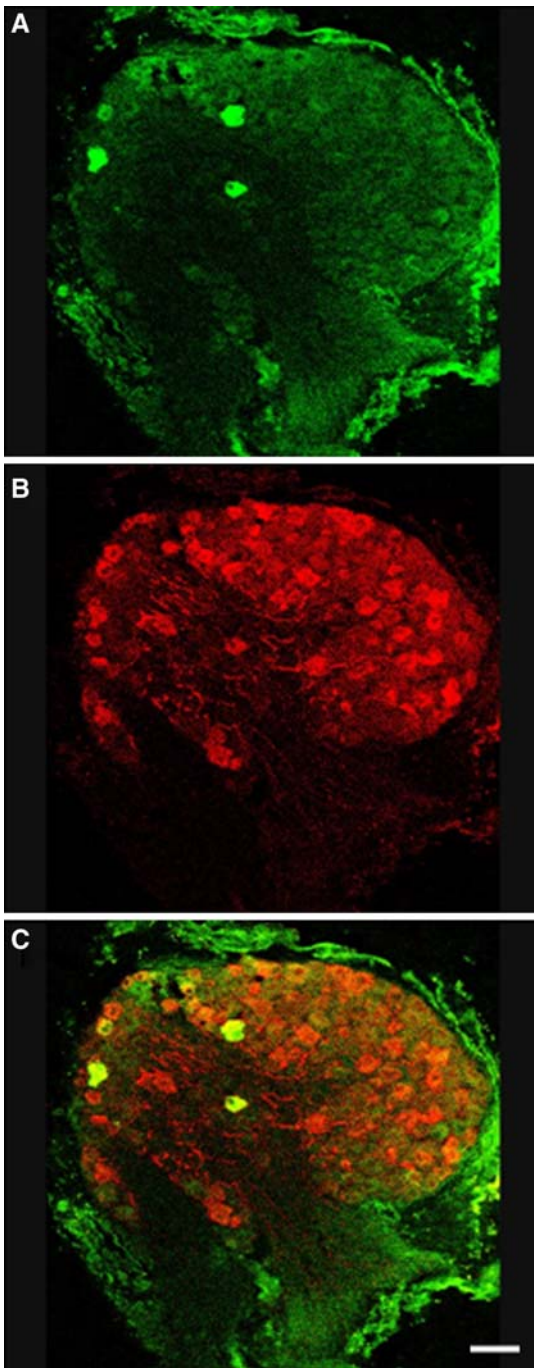

Fig. 2 Double labeling of nodose neurons innervating gastric fundus or corpus. Neurons projecting to the fundus (**A**) or corpus (**B**) were seen throughout the ganglion. *The merged image* (**C**) shows double-labeling in cells innervating the corpus (calibration bar 50 μm)

populations ($F = 9.0$, $P < 0.01$). The percentage was also similarly lower within the nodose ganglia, but the difference was not significant ($F = 1.5$, $P > 0.05$). The distribution of double-labeled cells along the rostro-caudal axis did not significantly differ between gastroduodenal DRG neurons ($P > 0.05$; Fig. 3).

Neurochemical phenotype of gastroduodenal sensory neurons

TRPV1

The heat- and proton-gated ion channel TRPV1 is often used as a surrogate marker for nociceptive neurons. TRPV1 immunoreactivity was present in 77 ± 2% and 59 ± 3% of the gastric and duodenal CTB-positive neurons, respectively, with no significant difference between the two populations (Fig. 4; $t = 2.68$; $P > 0.05$). The percentage of TRPV1-positive DRG neurons innervating skin or muscle was significantly lower, with 26 ± 4% and 40 ± 4%, respectively (skin vs stomach $t = 7.54$, $P < 0.01$; skin vs duodenum $t = 4.85$, $P < 0.01$; muscle vs stomach $t = 5.48$, $P < 0.01$; muscle vs duodenum $t = 2.79$; n.s.). Labeled somatic neurons with TRPV1 immunoreactivity were significantly smaller than TRPV1-negative cells (25.2 ± 1.0 μm vs 35.2 ± 1.0 μm, respectively; $P < 0.01$), whereas there was no size difference between TRPV1-positive and

