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Vagal Intramuscular Arrays: The Specialized Mechanoreceptor Arbors That Innervate the Smooth Muscle Layers of the Stomach Examined in the Rat

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Abstract

The fundamental roles that the stomach plays in ingestion and digestion notwithstanding, little morphological information is available on vagal intramuscular arrays (IMAs), the afferents that innervate gastric smooth muscle. To characterize IMAs better, rats were given injections of dextran biotin in the nodose ganglia, and, after tracer transport, stomach whole mounts were collected. Specimens were processed for avidin–biotin permanent labeling, and subsets of the whole mounts were immunohistochemically processed for c-Kit or stained with cuprolinic blue. IMAs ($n = 184$) were digitized for morphometry and mapping. Throughout the gastric muscle wall, IMAs possessed common phenotypic features. Each IMA was generated by a parent neurite arborizing extensively, forming an array of multiple (mean = 212) branches averaging 193 μm in length. These branches paralleled, and coursed in apposition with, bundles of muscle fibers and interstitial cells of Cajal. Individual arrays averaged 4.3 mm in length and innervated volumes of muscle sheet, presumptive receptive fields, averaging 0.1 mm^3 . Evaluated by region and by muscle sheet, IMAs displayed architectural adaptations to the different loci. A subset (32%) of circular muscle IMAs issued specialized polymorphic collaterals to myenteric ganglia, and a subset (41%) of antral longitudinal muscle IMAs formed specialized net endings associated with the serosal boundary. IMAs were concentrated in regional patterns that correlated with the unique biomechanical adaptations of the stomach, specifically proximal stomach reservoir functions and antral emptying operations. Overall, the structural adaptations and distributions of the IMAs were consonant with the hypothesized stretch receptor roles of the afferents.

INDEXING TERMS

antrum; corpus; forestomach; nodose; vagus; visceral afferent; RRID:AB_354750;
RRID:nif-0000-10294

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA ACCESSIBILITY

Vector Laboratories catalog No. PK-6100 RRID:AB_2336819; R&D Systems catalog No. AF1356 RRID:AB_354750; Hsd:SD Sprague Dawley RRID:RGD_737903; Vector Laboratories catalog No. SK-4700 RRID:AB_2314425; NeuroLucida RRID:nif-0000-10294; GraphPad RRID:rid_000081.

The extrinsic innervation of the stomach has not been fully characterized. In spite of the critical roles that the stomach plays in controlling both ingestion and digestion, the vagal sensory projections to the gastric wall are among the elements that have been less thoroughly described. The lack of such basic information is paradoxical in view of the fact that major gastrointestinal (GI) disorders such as gastroparesis, gastroesophageal reflux disease, and obesity are currently treated with gastric interventions predicated only on incomplete descriptions of stomach innervation. To address, in part, the relative lack of information available for the extrinsic sensory projections to the stomach, specifically the dearth of details on the recently discovered vagal afferent endings called *intramuscular arrays* (IMAs), the present experiment inventories and characterizes more fully these afferents that innervate smooth muscle fibers in the gastric wall.

Traditionally, the vagus, the nerve supplying the bulk of the nonnociceptive extrinsic innervation of the stomach (Iggo, 1955; Paintal, 1973; Brierley et al., 2012), was thought to project a single type of afferent ending to the muscle wall of the stomach. That ending, the intraganglionic laminar ending (IGLE), was initially described by Lawrentjew (1929; see also Nonidez, 1946) and later named by Rodrigo and coworkers (1975). As the name implies, IGLEs are associated with myenteric ganglia. The sensory terminals arborize into flattened plates of contacts at the ganglionic surfaces where the myenteric plexus is situated between the longitudinal and circular muscle sheets, but not actually within either of the muscle sheets. During the 8 decades in which IGLEs have been recognized and examined, the endings have been described in multiple surveys (see, e.g., Rodrigo et al., 1982; Christensen et al., 1987; Neuhuber, 1987; Lindh et al., 1989; Berthoud and Powley, 1992; Kressel et al., 1994; Berthoud et al., 1997; Neuhuber et al., 1998, 2006; Fox et al., 2000; Phillips and Powley, 2000; Wang and Powley, 2000; Wang et al., 2012).

In contrast, a second type of vagal afferent ending, the IMA, was discovered more recently (Berthoud and Powley, 1992; Wang and Powley, 2000). As its name suggests, and in distinct contrast to the IGLE projections, this second class of vagal afferents in the stomach wall actually distributes within the muscle sheets. Since the initial description of IMAs, the structure and regional distribution of this second type of ending have been partially but far from completely characterized (Berthoud and Neuhuber, 2000; Phillips and Powley, 2000; Wang and Powley, 2000; Powley and Phillips, 2002).

The limited observations on the morphology and distributions of vagal IMAs suggest that IMAs are organized as stretch receptors (Phillips and Powley, 2000; Powley et al., 2008; Powley and Phillips, 2011), whereas the more extensively studied IGLEs have the architecture of tension receptors (Neuhuber, 1987; Neuhuber and Clerc, 1990; Zagorodnyuk et al., 2003; Neuhuber et al., 2006).

Because IMAs have been incompletely characterized and have structural features and distributions that appear consistent with stretch (or length) receptor functions, we recently evaluated morphometrically the IMAs in the gastric sphincters. Densely arrayed vagal IMAs constitute the primary afferent innervation to the sling and clasp muscle fibers forming the lower esophageal sphincter (Powley et al., 2012). Similarly, short, tightly packed, and extensively interdigitated IMAs that appear to be adapted to yield high spatial resolution

comprise a continuous sensory annulus within the circular muscle thickening that constitutes the pylorus (Powley et al., 2013). Thus, IMAs form the principal afferent innervation in both of the gastric sphincters, and, significantly, the architecture of these afferent arrays varies to adapt to the different local tissue environments. The prominence of these rings of afferents in the sphincters underscores the critical participation of IMAs in stomach function, and the fact that the arbors in the sphincters exhibit conspicuous specializations suggests that IMAs in the other regions of the gastric wall may also display structural adaptations.

In contrast to these recent analyses of IMAs in the sphincters, however, the IMAs throughout the stomach proper (antrum, corpus, and forestomach) have not yet been systematically evaluated. For the most part, descriptions of IMAs in the antrum, corpus, and forestomach have remained limited to qualitative and generic descriptions drawn from the initial characterizations of the afferents (Berthoud and Powley, 1992; Wang and Powley, 2000). Little has been done to evaluate potential regional specializations of the IMAs within the stomach, differences of the endings in the two muscle sheets, potential variations in architecture of the endings, or density of the afferents in different gastric regions. Thus the present experiment inventories and characterizes the IMAs found in the separate muscle sheets of the antrum, corpus, and forestomach in sufficient detail to address such issues.

MATERIALS AND METHODS

Animals

Two- to four-month-old male Sprague-Dawley rats (n = 66; RRID:RGD_737903; Harlan, Indianapolis, IN) weighing 278.6 ± 51.6 g at the time of tracer injection were housed individually in hanging wire cages in an Association for Assessment and Accreditation of Laboratory Animal Care-approved temperature (22–24 °C)-and humidity (40–60%)-controlled colony room. The room was maintained on a 12-hour light–dark schedule. Pelleted chow (diet No. 5001; PMI Feeds, Brentwood, MO) and filtered tap water were provided ad libitum, except for the night before tracer injection, when food but not water was removed. All husbandry practices conformed to the NIH *Guide for the Care and Use of Laboratory Animals* (8th edition) and were reviewed and approved by the Purdue University Animal Care and Use Committee. All efforts were made to minimize any suffering as well as the number of animals used.

Neural tracer injections

Overnight-fasted animals were anesthetized with isoflurane (Fluriso; MWI, Boise, ID). After anesthesia, glycopyrrolate ($0.2 \text{ mg} \cdot \text{ml}^{-1}$, s.c.; American Regent, Shirley, NY) was injected to minimize secretions.

For dextran injections, each anesthetized animal was placed in a supine position, and a midline incision of the skin of the ventral neck was made. Both nodose ganglia were then exposed by blunt dissection of the overlying muscles. Each animal received bilateral injections of the dextran tracer (1 μl per ganglion) of lysine-fixable dextran biotin solution consisting of a 1:1 mixture of 3K and 10K MW dextrans in ultrapure water [final concentration 15% dextran biotin consisting of 7.5% D7135 (3K) and 7.5% D1956 (10K);

Invitrogen, Carlsbad, CA]. Injections were performed with a 35-gauge NANOFIL needle with syringe (NF35BV; World Precision Instruments, Sarasota, FL). To control the injection placement and to check the distribution of the tracer within the ganglia under direct visual control, Fast Green FCF (Sigma-Aldrich, St. Louis, MO) was added to the dextran tracer (0.01 mg per 100 μ l solution).

After both ganglia had been injected, the skin incision was closed with interrupted sutures. The animal was transferred first to a circulating-water heating pad until its righting reflexes had returned and then to its home cage. Buprenorphine (Buprenex; Reckitt Benckiser Pharmaceuticals, Richmond, VA) was given s.c. prior to suturing and again the following morning for analgesia.

Tissue fixation and GI dissection

After a survival period of 14 days for optimal tracer transport, each rat was given a lethal dose (180 mg/kg⁻¹, i.p.) of sodium pentobarbital. The animals had food available ad libitum until they were anesthetized, to facilitate the stomach being full and relaxed in accommodation. When each animal was completely unresponsive to nociceptive pinching and prodding, the GI tract was exposed with a midline abdominal incision. To ensure that the stomach was normally distended at the time of fixation, the organ was inspected for normal distension or accommodation, and, as required, physiological saline that had been warmed to body temperature was slowly infused into the stomach by gavage catheter. With the stomach normally dilated, the animal was first transcardially perfused through the vasculature with physiological saline and then with 4% paraformaldehyde in 0.1 mol liter⁻¹ phosphate-buffered saline (PBS; pH 7.4).

After the perfusion, the distal esophagus and the proximal duodenum were transected, and the stomach was freed and removed. The organ was then opened with a longitudinal cut along the greater curvature, and the material in the stomach was gently rinsed away with tap water. To ensure that the entire stomach was preserved and sampled, the specimen was then trimmed to include the distal lower esophageal sphincter and the proximal pylorus. Next, the ventral and dorsal stomach walls were separated by an incision along the lesser curvature, thus yielding two whole mounts per animal. The external muscle wall of the stomach was then isolated as a whole mount by removing the gastric mucosa and submucosa with forceps.

Staining

Whole mounts were processed free floating for all tracer processing, immunohistochemistry, and neuronal counterstaining. Processing for the dextran label consisted of treating the tissue with a hydrogen peroxide–methanol block (1:4) to quench endogenous peroxidase activity, followed by 3–5 days in PBS containing 0.5% Triton X-100 and 0.08% Na azide to facilitate penetration of all reagents through the muscle sheets. Whole mounts were then incubated for 60 minutes in avidin–biotin–horseradish complex (PK-6100; Vectastain Elite ABC kit, standard; RRID:AB_2336819; Vector Laboratories, Burlingame, CA). After the avidin–biotin complex was established, the specimens were rinsed in PBS and then reacted with

diaminobenzidine and H_2O_2 for 5 minutes to yield a permanent gold-brown stain of all labeled afferent neurites.

One series of whole-mount specimens with dextran labeling ($n = 42$) was coverslipped without further staining to maximize tissue transparency and differential interference contrast (DIC) imaging. Another group of specimens with labeled vagal afferents ($n = 13$), however, was counterstained with the panneuronal chromogen cuproinic blue (170521; Polysciences, Inc., Warrington, PA) according to an established protocol (Phillips et al., 2004; Walter et al., 2009) for labeling enteric neurons. Another subset of whole mounts processed for dextran-labeled vagal afferents ($n = 15$) was double labeled for c-Kit (1:1,000; AF-1356; RRI-D:AB_354750; R&D Systems, Minneapolis, MN) for examining the interstitial cells of Cajal (Powley et al., 2012; see Table 1). The c-Kit was processed with the steel gray chromogen (SK-4700; Vector SG peroxidase; RRID:AB_2314425; Vector Laboratories) as the substrate, according to our previously published protocol (Powley et al., 2012).

After the labeling protocols, all whole mounts were rinsed and mounted with circular muscle side up on gelatin-coated slides. Specimens were air dried, dehydrated with alcohols, cleared with xylenes, and cover-slipped with DPX (317616; Sigma-Aldrich) or Cytoseal XYL (8312-4; Richard-Allen Scientific, Kalamazoo, MI).

Antibody characterization

Table 1 summarizes information about the primary antibody used in this study to identify gastric intramuscular interstitial cells of Cajal within the circular and longitudinal muscle layers. The specificity of the polyclonal goat CD177/c-Kit antibody (RRID:AB_354750; R&D Systems) was confirmed by direct ELISAs and Western blots; approximately 15% cross-reactivity with recombinant human CD117 is observed (manufacturer's information). Additionally, in the present study, cells that had been labeled with the CD117/c-Kit antibody ran parallel to the smooth muscle cells and were spindle shaped with a prominent nuclear region, consistent with the morphology and distribution of gastric intramuscular interstitial cells of Cajal described in previous reports (Fox et al., 2000, 2001; Song et al., 2005; Powley et al., 2008; Kito et al., 2009; Powley and Phillips, 2011).

Afferent terminal inventories, image analysis, morphometry, and photography

All whole mounts were searched systematically in progressively more stringent steps for labeled IMAs. In a first scan at $\times 100$ and $\times 200$, all apparently isolated and well-labeled afferent arbors in the smooth muscle sheets were identified with brightfield and DIC optics on a Leica (Wetzlar, Germany) DMRE or DM5500 microscope equipped with long-working-distance objectives. All arbors were inventoried according to location and (provisionally) as to type (e.g., IMA, IGLE). Practically, when the vagal afferents in the longitudinal and circular muscle sheets were evaluated, IMAs were the only type of vagal afferent terminal found running with or contacting muscle fibers.

In a second survey, the well-labeled afferent terminals were then re-evaluated at higher magnification ($\times 400$ – 630) for the completeness of all terminal endings (arbor continuity; e.g., all branches with normal terminations) and the adequacy of the specimen for image

analysis (i.e., lack of tissue folds, absence of tears, lack of artifacts obscuring neurite). In this second survey with the higher magnifications, we also weighed an additional factor: although the parameters of our nodose injection protocol had been chosen specifically to limit the number of labeled afferents per animal and thereby to avoid the frequent labeling of immediately neighboring terminals that could potentially confuse an effort to distinguish and digitize all elements of an individual arbor, we did encounter cases in which neighboring arbors were labeled and intertwined, making morphometry problematic. Such neighboring arbors were dropped from the population of terminals used for full morphometric reconstructions.

In a final inventory step, an attempt was made to digitize, evaluate, and analyze formally all muscle sheet afferents that had satisfied the criteria of the first two surveys. In this morphometric step, we examined all IMAs that had been screened and identified and that we were able to follow throughout their full arbors. For morphometry, Neurolucida software (RRID:nif-0000-10294; Microbrightfield, Williston, VT) controlling the motorized stage of a Zeiss (Oberkochen, Germany) Axio Imager Z2 microscope equipped with DIC optics and long-working-distance ($\times 40$ and $\times 63$ water) objectives was used to trace parent axons as they entered a smooth muscle sheet and then formed a terminal arbor. All branches of an arbor were digitized in three dimensions as the parent neurite repeatedly bifurcated, arborized, and finally terminated. At this morphometry step, however, another percentage of the provisionally designated terminals had to be dropped because comprehensive Neurolucida digitization encountered a flaw, artifact, discontinuity, or intermingled branch of a neighboring arbor not seen in the earlier scanning that made identification of the target arbor problematic. Where appropriate for more qualitative observations, the arbors dropped from the morphometry analyses were retained and used (e.g., for the distribution of IMAs illustrated in Fig. 3).

Single-field, single-plane-of-focus photomicrographs were acquired digitally with a Spot Flex camera controlled in the Spot software Advanced Plus V4.7 (Diagnostic Instruments, Sterling Heights, MI; www.spotimaging.com). For all-in-focus images merged in the z-dimension, Helicon Focus Pro X64 software V5.3.7 (HeliconSoft, Kharkov, Ukraine; www.heliconsoft.com) focus stacking was performed. For neurites and arbors that had more extensive distributions in the x- and/or y-plane (as well as the z-plane), multiple-field composites or mosaics of high-magnification views were collected and merged in Surveyor with Turboscan software V6.0.5.3 (Objective Imaging, Cambridge, United Kingdom; www.objectiveimaging.com) running on a Leica DM5500 workstation.