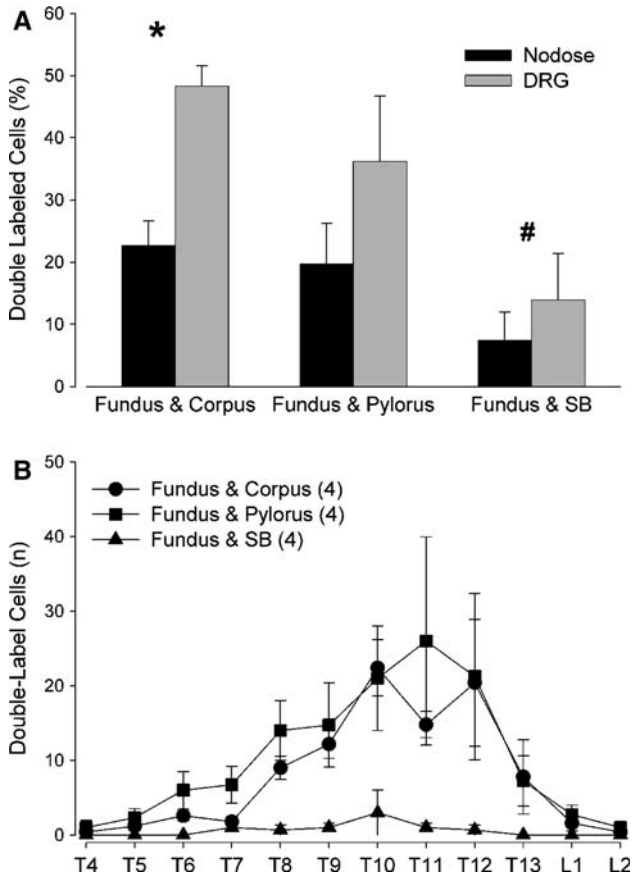

Fig. 3 Double-labeling of gastroduodenal afferent neurons. Two distinct retrograde labels were injected in fundus and corpus (distance 1 cm), fundus and pylorus (distance 2 cm) and fundus and duodenum (*SB*; distance >3 cm). The percentage of double-labeled neurons in nodose (*black bars*) and T-4—L1 DRG neurons (*gray bars*) is displayed in panel **A**. The distribution of double-labeled DRG neurons along the rostro-caudal axis is shown in panel **B**. $^{*}P < 0.05$ compared between nodose and DRG neurons. $^{\#}P < 0.05$ compared between gastric and duodenal double-labeling of DRG neurons

TRPV1-negative gastroduodenal neurons [35.3 ± 1.4 μm vs 36.2 ± 1.9 μm; not significant (n.s.)].

TRPV1-positive neurons were detected throughout the nodose ganglion. TRPV1 immunoreactivity was observed in 51 ± 9% of the gastric and 27 ± 4% of the duodenal CTB-positive neurons (Fig. 4; $t = 3.51$, $P < 0.01$ for gastric vs duodenal neurons). The diameter of gastroduodenal TRPV1-positive (31.0 μm ± 1.4) and negative (29.7 μm ± 1.2) neurons did not significantly differ ($t = 0.45$; $P > 0.05$).

N52

The presence of N52 immunoreactivity was seen in 21 ± 5% of the gastric and 39 ± 7% of the duodenal CTB-positive DRG neurons, respectively, with no significant difference between the two populations (Fig. 4; $t = –2.0$; $P > 0.05$). A similar percentage of skin (23 ± 4%) and muscle (23 ± 2%) afferent neurons labeled positive for N52. N52-positive neurons were significantly larger than cells not labeled by N52 (visceral neurons 42 ± 4 μm vs 35 ± 1 μm; $t = 2.0$, $P < 0.05$; somatic neurons 45 ± 1 μm vs 29 ± 1 μm; $t = 10.3$, $P < 0.01$).