Two different protocols were employed to generate two-dimensional (2D) projections of the afferent arbors. The Neurolucida software, in addition to capturing all morphometric information on arbors, was also used to collapse the tracings across one dimension (typically the z-axis of the IMAs because the arrays were flattened near-planar structures parallel to and within a muscle sheet) and to project the arbor in the other two dimensions (x and y planes). Alternatively, because Neurolucida reduced the tracings to stick-figure renderings that ignored the finer structural detail (e.g., lamellar plates), extensive “virtual tissue sections” or photomicrographic mosaics of entire arbors in three dimensions collected at high power ($\times 400$ – 630) were prepared with the Surveyor software and exported to

Photoshop CS5 (Adobe Systems, San Jose, CA; www.adobe.com) and then used as a volume of tissue containing an arbor that could be traced realistically in Photoshop. These Photoshop tracings were translated from three-dimensional (3D) representations to 2D projections for graphic representation. For final figure production of photomicrographs, Photoshop CS5 was used to adjust brightness, contrast, and sharpness; to organize layouts; and to apply text and scale bars.

Statistical analysis

Formal statistical comparisons, commonly two-way ANOVAs (region $\times$ muscle sheet) with Tukey's post hoc tests but where more appropriate unpaired *t*-tests and nonparametric Kruskal-Wallis ANOVAs by ranks, were performed in Statistica (StatSoft, Tulsa, OK; www.statsoft.com). Grapher 10 (Golden Software, LLC, Golden, CO; www.goldensoftware.com) was used to generate polar plots. Bar graphs were created in Prism 6 (Graph-Pad Software, La Jolla, CA; RRID:rid_000081).

RESULTS

Throughout the stomach wall, IMAs were defined by a set of phenotypic morphological features. These basic structural features were then, in some cases, modified and adapted to the local tissue environments in the different gastric regions. Figure 1 illustrates a circular muscle array exhibiting the common morphological hallmarks of the IMAs. Similarly, Figure 2 displays a longitudinal muscle arbor exhibiting the phenotypic features of IMAs.

In the gastric whole-mount series, 184 IMA afferents satisfied all criteria for complete evaluation. This sample included substantial numbers of IMAs innervating the antrum ($n = 64$), corpus ($n = 43$), and forestomach ($n = 77$). Similarly, the pool of IMA arbors contained balanced subsets of arrays from the circular ($n = 84$) and longitudinal ($n = 100$) muscle sheets. Each of the afferents in the sample was examined structurally; each was also digitized, reconstructed, and assessed morphometrically. Figure 3 summarizes the locations (i.e., symbols at loci of first bifurcations) of the individual arbors in the sample.

General IMA architecture

Parent neurites of vagal IMAs fanned out from the area of the cardia, over the stomach wall in the gastric fascicles issued by the vagal trunks. As the neurites traveled centrifugally, they traversed the thin longitudinal muscle sheet to reach and then continue traveling within the myenteric plexus. In their initial trajectories through the longitudinal muscle and myenteric plexus, parent fibers that eventually formed IMA arbors typically displayed few to no small spurs or collateral branches.

Relatively close to the site where an IMA afferent formed its terminal arbor, the parent fiber would change its trajectory and leave the myenteric plexus either to re-enter the more superficial longitudinal muscle sheet or to course into the deeper circular muscle layer. After coursing from the plexus into its muscle sheet, the individual IMA afferent would then soon arborize and bifurcate repeatedly in a pattern that generated an elongated, narrow, and near-planar array (parallel with muscle sheet) of higher order branches that coursed in

conjunction with smooth muscle fibers and the interstitial cell network (ICC-IM) of the muscle sheet.

Such parent neurites formed their IMA arbors by initially (i.e., at the first, or occasionally a later, branch point) bifurcating, with one set of ramifying branches running in one direction parallel to the smooth muscle fibers and a second set of branches traveling also parallel to the smooth muscle fibers, but in the opposite direction. Within an arbor, those branches (principal branches) paralleling the muscle fibers were relatively long, whereas orthogonal branches (bridging branches) interconnecting the principal branch segments that ran in parallel with the muscle bundles were extremely short, just long enough to span from one bundle of muscle fibers to an adjacent fascicle of muscle fibers. These details of the IMA branching patterns were effectively illustrated by polar plots of the cumulative lengths of neurites running in different directions from the initial bifurcation of the afferent (see Fig. 4).

The basic IMA organization conformed to two particular local tissue features. First, the rectilinear and parallel principal branches of IMA neurites coursed on, or in conjunction with, the networks of interstitial cells of Cajal of the intramuscular type (ICC-IMs) intercalated among smooth muscle fibers. More specifically, IMA branches alternated between coursing in tight registration with ICC-IMs, seeming to form appositions with, and sometimes terminating on, the ICC-IM network, and, alternatively, coursing directly on the bundles of smooth muscle fibers for some length. As Figure 5 illustrates, the afferent neurites formed appositions or apparent contacts on ICC-IMs as the two types of cells coursed together. Although these complexes of IMA neurites with ICC-IMs have been reported previously (see, e.g., Berthoud and Powley, 1992; Phillips and Powley, 2000; Powley et al., 2008), it is noteworthy that in the present survey, in which a considerable sample of whole mounts ($n = 15$) was c-Kit immunostained for ICC-IMs, IMAs were invariably found to course in conjunction with the ICC-IM network. In this inventory, which systematically included all gastric regions and both muscle layers, no IMAs were observed remaining completely independent of the ICC-IM network, although some polytopic collaterals and specialized branches of an IMA did course independently of the ICC-IM network.

The second common tissue feature that appeared to mold the IMA arbor structure was the organization of the fascicles or bundles of smooth muscle fibers themselves. When DIC optics were used to identify unstained muscle fibers, it was clear that the individual IMA principal branches running with the long axis of the overall arbor actually were lying on, and potentially establishing some type of apposition with, smooth muscle bundles as well as with ICC-IM networks (see Figs. 6 and 7C). The tightly aligned and parallel principal branches of the arbors of the IMAs that give them their characteristic form reflect this vagal afferent–smooth muscle juxtaposition insofar as an individual principal neurite would course on one bundle of smooth muscle, and that neurite's closest neurite neighbor would course on a second bundle of smooth muscle fibers adjacent to the first bundle. Overall, the neurite elements forming the array of an IMA typically corresponded to a similar number of neighboring smooth muscle fiber bundles.

Although single-array arbors were the most common form of the IMA afferent terminal, multiple-array arbors also occurred. As some parent neurites began to arborize, the parent would form a conspicuous fork or major branch bifurcation that would then effectively split the arbor and produce a pair (or rarely a triplet) of close, parallel IMA arrays separated by one or more bundles of smooth muscle fibers. Figure 7 illustrates a typical IMA with tandem arrays. With the exception that these forked afferents tended to span wider terminal fields and thus, presumably, wider receptive fields, the tandem-array IMAs were otherwise unremarkable and conformed to the basic core IMA architecture.

Morphometry of basic IMA architecture

The morphometric assessments of the sample of IMAs reinforced the observations that a core IMA architecture was found throughout the stomach wall. Morphometry also indicated that the basic IMA architectural pattern was incrementally and quantitatively, but still systematically and significantly, adapted to each of the three gastric regions and the two different muscle sheets.

To produce their arbors, IMA afferents bifurcated an average of 105 ± 5.22 times, producing an average of 212 ± 10.4 total branches per arbor. For both the measures of bifurcations and the branch number, there were significant differences between the IMAs in the two muscle sheets, with the longitudinal muscle vs. the circular muscle IMAs having significantly fewer bifurcations (93 ± 4.9 vs. 120 ± 9.6 , respectively; $P = 0.004$) and branches (187 ± 9.8 vs. 241 ± 19.3 , respectively; $P = 0.004$) than the arbors in the circular muscle sheet. The pattern resulted from significantly fewer numbers of bifurcations and branches in the longitudinal muscle sheet of both forestomach and corpus ($P < 0.001$) but not antrum ($P > 0.5$).

The principal branch segments that constituted the arbors (bridging elements excluded from calculation) averaged roughly $193 \mu\text{m}$ in length, with a significant ($P < 0.0002$) difference in averages between the muscle sheets. The longitudinal sheet was innervated by longer average principal branches than the circular muscle sheet ($242 \pm 10.9 \mu\text{m}$ vs. $152 \pm 10.7 \mu\text{m}$, respectively; $P < 0.0001$). The pattern of longitudinal muscle IMAs having longer principal branches held for both forestomach and corpus ($P < 0.05$). Antral longitudinal IMAs, as described below, were specialized in a manner that precluded a simple comparison of the IMA subgroups in the two muscle sheets. Nonetheless, the principal branches of the IMAs in the circular muscle of the antrum were similar ($156 \pm 8.3 \mu\text{m}$) to the overall circular muscle IMA estimate ($152 \pm 10.7 \mu\text{m}$).

Individual IMA arbors or branched arrays spanned or averaged $\sim 4.3 \text{ mm}$ in length (i.e., parallel to the vector of their muscle fibers). Within each of the three major gastric regions, these arbors tended to be relatively similar in length in the longitudinal and circular sheets. Arbor lengths were also relatively similar for the forest-omach and the corpus.

Because the degree of muscle contraction or relaxation at the time of tissue fixation could affect the length of the arrays, the dimensions of the stomach whole mounts were also used as relative gauges of the lengths of the arrays. Longitudinal IMA arbors in the stomach spanned an average of roughly 20% of the longitudinal dimension of the stomach wall (21%

for the forestomach, 19% for the corpus). Circular IMAs spanned roughly 14% of the circular circumference of the organ (15% for the forestomach, 12% for the corpus).

A calculation of the “receptive fields” of the individual afferents was used to condense the several morphometric parameters into a composite estimate relevant to function. For these putative receptive fields, the volume of muscle sheet innervated by each of the IMA arbors was calculated. These volumes were computed in two steps. In the first step, a 2D (length $\times$ width) area of the planar arbor was extracted with NeuroLucida’s convexhull algorithm. In the second step, a depth or z-measurement (serosa–mucosa axis) of each arbor was determined by scanning through all levels of the IMA and identifying the highest and lowest optical planes occupied; this measure of depth had to be determined “manually” because many of the whole mounts had minor wrinkles or folds somewhere within the field of the arbor that substantially distorted the 3D convex-hull calculation automated within NeuroLucida. These volume or “receptive field” values are summarized in Figure 8A, which illustrates that there was a conspicuous progression ($P < 0.002$) in the size of the receptive fields across the regions (forestomach $>$ corpus $>$ antrum) with only nonsignificant differences between the two muscle sheets within each of the gastric regions.

The total or composite length of all the branches of the individual parent neurite that ramified or “folded” into a receptive field (i.e., the cumulative length of all branches of an individual arbor) added additional perspective with respect to how the IMA arbors were organized and may function. Whereas the convex-hull or volume-innervated calculations described the space that an arbor spanned or innervated, those spatial estimates of the span of the receptive field did not reflect the neurite packing density within the field. As summarized in Figure 8B, the average cumulative length of all neurite segments within an IMA arbor was 26 ± 1.1 mm, and the cumulative lengths within the forestomach (30 ± 1.9 mm) and corpus (29 ± 2.6 mm) were quite similar, even though the volumes innervated were considerably smaller in the corpus (cf. Fig. 8A). Such differences in receptive field volumes were achieved by the IMA arbors varying the packing density, that is the numbers and the lengths of individual branch segments, within the arrays. In contrast, IMAs in the antrum innervated smaller volumes (cf. Fig. 8A) and had somewhat shorter overall or cumulative lengths of neurites within those volumes (cf. Fig. 8B). Within each of the three gastric regions, the IMAs innervating the two muscle sheets occupied relatively similar volumes (although there was a significant difference in the antrum) and were formed from neurites of comparable total or cumulative lengths that branched within the innervation volume.

Regional concentrations and specializations of the IMA architecture

Although the gastric IMAs shared a common phenotypic architecture and this structure varied incrementally to adapt it to the two muscle sheets and the three gastric regions, as just summarized, a minority of IMAs also exhibited specializations that were more categorical. Two of the modifications entailed simply adjustments in the distribution of some branches, or arbors in their entirety, of the IMA arrays. Two other modifications, however, were more conspicuous and categorical, involving one or more branches of an IMA distributing polytopically, to unconventional targets, and polymorphically, with unconventional terminals.

IMAs with orthogonal tandem arrays—As discussed above, a subset of IMA arbors bifurcated and formed two neighboring and parallel arrays separated by one or more muscle bundles in a single smooth muscle sheet. In a variant of the tandem array pattern, however, a smaller subset of IMAs actually branched and distributed one of the arrays, or part of the divided arbor, not to innervate the same muscle sheet as its “twin” array or other part of the arbor, but rather to innervate the orthogonal muscle sheet.

In these orthogonal cases, a parent neurite left the myenteric plexus and coursed into one of the muscle layers where it issued one or more “primary” arrays innervating the smooth muscle bundles of the sheet. Creating the orthogonal specialization, however, at or near the initial branch point of the primary arbor(s), a distinct collateral emerged and coursed perpendicular to the first muscle sheet to enter the second sheet and establish a “secondary” array associated with the smooth muscle fibers of that second sheet. The effect was that the single parent neurite created “crossed” or orthogonal arbors in the two muscle sheets. Figure 9 illustrates an IMA with such an orthogonal specialization.

In all, 10 of the 184 IMAs evaluated morphometrically had orthogonal arbors. Parent neurites with orthogonal arbors were observed in both muscle sheets; three of the parent neurites and associated primary arrays were situated in circular muscle, whereas seven of the parent fibers and primary arrays were observed in the longitudinal muscle sheet.

Clustering of IMAs by regions of the stomach—Figure 3 illustrates the distribution of the sample of IMAs evaluated with NeuroLucida morphometry. This figure incorporates the 184 IMAs that were successfully digitized, and it also includes as a second subpopulation sample, those well-labeled candidate IMAs that ultimately were not used for morphometry because of tissue artifacts or intertwined neurites that might have potentially produced some ambiguity with respect to identifying every branch of the array. The manner in which the inventory was generated and the sizeable sample that was quantified provided a quasirandom survey of the gastric IMA distribution.

Overall, IMAs were widely distributed through the stomach wall, although two aspects of the distribution revealed that the IMAs were not distributed uniformly. First, the distal corpus was largely devoid of IMA arbors, whereas the proximal stomach (i.e., forestomach plus proximal corpus) and the distal antrum were densely innervated by IMAs. Second, within the proximal stomach, the concentrations of IMAs within the longitudinal and circular muscle sheets were partially out of registration. Longitudinal IMAs in the proximal stomach were heavily distributed near and along the greater curvature. In contrast, circular muscle sheet IMAs in the proximal stomach were more numerous in a “pocket” of the gastric wall midway between the lesser and greater curvatures.

Circular muscle IMAs with collaterals to myenteric ganglia

One-third (27 of 84) of the IMAs innervating the sheet of circular muscle issued one or more (mean = 5) polymorphic collaterals that separated from the main array of the IMA, which articulated with the ICC-IM network and muscle fibers, and coursed back into the myenteric plexus to articulate with the enteric neurons in one or more myenteric ganglion.

These polymorphic collateral branches typically emerged from the parent neurite near the IMA origin or from lower order branch points (mean = 8.5) within their corresponding IMA arbors. As illustrated in Figures 10 and 11, these polymorphic branches issued by some circular muscle IMAs consisted of neurites that intermingled with the myenteric ganglion neurons. In contrast to the primary intramuscular branches of the arrays that coursed with and made appositions on the ICC-IM networks (cf. Fig. 5), these polytopic/polymorphic collaterals of the arrays had no particular association with ICC-IMs. Similarly, the collaterals that extended into the myenteric ganglia had no obvious affinity for ICCs of the myenteric type (i.e. "ICC-MYs;" see Fig. 12).