Immunoreactivity for N52 was also seen throughout the nodose ganglion and in 23 ± 10% of the gastric and 19 ± 7% of the duodenal CTB-positive neurons, with no significant difference between the two populations (0.3, $P > 0.05$). Gastroduodenal neurons with (32 μm ± 2) and without (31 μm ± 1) N52 immunoreactivity did not differ in diameter ($t = 0.4$, $P > 0.05$).

A small number of TRPV1-/CTB-positive DRG neurons also exhibited N52 immunoreactivity (stomach 11 ± 3%; duodenum 13 ± 7.2%). While the percentage of similarly triple-labeled neurons was higher in the nodose ganglia (stomach 25 ± 11%; duodenum 31 ± 12%) than in DRG, the difference was not significant ($F = 1.2$; $P > 0.05$).

CGRP

Antibodies against the calcitonin gene-related peptide (CGRP) are frequently used to label peptidergic neurons, which play a role in peripheral vasodilation and nociception [24]. Within the DRG, most CGRP-positive cells were small or medium in diameter. CGRP immunoreactivity was present in 83 ± 6% and 82 ± 5% of the gastric and duodenal CTB-positive neurons, respectively (Fig. 4). The percentage of CGRP-positive DRG neurons innervating skin or muscle was significantly lower, with 18 ± 2% and 39 ± 3%, respectively (skin vs stomach $t = 10.7$, $P < 0.01$; skin vs duodenum $t = 11.7$, $P < 0.01$; muscle vs stomach $t = 7.7$, $P < 0.01$; muscle vs duodenum $t = 7.2$, $P < 0.01$). There was no size difference between CGRP-positive and -negative gastroduodenal neurons (29 ± 1 μm vs 31 ± 1 μm; $t = –1.7$, n.s.), while somatic CGRP-positive neurons were significantly smaller than CGRP-negative cells (28 ± 2 μm vs 32 ± 1 μm; $t = –2.3$, $P < 0.05$).

In the nodose ganglion very few neurons showed immunoreactivity for CGRP, and the majority of these cells were located in the rostral third of the ganglion. Significantly fewer gastric (10 ± 6%) and duodenal (12 ± 5%) neurons were CGRP positive compared to gastroduodenal DRG neurons ($t = 12.6$; $P < 0.05$). There was no significant difference in diameter between CGRP-positive (30 ± 1 μm) and negative (32 ± 1 μm) gastroduodenal neurons within the nodose ganglion ($t = –0.5$, $P > 0.05$).

IB4 binding

The plant lectin IB4 preferentially binds to small non-peptidergic neurons, which may contribute to nociceptive