In contrast to IGLEs, which also innervate the myenteric ganglia (see Fig. 13A), the polymorphic collaterals originating from the circular muscle IMAs distributed within the myenteric ganglia, not at the surfaces of the ganglia. The polymorphic collaterals from the circular muscle IMAs formed a striking heterogeneity of contacts, terminals, or plates as the fibers coursed around the ganglion cells of the plexus. Some of the presumptive contacts on the polymorphic collaterals were flattened, somewhat irregular, lamellar plates most reminiscent of the lamelliform leaflets seen on the branches of IMAs within the arbors in smooth muscle (cf. Figs. 11B2, 12, and 13). These lamellar plates were often formed on small hooks or spurs issued by the collateral; in other cases the plates occurred at the terminal tips of the collaterals. In contrast, other contacts on the polymorphic collaterals issued by the circular muscle IMAs formed contacts within the myenteric ganglia that were more spherical and regular in shape and that were somewhat more varicose and bead-like in appearance. Overall, the polymorphic branches of the circular muscle IMAs ended within the ganglia in a distinctive, perhaps unique, pattern of relatively simple neurites with limited varicosities winding among the myenteric neurons (cf. Figs. 11A1 and 11B1; also Fig. 13). Thus, the distinctive IMA polymorphic collateral pattern did not duplicate the phenotypes of either IGLEs (compare Fig. 13 with inset A of the same figure) or vagal preganglionic motor terminals (compare Fig. 13 with inset B of the same figure).

Such branch polymorphisms were observed in the circular muscle sheet of all three gastric regions, accounting for 20% of antral, 41% of corpus, and 41% of forestomach IMAs. In contrast, such a polymorphic adaptation was observed only once (1 of 100) in the case of the IMAs in the longitudinal muscle sheet.

Although polymorphic collateral branches coursing from IMA arbors to myenteric ganglia were routinely observed in a portion of the afferents projected to the circular muscle, neither IMA nor IGLE afferent collaterals were observed coursing to the dissected junction of the smooth muscle with the submucosa (and thus presumably continuing into the submucosa/mucosa). Because, however, the submucosa/mucosa was not preserved in the present whole-mount series and because the specimens were not prepared optimally for a thorough inventory of collaterals extending into the nonmuscular tissues of the gastric wall, it was impossible to reject categorically the possibility that some afferents to the smooth muscle layers also issued collaterals to the deeper tissues.

Antrum: regional specializations

Most of the subsample of IMAs innervating the antrum evidenced the general characteristic features of IMAs, but the antral IMAs did display some unique features that presumably adapted them to the biomechanical specializations of the muscle wall in the antrum. Most conspicuously, antral IMAs in the longitudinal muscle sheet often had exceptionally lamelliform neurites. Whereas gastric IMA arbors were commonly composed of neurite branches that appeared relatively rounded, sometimes with inconspicuous flattenings that apparently corresponded to the sites of apposition with ICC-IMs and/or smooth muscle (compare Fig. 1C with Fig. 5A,C,D), in contrast, antral IMA arbors were often composed of lamella-like flattened segments of the neurites (cf. Fig. 14B, see also Fig. 5B). Indeed, the specializations of antral IMAs appeared to occur in a continuum, becoming progressively more lamellar and grading into the highly localized “web endings” previously described (Powley et al., 2012), because the arbors were found progressively closer to the antral lesser curvature region where sling muscle fibers appear to attach.

DISCUSSION

The present experiment expands the earlier descriptions of vagal IMAs (see, e.g., Berthoud and Powley, 1992; Wang and Powley, 2000) by characterizing more completely these mechanoreceptor afferents that innervate the stomach smooth muscle wall. The survey adds details about the structure, as well as morphometry, of IMAs. The inventory also provides information about the distribution of IMAs in each of the different gastric regions as well as in the two muscle sheets. In addition, the present article supplies a description of the specializations that adapt the basic architecture of the afferent to the different gastric regions.

These newly observed details of IMA structure and its specializations allow comparisons with recent advances in characterizing and modeling the complex biomechanics of the stomach and in investigating the electrophysiology of the vagal mechanoreceptors. These comparisons provide a means of evaluating the hypothesis that IMAs are stretch (or length) receptors (see, e.g., Phillips and Powley, 2000; Powley and Phillips, 2011). More generally, the additional morphological information establishes a foundation to inform the design of future experiments to assess the physiology of the afferents. Before discussing some of the functional implications of the present observations, however, the phenotypic architecture and specializations that the present experiment elucidates should first be briefly discussed.

Fine architecture of IMAs and their specializations

Both the extent and the frequency of the heterogeneity of vagal IMAs has been difficult to evaluate, given that past observations (see, e.g., Wang and Powley, 2000; Berthoud and Powley, 1992) used different (and typically lower resolution) tracers, focused on limited and different regions of the stomach, and collected relatively small samples of endings to generate descriptions of the afferents. In contrast, the present experiment employed a tracer protocol that provided high-definition labeling and collected morphometric descriptions on a relatively large sample of endings from all three major gastric regions and both muscle sheets to clarify the elements of IMA architecture and evaluate its specializations.

The present survey establishes that all IMAs in the stomach wall share common phenotypic features. Notably, all IMAs terminate in one or the other smooth muscle sheet, and IMAs are the only vagal afferents that terminate within the muscle sheets. Structurally, individual IMAs consist of arrays of long (average length of principal branches = 193 μm) parallel neurite branches (average number > 100 principal branches/arbor) interconnected by short bridging branches. The neurite branches within an array have varicosities, although the varicosities vary widely in number and morphology. Some branches can be relatively smooth simple neurites (see Figs. 2–9), some contain dense spherical en passage varicosities (see Figs. 1C, 5A,D), and others express puncta occurring as flattened, highly lamelliform leaves, often on short spurs or spines (see Figs. 1D, 5B, 14). The IMA branches within an array run in tight conjunction both with bundles of smooth muscle fibers (Fig. 6) and with the network of interstitial cells of Cajal intercalated among the smooth muscle fibers (see Powley et al., 2008; Powley and Phillips, 2011; Fig. 5). The long principal neurite branches are distributed onto the muscle fibers and ICC scaffolds by interconnecting short bridging branches of the arbor that course orthogonally from muscle bundle to muscle bundle.

Subsets of IMAs within different gastric regions then exhibit adaptations of the basic array elements, apparently to adapt the afferents to local tissue requirements and functions. Several of these variations in IMA architecture are graded, incremental, and quantitative changes in the arbor array. These adjustments include changes in the lengths of arrays, separations of the principal array into two or more distinct daughter arrays, variations in the density of the arborization patterns within the arrays, and variation in the number and extent of lamelliform puncta and spines elaborated by the neurite branches. In the main, such graded adjustments of IMA structure do not dramatically affect the structural appearances of IMAs. A few specializations of IMA structure or regional distributions, however, are more dramatic, and two variants in particular are distinctive enough and potentially specialized enough functionally to merit discussion.

Circular muscle IMAs with polymorphic collaterals—In the original description of IMAs (Berthoud and Powley, 1992), it was noted that some IMAs extended collateral branches to myenteric ganglia. Since the original observation, and in spite of the signal importance of the observation for any comprehensive understanding of IMA physiology, however, this particular feature of IMAs has not been subsequently examined more systematically.

The present experiment replicates the basic observation of these collateral branches and provides a more detailed characterization of the polytopic specializations. Two salient features have been described. First, whereas the initial report of the collaterals made no attempt to examine where, with respect to the major gastric regions or muscle sheets, the collaterals occurred or in what percentage of IMAs they occurred, the present survey addressed these issues. Polytopic collaterals projecting to myenteric ganglia occurred in 32% of all circular muscle sheet IMAs (and in only a single outlier within the longitudinal muscle), and those specialized afferents were found in similar proportions in the circular muscle of all three major gastric regions.

The present observations also have clarified the structure of the terminal projection these polytopic IMAs issue to myenteric ganglia. The original report (Berthoud and Powley, 1992) of the polytopic projections provisionally suggested that the ganglionic projections formed IGLEs. That initial inference, which was based on the granular labeling pattern of an in vivo DiI-labeling protocol and which was shaped by the fact that, at the time, the only known ganglionic projections were IGLEs, proved to be incorrect. As detailed in the present results, a fuller examination of the collateral terminals labeled with the high resolution provided by the dextran–biotin protocol establishes that the IMA collaterals to myenteric ganglia form distinctive and heterogeneous terminals that differ from both contacts of IGLEs and the varicose neurites of preganglionic motor fibers (see Fig. 13).

The fact that approximately one-third of all IMAs in the circular muscle of all three gastric regions elaborate polytopic collaterals innervating the myenteric plexus suggests that the specialization is not anomalous. This finding also suggests that the polymorphic feature does not correlate with a functional operation that is limited to a single gastric region.