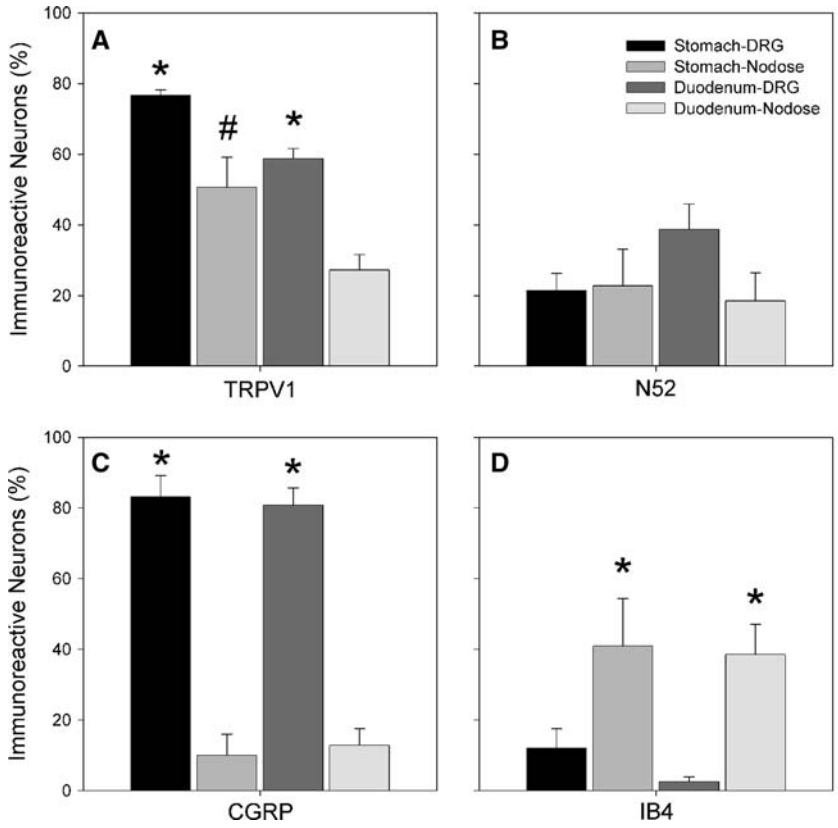

Fig. 4 Immunoreactivity of gastroduodenal sensory neurons. Vagal and spinal gastroduodenal sensory neurons differ, with higher percentages of TRPV1 (**A**) and CGRP (**C**) immunoreactivity in DRG neurons, while more nodose neurons than DRG neurons bind the plant lectin IB4 (**D**). The percentage of N52 positive neurons did not differ between vagal and spinal neurons innervating stomach or duodenum (**B**). $^{*}P < 0.01$ DRG compared to nodose neurons. $^{\#}P < 0.05$ gastric compared to duodenal nodose neurons

signaling. Within the DRG, IB4 labeling was present in 12 ± 5% and 3 ± 1% of the gastric and duodenal neurons, respectively, with no significant difference between the two populations (Fig. 4; $t = 0.3$; $P > 0.05$). A similarly small percentage of IB4-positive neurons was seen after the labeling of skin or intercostal muscle (7 ± 3% vs 6 ± 2%, respectively). IB4-positive somatic neurons were significantly smaller than cells not labeled by the plant lectin (24.6 ± 1.5 μm vs 36.3 ± 1.4 μm; $t = -4.4$, $P < 0.01$), while there was no size difference between IB4-positive and IB4-negative gastroduodenal neurons (29 ± 1 μm for both groups; $t = -0.2$, n.s.).

In contrast to DRG neurons, 41 ± 13% of gastric nodose and 39 ± 9% of duodenal nodose neurons bound IB4 (Fig. 4; $t = 4.5$, $P < 0.01$ compared to spinal gastroduodenal neurons). There was no significant difference in the diameter of IB4-positive (32 μm ± 2) and negative (32 μm ± 1) gastroduodenal nodose neurons ($t = -0.02$, $P > 0.05$).

Triple-labeling for IB4 and CGRP was seen in 10 ± 5% and 4 ± 3% of the CTB-positive DRG neurons projecting to stomach and duodenum, respectively. Similarly, 4 ± 1% and 7 ± 5% of gastric or duodenal nodose neurons were positive for both IB4 and CGRP ($F = 0.4$, $P > 0.05$).

Discussion

Behavioral and physiological studies have demonstrated significant differences between spinal and vagal afferents innervating the proximal gastrointestinal tract [6, 9, 25–27]. Using retrograde labels and immunohistochemistry, we characterized the afferent innervation of the mouse stomach and duodenum. Consistent with the physiological studies, our data show that gastroduodenal nodose and DRG neurons have distinct neurochemical properties. In addition, we were able to demonstrate that single sensory neurons may project to anatomically and physiologically distinct areas, such as the proximal stomach and distal duodenum, suggesting the presence of dichotomizing axons.

Projections of gastroduodenal sensory neurons

Injection of retrograde tracers into the gastric wall of mice labeled dorsal root ganglion neurons from T4 through L2 with a peak around T10 and T11, as has previously been reported for other species [9, 28–30]. We extended these studies by comparing the rostrocaudal distribution of sensory neurons labeled by tracer

injection into different areas of the stomach or the proximal small intestine. While the innervation density of the small bowel was significantly lower than that of the stomach, the highest number of neurons innervating stomach or duodenum was seen around T10 and T11. Within the nodose ganglion, labeled cells were seen throughout the entire ganglion, without an apparent organotypic representation, which is consistent with physiological data and results obtained in rats [31, 32]. Vagal fibers branch in the periphery, forming specialized endings that either intercalate with cells within myenteric ganglia (intraganglionic laminar endings) or form parallel arrays along smooth muscle cells (intramuscular arrays) [33]. Owing to extensive arborization, a single neuron may give rise to several of these distinct endings [22, 34]. These anatomical findings correspond with physiological characterizations of vagal sensory fibers, which demonstrated several distinct receptive fields for vagal afferents [14, 15, 32]. Using a different approach by retrogradely labeling sensory neurons from different sites, one can see double-labeling in neurons, suggesting the existence of peripheral projections to distinct areas within the same organ or even to adjacent viscera, such as colon and bladder [16, 17]. Consistent with these results, we noted double-labeling of nearly 40% of nodose and more than 70% of DRG neurons innervating the stomach after injecting differently conjugated fluorescent tracers into the fundus and corpus of the mouse stomach. While these data may be confounded by diffusion and overlap of the tracer within the small mouse stomach, similar findings were obtained when labels were injected into fundus and prepyloric antrum, where no peripheral overlap of the label was present. We saw a lower, but still significant, percentage of double-labeled cells projecting to fundus and duodenum. As vagal fibers extend from the lesser to the greater curvature [34], we injected the tracer close to the greater curvature to minimize contributions due to en passant labeling of afferent fibers. It is possible that such labeling of passing fibers is responsible for the higher percentage of double-labeled DRG neurons, because the course of spinal afferents within the murine stomach is not as well characterized. Nonetheless, our data suggest that a fraction of visceral afferents project to distinct areas within the gastrointestinal tract, which may contribute to the poor spatial discrimination of visceral afferent input in humans. Considering the small size of the mouse stomach, diffusion of injected tracer may certainly affect the results, as already mentioned above. Despite this theoretical problem, we chose to perform experiments in mice, as several recent investigations have demonstrated the powerful role of knockout or transgenic animals [35–38].

Neurochemical properties of gastroduodenal sensory neurons

Spinal and vagal sensory neurons innervating the proximal gastrointestinal tract have distinct neurochemical properties, with most spinal neurons being peptidergic and expressing the heat sensitive ion channel TRPV1, while only about 10% of gastroduodenal nodose neurons showed immunoreactivity for CGRP. The high percentage of TRPV1 and CGRP-positive neurons is in line with findings obtained by labeling esophageal, gastric, colonic, and bladder afferent neurons in different species [16, 29, 30, 39–41]. Consistent with previously published findings [42, 43], a significantly lower percentage of neurons labeled by skin and muscle injection of the retrograde tracer showed CGRP or TRPV1 immunoreactivity. Cutaneous nociceptive neurons are characterized by a high threshold to mechanical stimulation [18, 44]. Yet, studies in different species demonstrated that only about 20% of spinal fibers innervating esophagus, stomach, colon or the urinary bladder, are high threshold fibers [8, 9, 45]. Thus, considering the discrepancy between the known fraction of high-threshold mechanosensitive fibers and the predominant labeling of spinal visceral afferent neurons by either TRPV1 or CGRP antibodies, these neurochemical markers do not identify nociceptive neurons. This is further supported by the presence of TRPV1 immunoreactivity in about 40% of nodose neurons innervating the stomach, as no high threshold and thus presumably nociceptive mechanosensitive fibers have been identified in the vagal afferents innervating the stomach [6, 7, 32, 46]. As already indicated above, our findings are in line with results obtained by other investigators studying the innervation of different viscera in various species. Nonetheless, many investigators continue to use size, expression of TRPV1, trkA or NaV1.8 or functional correlates as surrogate markers to identify cells that contribute to nociception [17, 47, 48].