The observation also underscores a structural feature of IMA afferents that should be considered and respected in electrophysiological investigations of gastric smooth muscle innervation. Berthoud and Powley (1992), in the original description of IMAs, speculated that the collaterals from IMAs to the plexus might constitute arms of local or axoaxonic reflexes, and such peripheral reflex arcs remain a viable possibility (cf. Wei et al., 1995). We have also proposed (Phillips and Powley, 2000; Powley and Phillips, 2011) that the complexes that IMAs establish with ICC-IMs and other neurites may establish smooth muscle analogues of the spindle organs that report striated muscle stretch or length. Within the framework of such a spindle-organ hypothesis, the IMA collaterals may provide a direct neural linkage that could more tightly cross-link or integrate myenteric operations with smooth muscle stretch reflexes.

Antral longitudinal muscle IMAs with web ending specializations—Another distinctive specialization of IMAs that had been noted in a previous report but not fully characterized is the web ending. In an earlier examination of the afferent innervation of the gastric sling muscles associated with the lower esophageal sphincter (Powley et al., 2012), we noted that vagal afferents projecting to the distal site of attachment of the sling muscles elaborated apparently unique webs or networks of flattened laminar puncta. That analysis, with its small sample and limited field surveyed, was unable to provide a full assessment and comparison of the web endings to IMAs throughout the antral region.

In the present inventory, several details about the web endings are brought into focus. Most essentially, the web endings, which are found near the lesser curvature at the distal antral insertion of gastric sling muscles, are specialized IMAs (as opposed to a unique species of afferents). As one surveys the IMAs of the longitudinal muscle sheet in the antrum and scans progressively closer to the most distal antrum near the lesser curvature, IMAs express more and more of their characteristic flattened lamelliform plates, spines, and spurs. In effect, the IMAs in this region of the distal antrum become more and more web-like in appearance. At the location where the distal processes of the sling muscles insert into the antral wall, the IMAs of the longitudinal muscle have effectively migrated to the interface of

the muscle sheet and serosa, and the arbor branches form a web of flattened, lamellar plates. Notably too, the specialization is limited to IMAs within the longitudinal muscle sheet. Antral IMAs in the circular muscle sheet, on the other hand, do not develop the unique web-like profiles seen in the longitudinal muscle IMAs in the region.

Functions of gastric IMAs

Given, when it is recognized that IGLEs innervate the plexus between the muscle layers and that IMAs constitute the only direct vagal innervation of the smooth muscle sheets of the stomach, the requirement for a sound understanding of the function of the afferents seems apparent. It is difficult to envision how any adequate understanding of the extrinsic reflex control of gut physiology will be achieved without thorough structural and functional characterizations of the first-order afferents that initiate the vagovagal reflexes controlling stomach muscle wall function. Comparing the details of IMA structure and distribution throughout the stomach characterized in the present experiment with available physiological and neurophysiological observations both suggests some provisional conclusions about the operations of the afferents and points out details of the organization of the mechanoreceptors that may help design additional experiments on the functions of IMAs.

Stomach biomechanics, GI physiology, and functions of IMAs—A biomechanical comparison of the stomach with the rest of the GI tract and then a correlation of the distinctive functional roles of the different gastric regions with the sharply different distributions of vagal IMAs and IGLEs is instructive.

With the exception of the stomach, most of the GI tract is essentially an open-ended tube. In terms of the distribution of mechanical forces, the nongastric organs are biomechanically considered “soft cylindrical shells” (cf. Gregersen, 2003; Miftahof and Nam, 2010). Practically, in these “shells,” forces tend to distribute and dissipate down as well as up the lengths of the tubes, and tension and stretch of the wall typically remain reasonably well correlated. In such circumstances, signals from a single type of mechanoreceptor may be able to reflect both tension and correlated stretch effectively. Consistent with such a consideration, the nongastric regions of the GI tract muscle wall are inclusively innervated by IGLEs (e.g., esophagus [Zagorodnyuk et al., 2003; Neuhuber et al., 2006] and intestines [Berthoud et al., 1997; Wang and Powley, 2000]) and are practically devoid of IMAs (except, arguably, in the vicinity of valves and sphincters). Additionally, it has been established that IGLEs transduce tension (Zagorodnyuk et al., 2001).

In contrast, the stomach operates biomechanically quite differently from the rest of the GI tract. Specifically, the stomach functions as a “biological shell” (cf. Gregersen, 2003; Miftahof and Nam, 2010) or, effectively, a bladder. Functionally, within this “gastric bladder,” tension and stretch are frequently uncorrelated. When food is ingested, the stomach reflexively executes motor patterns to receive, store, grind, mix, and eventually empty nutrients into the intestines for subsequent digestion and absorption (Mayer, 1994; Camilleri, 2006). During these coordinated programs, the organ is subject to multiple forces (see, e.g., Gregersen, 2003; Miftahof and Nam, 2010), and tension and stretch of gastric muscle fibers are often independent. For example, during a meal, as nutrients are ingested in

successive swallows, the proximal stomach relaxes incrementally in a progression of vagovagally mediated adjustments. As the gastric wall undergoes receptive relaxation or accommodates, the smooth muscle fibers relax and lengthen, thus increasing the circumference of the organ so that the volume of ingested material is accepted and stored temporarily, with minimal to no change in tension. Stretch and tension not only are uncoupled in this situation, the two forces can actually independently affect different perceptions and responses (see, e.g., Notivol et al., 1995; Penagini et al., 2004; Carmagnola et al., 2005).

Similarly, in the case of the antrum, with its adaptations for triturating and emptying digesta into the duodenal bulb, tension and stretch can be uncorrelated. Because gastric contents vary in consistency, they can vary in the resistance or tension that they generate, while the circumference of the antral lumen (i.e., stretch or length of the muscle sheets) does not necessarily covary. As discussed elsewhere (Powley et al., 2014), the same dissociation of the different forces can also occur in the stomach sphincter orifices as well.

Those regions of the stomach in which tension and stretch are frequently and conspicuously uncorrelated are the same regions in which vagal IMAs are densely distributed. As illustrated in Figure 3, gastric IMAs were concentrated in two functionally specialized areas; the arbors were massed in the proximal stomach, the compartment consisting of the forestomach and proximal corpus adjacent to the forestomach, and in the distal antrum. Although they are not the subject of the present experiment, IMAs are also densely distributed in the smooth muscle of the lower esophageal sphincter (Powley et al., 2012) and in the pylorus (Powley et al., 2014) as well. Thus, those gastric sites (i.e., the lower esophageal sphincter, proximal stomach, antrum, and pylorus) in which mechanical forces associated with ingestion and digestion regularly dissociate are the sites heavily innervated by IMAs (as well as IGLEs).

Electrophysiology and vagal mechanoreceptive function—During the several decades in which neurogastroenterology recognized only a single type of vagal afferent (i.e., IGLEs) in the stomach muscle wall, the facts that 1) tension and stretch of the gut wall are distinct forces and 2) these two forces are regularly uncorrelated during gastric activity, substantially complicated attempts to explain the vagal afferent operations in ingestion and digestion. Thus, the recent recognition that the vagus supplies a second and independent type of sensory terminal, namely, IMAs, provides the prospect that tension and stretch (or length) might be separately transduced by the two types of vagal afferents.

With regard to the issue of experimentally distinguishing tension from stretch in the GI tract, the results of early efforts to explore gastric mechanoreceptors with electrophysiology were problematic. In addition to the confusion caused by the presumption that a single type of mechanoreceptor innervated the gastric wall, it was generally impractical to vary tension and stretch independently while maintaining physiological conditions, including baselines of both myogenic and neurogenic tone. In addition, these complications confounding neurophysiological analyses of gastric mechanoreceptors were further compounded by uncertainties with respect to what were adequate or physiological stimuli of the GI tract rather than nonphysiological or supernormal stimulus conditions. Furthermore, little

information on the complex biomechanics of the intact stomach (see Gregersen and Christensen, 2000; Simonian et al., 2004; Zhao et al., 2005) was available to guide the early electrophysiology. Without adequate information on such biomechanics, the exposed, reduced, and in vitro preparations often used to achieve adequate access and stimulus delivery almost certainly compromised at least some reflexes required to establish normal smooth muscle tone. Finally, early GI neurophysiology was impeded by a lack of information with respect to details on the architecture and distributions of any putative mechanoreceptors, even IGLEs. Overall, with the absence of critical information and with the experimental challenges to preserving physiological conditions, the status of IMA electrophysiology was aptly summarized by Brookes and colleagues (2013) in their review, “[t]o date, no electrophysiological activity has been recorded that can confidently be attributed to vagal intramuscular arrays.”