Only about 10% of nodose neurons projecting to stomach or duodenum were peptidergic. This confirms previous reports showing limited CGRP immunoreactivity in the nodose ganglion [49, 50] and in nodose neurons innervating esophagus or stomach [29, 30, 40, 51]. It is unclear whether these peptidergic neurons have a distinct role, as no study to date has combined functional and neurochemical characterization of vagal afferents.

The plant lectin IB4 preferentially labels small non-peptidergic neurons, which may contribute to nociception [20]. We identified relatively small fractions of visceral and musculo-cutaneous afferents with IB4 staining. This may, in part, be due to differences in methodology, as antibody-based assays appear to demonstrate more neurons

with IB4 binding [52, 53]. Our findings for spinal gastric neurons are consistent with results reported previously [16, 54]. The relatively small fraction of IB4-positive muscular and cutaneous afferents may be due to the larger size of these cells, as IB4 binding has been preferentially seen in small, non-peptidergic neurons [20, 53]. There is a substantial overlap between IB4 binding and presence of purinergic P2X receptors, a family of non-selective cation channels activated by ATP [55]. Consistent with the limited overlap between IB4 and peptidergic neurons, only few DRG neurons innervating stomach or duodenum were IB4-positive. Similar findings have been reported for the colon [16, 39, 54]. In contrast, about 40% of gastroduodenal nodose neurons bound the plant lectin. We did not directly address expression of purinergic receptors with immunohistochemistry. However, essentially all of the rat gastric nodose neurons responded to selective ligands of P2X receptors [56]. Therefore, it is unlikely that IB4 binding overlaps as closely with P2X receptor expression as reported for DRG neurons [55]. Additional studies will be needed to determine the properties of IB4-labeled visceral sensory neurons.

About 20% of spinal and vagal neurons projecting to the proximal gastrointestinal tract were N52 positive. Expression of the neurofilament heavy chain is typically used as a surrogate marker for the presence of myelinated axons [21]. Detailed anatomical studies of the abdominal vagus demonstrated that nearly all afferent fibers are unmyelinated [57]. Consistent with these morphological data, vagal afferents innervating the stomach conduct in the C fiber range [6, 9, 32]. By using intracellular recordings of nodose neurons activated by electrical stimulation of the thoracic nerve trunk, we only determined that 98% of the vagal fibers in mice were C fibers [32]. Interestingly, a small group of myelinated vagal afferents with distinct properties has been observed in the guinea pig esophagus [58]. These fibers are very sensitive to mechanical stimulation and are likely activated by luminal stimuli. It is unclear whether these mechanosensitive esophageal afferents correspond to the mucosal afferents identified by light mucosal stroking of the gastric mucosa [13].

Considering the finding of potentially dichotomizing axons, it would be appealing to determine whether these cells have distinct neurochemical properties. We did not attempt to address this question for several reasons. First, we have not been successful in performing more than triple-labeling, thus requiring a very high number of labor-intensive experiments. Second, at least spinal neurons appear to be less heterogeneous, with most cells falling into the category of peptidergic neurons which typically also express TRPV1. Thus, realistically feasible experiment will likely lack the statistical power to provide a conclusive answer.

In conclusion, our data clearly demonstrate distinct neurochemical characteristics of vagal and spinal afferents innervating the proximal gastrointestinal tract. Consistent with functional data, many visceral sensory neurons appear to send dichotomizing axons to distinct areas within the proximal gut. While some of these differences are consistent with functionally distinct subgroups that have previously been identified, the results raise questions about the utility of surrogate markers for neuron function in visceral sensory neurons. Combined physiologic and neurochemical studies will be needed to relate functional properties to such anatomical markers. These findings will form a basis for additional investigations to address changes in visceral innervation during aging or disease processes, such as inflammation.

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