IMA stretch receptor inference: observations on associated tissues and ion channels

—Without unambiguous electrophysiological characterizations of vagal IMAs under physiological conditions, provisional conclusions have relied primarily on the evidence from the limited structural observations available and from gastric loading experiments, as previously discussed. Other indirect observations, based on the tissue associations and ion channel characteristics, are also consistent with the working assumption that IMAs transduce stretch, whereas IGLEs transduce tension.

The associations of vagal IMAs with nonneural tissues in the muscle sheets appear to implicate the afferents in stretch transduction. By light microscopic criteria, IMAs contact both ICCs (e.g., Fig. 5; Powley and Phillips, 2011) and smooth muscle (e.g., Figs. 6 and 5B; also see Powley and Phillips, 2011). Furthermore, IMA-ICC associations have also been examined ultrastructurally, and IMA varicosities form synapse-like contacts (i.e., the varicosities of the IMA branches exhibit prejunctional thickenings and contain vesicles) with ICCs (Powley et al., 2008; see also Huizinga et al., 2008). In addition, both of these nonneural elements of the muscularis externa, i.e., ICC-IMs and smooth muscle, that complex with IMAs express mechanoreceptive channels (Mazet, 2015). Additionally, the network of ICC-IMs has stretch-receptor features (Won et al., 2005; Kraichely and Farrugia, 2007), and the GI smooth muscle fibers also have stretch-sensitive properties (Sanders and Koh, 2007). Thus the IMA/ICC/muscle architecture suggests that IMAs are strategically situated to operate as primary afferents of a stretch-detection complex or network.

In addition, a subpopulation of mechanosensitive nodose ganglion neurons expresses ion channels that are specifically stretch sensitive. A substantial percentage ($\approx 62\%$) of nodose neurons contains mechanosensitive cation channels through which calcium enters the cell in response to mechanical stimulation, and a subset of those neurons expresses channels that are blocked by gadolinium (Sharma et al., 1995), the element used to identify stretch-activated channels specifically and to distinguish them from other types of mechanoreceptors. Also, the fact that the nodose ganglia supply stretch-sensitive fibers to the lungs (Yu, 2005; Kwong et al., 2008; Cutz et al., 2013) and to the aortic baroreceptors (Cunningham et al., 1997; Sullivan et al., 1997) as well as the fact that no evidence currently indicates these pulmonary and cardiovascular afferents constitute the entire pool of gadolinium-sensitive afferents in the ganglia renders moot the possible extrapolation that

IMAs also express the same type of stretch-sensitive channels. Such a possibility seems stronger in light of the complementary observation that vagal mechanoreceptive IGLEs, which appear to respond to tension rather than to stretch, express gadolinium-insensitive mechanoreceptive channels (Zagorodnyuk et al., 2003). This lack of gadolinium sensitivity also reinforces the inference that IGLEs do not serve as stretch detectors as well as confirms, by process of elimination, that these endings are tension transducers.

Regardless of the eventual assessment, including any revisions, of the provisional inferences that IMAs transduce stretch and IGLEs transduce tension, the associated issues and remaining questions underscore the requirement for a detailed understanding of the architecture, tissue associations, distributions, and specializations of IMAs (as well as any other afferents) if physiology is to understand fully the stomach and its operations in ingestion and digestion. The results of the present experiment add several conclusions about such structural details for the vagal IMA innervation of the gastric wall, and such details should facilitate the design of both more conclusive physiological assessments and more refined GI surgeries.

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ROLE OF AUTHORS

TLP and RJP participated in the design of the experiment and in the writing of the article. TLP, CNH, JLM, EAB, and RJP participated in developing and designing the sampling criteria and morphometric evaluations employed. EAB performed the nodose exposure surgery and tracer injections. CNH, JLM, and EAB performed the histology, tracer labeling, immunohistochemistry, and counterstaining. RJP produced the final micrographs and processed the images in Photoshop.

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Figure 1.

Representative IMA in corpus circular muscle of a whole mount of the ventral stomach wall. A: Photoshop tracing of the IMA arbor (parent neurite entering the field from 12:00) from a virtual 3D section collected with Surveyor software. The position of the arbor is illustrated in the outline tracing of the whole mount at lower right. **B–E**: All-in-focus photomicrographs of fields within the overall IMA; each lettered photomicrograph refers to the region of the tracing (in A) with the same letter designation. As the photomicrographs illustrate, IMA branches contained varicosities that varied, even in the same arbor, from relatively simple, spherical puncta (e.g., C) to more flattened, lamellar varicosities (e.g., B,E). Scale bars = 250 μ m in A; 8 mm in outline of whole mount in A; 20 μ m in D (applies

to B–E). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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Figure 2.

Representative IMA in forestomach longitudinal muscle of a whole mount of the ventral stomach wall. **A:** Photoshop tracing of the arbor from a virtual 3D section collected with Surveyor software. The parent neurite (at 12:00 in the tracing) travels radially through the stomach wall to arborize near the greater curvature. The position of the arbor is illustrated in the outline tracing of the whole mount at lower left. **B:** All-in-focus image of the region of the IMA's projection field designated with a "B" in the tracing (in A). The arbor illustrates the branching or arborizing pattern characteristic of gastric IMAs. Scale bars = 250 μ m in A;

8 mm in whole-mount outline; 50 μ m in B. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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Figure 3.

Location of the full sample of 369 IMAs in the present survey (from both sides of stomach) mapped onto the contour of a dorsal stomach whole mount. IMAs were concentrated in the proximal stomach and the antrum. Circles designate the location of the first bifurcation of a parent neurite into an IMA arbor. The full sample consisted of 184 complete and intact IMAs that were successfully digitized and used for morphometry plus an additional 185 complete IMAs that were not digitized for morphometry because of a significant tissue fold or other artifact that compromised digitization. Checks for penetration problems or other factors that could bias the inventory indicated that the sample of complete IMA arbors was a representative and (effectively) random sample of the distribution of IMAs in the stomach. LES, lower esophageal sphincter. Scale bar = 8 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 4.

Polar plot in bins of 10° of arc of a subset ($n = 44$) of forestomach longitudinal IMAs used for morphometry. Length of each bar in the plot represents the average cumulative length of IMA branches that were oriented in the respective 10° bin illustrated. Longitudinal muscle fibers were oriented from 90° to 270° , and all IMAs thus aligned with the axis of longitudinal smooth muscle fibers. The plot illustrates both how tightly aligned the principal branches of the IMA are with respect to the muscle orientation and how little cumulative neurite length is allocated to the bridging branches (which in the plot are oriented to $\sim 0^\circ$ or $\sim 180^\circ$, the orientation of the circular muscle fibers). Scale bar at 130° refers to the average

cumulative length of branches per IMA in the different bins. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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Figure 5.

Branches of IMAs (brown, 3,3'-diaminobenzadine stained) run in tight apposition with ICC-IMs (blue-gray, c-Kit immunolabeling with SG peroxidase). Branches of IMA arrays vary in their degree of varicosity and in the tightness of apposition. **A:** Four neighboring principal branches of an IMA array coursing in tight apposition with a network of neighboring ICC-IMs intercalated among smooth muscle bundles (unstained). In this example, the IMA branches express modest swellings or varicosities, most of which are in close proximity to the somata and processes of ICC-IMs. **B:** Two neighboring principal branches of an IMA array course near to, and appear to contact intermittently, the local ICC-IM network. In

contrast to the array branches shown in A, those shown in B are more lamelliform, the apparent contacts with the ICC-IMs are more intermittent, and many of the IMA lamellae appear to lie on the smooth muscle bundles (unstained) adjacent to the ICC-IM network.

C,D: Two examples of principal IMA branches that course in tight conjunction with ICC-IMs and form swellings or varicosities on both ICC-IM somata and fibers. Scale bar = 10 μm . [Color figure can be viewed in the online issue, which is available at

wileyonlinelibrary.com.]

Figure 6.

Close association of IMA principal branches (brown, 3,3'-diaminobenzadine stained) with smooth muscle fiber bundles (unstained, but imaged with DIC). **A:** A dense field of branches within an IMA array. The closely packed branches course on the textured surfaces of smooth muscle fascicles. **B:** Two (compared with the example in A) less densely branched fields of IMA branches that originate (out of view) from a single parent neurite and that course on the surface of smooth muscle fascicles. Scale bar = 100 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 7.

Some IMA parent neurites formed arbors that divided into double or tandem arrays. **A:** Digitized tracing of a single neurite that enters the field from the top of the figure and forks conspicuously into two second-order branches that travel to two sites where they then branch profusely into two arrays that distribute in parallel but clearly separate loci in the circular smooth muscle sheet. **B:** High-power photomicrograph of the major fork (site designated by a “B” in the tracing in A) dividing the IMA arbor into two separate arrays. **C:** Branches of the IMA array on the right side of the tracing at the location indicated with a “C” on the tracing in A. The DIC illumination of the photomicrograph also reveals the

texture of the unstained smooth muscle bundles on which the IMA branches run. **D:** Branches of the IMA array at the bottom of the tracing at the location indicated with a “D” in A. This example illustrates the extensive, tightly packed nature of IMA arrays and contains representative examples of both short bridging branches (running roughly left to right) and long principal branches (running top to bottom, parallel to the muscle fibers) that collectively create IMA arrays. Scale bars = 500 μm in A; 8 mm in contour in A; 5 μm in B; 10 μm in D (applies to C,D). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 8.

A: Volumes within gastric smooth muscle sheets innervated by IMA terminal arbors, the presumptive receptive fields of the arrays, varied by a factor of essentially 3 across the three gastric regions. **B:** Total cumulative lengths of all arbor branches within the corresponding receptive fields were more stable across regions and varied by only a factor of 0.5. IMAs in a given region tended to have similar values in both longitudinal and circular muscle. The one exception, perhaps reflecting the lamelliform “net ending” specialization of IMAs in antral longitudinal muscle, was that antral longitudinal IMAs tended to have smaller

innervated volumes or receptive fields than did the IMAs in the corresponding circular muscle sheet.

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Figure 9.

A small subset of IMAs formed one array in one smooth muscle sheet and a second array in the orthogonal smooth muscle sheet. The IMA tracing in the central region of the figure illustrates one of the orthogonal tandem array IMAs with the parent neurite entering the innervated field from roughly 4:00, forming an array (roughly vertical in figure) running in the longitudinal muscle layer and then branching and continuing on to produce a second array (roughly horizontal in figure) that innervated the circular muscle layer. **A,B:** All-in-focus photomicrographs of the array running with the longitudinal muscle fibers (locations in the tracing designated with the same letters). **A** also illustrates the distinctive fork in the neurite that generates the second array, which runs in the circular direction. **C:** Photomicrograph of the segment of the second array, running in the circular muscle, designated with the same letter. Scale bars = 250 μm in IMA tracing; 8 mm in whole-mount contour; 20 μm in C (applies to A–C). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 10.

Roughly one-third of IMAs that innervated the circular muscle sheet issued collaterals from the arbor. These collaterals returned to the myenteric plexus and innervated ganglia more or less in registration with their smooth muscle arbors. Photoshop-rendered tracing from a 3D virtual section collected with Surveyor software. The parent neurite enters the tracing at the top of the page, near 1:00, and the location of the arbor, in the proximal corpus, is designated in the outline of the whole mount at extreme upper left. The parent neurite formed an extensive circular muscle IMA (black branches) that issued several collaterals (green branches), which projected to and innervated (orange terminal fields) several of the

local myenteric ganglia stained with cuproline blue (gray-blue somata). **Insets A,B**, taken from the sites in the lower power tracing that are designated with the same letters, illustrate at higher power the details of the tracing with examples of the array (black) in smooth muscle, an IMA collateral (green) coursing into a local ganglion, and the dense innervation (orange) of the myenteric ganglion cells (gray-blue). Photomicrographs corresponding to the fields in the insets appear in Figure 11. Scale bars = 8 mm in the whole-mount contour; 250 μm in full low-power tracing at left; 63 μm in inset B (applies to insets A,B). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 11.

Photomicrographs keyed to the tracing in Figure 10 that illustrate the different layers and tissues innervated by the same polytopic and polymorphic IMA. **A1:** Photomicrograph taken at the optical plane of the myenteric ganglion from Figure 10A. **A2:** Photomicrograph in x- and y-coordinate registration with A1 (as well as Fig. 10A) taken at the optical plane of the underlying circular muscle array of the IMA. **B1:** Photomicrograph taken at the optical plane of the myenteric ganglion illustrated from Figure 10B. **B2:** High-power photomicrograph illustrating the varicosities the polymorphic IMAs developed in their myenteric ganglion collaterals. Scale bars = 20 μ m in A2 (applies to A1,A2); 20 μ m in B1; 10 μ m in B2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 12.

The collaterals that the subset of polymorphic circular muscle IMAs issued into the myenteric ganglia coursed to the ganglia without any ICC association, although, as illustrated earlier, the smooth muscle arrays of the IMAs were complexed with ICC-IMs and smooth muscle fascicles. **A–C:** All-in-focus photomicrographs that illustrate three examples of polymorphic IMA collaterals that assume the contours of (unstained, in this case) myenteric ganglia that they innervate but show no apparent relationship to the local ICCs (gray-blue c-Kit immunostaining). Photomicrographs also illustrate the heterogeneity and complexity of the varicosities that IMA collaterals express in the myenteric ganglia. Scale

bar = 25 μ m. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

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Figure 13.

The subset of circular muscle IMAs that were polymorphic and polytopic, issuing collaterals to myenteric ganglia, formed an innervation pattern within the ganglia that was distinctly different from the myenteric ganglionic projections of both vagal IGLE afferents and vagal preganglionic efferents. Collateral of a circular muscle IMA that courses into a cuprolinic blue-stained myenteric ganglion from the circular muscle layer (arrow at bottom left shows the collateral fiber coming into the focal plane of the ganglion) and then ramifies and forms apparent contacts throughout the ganglion. **Inset A:** For comparison, a vagal IGLE afferent (brown, dextran–biotin labeled) terminal plate of lamellar puncta situated between muscle

sheets, at the surface of myenteric ganglia (blue somata, cuprolinic blue), illustrates the differences between the profiles of IGLE vs. IMA collateral (central panel) projections to myenteric ganglia. **Inset B:** Vagal preganglionic motor fiber (brown, dextran-biotin labeled, from an injection into the dorsal motor nucleus of the vagus) innervating a myenteric ganglion (blue somata, cuprolinic blue). Whereas the collaterals of circular muscle IMAs meandered through the ganglia forming irregular lamelliform and spiny contacts both on neuronal somata and regions between the somata (presumably making contacts with dendrites and/or axons that were unstained), vagal efferents tended to form simple, relatively spherical varicosities in fibers encircling the myenteric somata. Scale bars = 25 μm ; 20 μm in insets A,B. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 14.

Antral longitudinal IMAs developed complex, flattened lamelliform varicosities on some branches. **A:** Photoshop tracing of an antral longitudinal IMA. The parent neurite enters the field at upper right and branches to form a longitudinal IMA near the greater curvature. Most of this IMA's array is relatively conventional in appearance, but a few branches near the bottom of the tracing are lamelliform in appearance. **B:** All-in-focus photomicrograph of the branches at the site in the tracing designated "B" in A. The contour at lower right illustrates the location of the IMA near the greater curvature of the dorsal gastric whole mount. Those antral longitudinal muscle IMAs that project to the vicinity of the antral lesser

curvature where sling muscle fibers attach displayed the lamelliform pattern on all or nearly all branches, producing the net ending pattern described previously (Powley et al., 2012). Scale bars = 250 μ m in A; 8 mm in whole-mount contour; 50 μ m in B. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1

Antibodies Used

Name	Immunogen	Host	Source	Dilution
CD117/c-Kit	Mouse myeloma cell line NSO-derived recombinant mouse CD117/c-kit, Gln25-Thr519 (Ala207Glu), accession No. P05532	Goat	AF1356, RRID:AB_354750, R&D Systems	1:1,000