Innervation of enteric mast cells by primary spinal afferents in guinea pig and human small intestine

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Running Head: Spinal Afferents, Mast Cells and Enteric Nervous System

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Abstract

Mast cells expressed the substance P NK1 receptor and the calcitonin gene-related peptide (CGRP) receptor in guinea pig and human small intestine. Enzyme-linked immuno assay (ELISA) showed that activation of intramural afferents by antidromic electrical stimulation or by capsaicin, released substance P and CGRP from human and guinea pig intestinal segments. Electrical stimulation of the afferents evoked slow excitatory postsynaptic potentials (slow EPSPs) in the enteric nervous system (ENS). The slow EPSPs were mediated by tachykinin NK1 and CGRP receptors. Capsaicin evoked slow EPSP-like responses that were suppressed by antagonists for protease activated receptor 2. Afferent stimulation evoked slow EPSP-like excitation that was suppressed by mast cell stabilizing drugs. Histamine and mast cell protease II were released by: i) exposure to substance P or CGRP; ii) capsaicin; iii) compound 48/80; iv) elevation of mast cell Ca\(^{2+}\) by ionophore, A23187; v) antidromic electrical stimulation of afferents. The mast cell stabilizers, cromolyn or doxantrazole, suppressed release of protease II and histamine when evoked by substance P, CGRP, capsaicin, A23187, electrical stimulation of afferents or compound 48/80. Neural blockade by tetrodotoxin prevented mast cell protease II release in response to antidromic electrical stimulation of mesenteric afferents. The results support a hypothesis that afferent innervation of enteric mast cells releases histamine and mast cell protease II, both of which are known to act in diffuse paracrine manner to influence the behavior of ENS neurons and to elevate the sensitivity of spinal afferent terminals.

Key words: mast cell degranulation, histamine, mast cell proteases, functional gastrointestinal disorders, irritable bowel syndrome, visceral pain
Introduction

Functional Pain Syndromes

Experimental antidromic stimulation of the sensory innervation of the airways or skeletal joints evokes neurogenic inflammation, associated with hypersensitivity in the respiratory system and arthritic pain in the joints (11, 21, 33, 41). O’Connor et al. reviewed evidence implicating afferent release of substance P (SP) as a mediator for neurogenic inflammation in skeletal joints and the respiratory system (51). Aside from the airways and joints, afferent sensitization is implicated as a factor in multiple abdominal and pelvic pain syndromes, which include chronic urologic pain, the irritable bowel syndrome (IBS), prostatitis, fibromyalgia and vulvodynia ((25, 26, 79, 92). Most of these fall into a so-called functional pain category because no abnormal physical or metabolic processes, which explain the symptoms, can be identified.

Overlapping morbidity is frequent for one or more of the functional pain syndromes. For example, symptoms associated with IBS have a high rate of overlap with symptoms of interstitial cystitis in the urinary tract (25). Francis et al. reported that one-third of patients attending urological clinics for symptoms of pelvic pain also had a diagnosis of IBS (26). Women are impacted in a majority of the cases and the symptoms can be sufficiently severe as to compromise ability to function in daily life (20). Comorbidity of IBS and fibromyalgia attracts attention in terms of translational sensory physiology, because IBS is a visceral pain syndrome with lowered threshold to bowel distension while fibromyalgia is somatic with lowered thresholds for tactile stimulation. A subset with both IBS and fibromyalgia experience a combination of visceral and somatic hypersensitivity (12, 13).

Afferents

Vagal and spinal afferent terminals in the digestive tract express receptors for several mast cell degranulation products including 5-hydroxytryptamine, bradykinin, ATP, adenosine, prostaglandins,
leukotrienes, histamine and mast cell proteases (5, 6, 10, 40). Any one of these inflammatory- or ischemia-related degranulation products, when applied experimentally, stimulates the terminals to fire. This endows mast cell degranulation products with a potential for elevating the sensitivity of an afferent to its preferred stimulus modality (e.g., mechanical, chemical, temperature or nociception) especially in disordered conditions associated with inflammation or ischemia. This possibility is reinforced by findings of a reduced threshold for painful responses to balloon distension in the large bowel associated with degranulation of mast cells in animal models (14, 54). Treatment with mast cell stabilizing drugs prevents lowering of the pain threshold to distension during mucosal inflammation in these models.

**Enteric Nervous System**

Neurons in the enteric nervous system (ENS) express the same array of receptors for mast cell degranulation products as found on vagal and spinal afferents (9, 30, 85, 87). Stimulation of neuronal excitability, presynaptic suppression of neurotransmitter release and activation of an ENS network that elevates mucosal secretion in concert with powerful aboral propulsive motility can result from mast cell immuno neural communication in the small and large intestine (81, 87, 91).

In view of the several kinds of evidence for functional interactions among sensory nerves, mast cells and the ENS, we aimed to investigate the effects of antidromic stimulation of intestinal spinal afferents on mast cell degranulation and electrophysiological and synaptic behavior of ENS neurons. Our results suggest that enteric mast cells receive input from spinal afferents, which in turn evokes the release of mast cell degranulation products that might feedback as paracrine mediators at receptors expressed by the same afferent terminals and simultaneously act in paracrine manner at receptors on ENS neurons.
Materials and Methods

Our methods for procurement of tissues for in vitro study were the same as we described elsewhere (77). Segments of ileum between 10 and 20 cm proximal to the ileocecal junction, segments of jejunum ~ 20 cm distal from the gastroduodenal junction and from the colon were obtained from male Hartley-strain guinea pigs (300-400g; Charles River, Wilmington, MA). The animal care and experimental protocols were approved by the Ohio State University Laboratory Animal Care and Use Committee and followed the USA National Institutes of Health Guide for the Care and Use of Laboratory Animals. Fresh preparations of healthy human small intestine were obtained from segments of jejunum discarded during Roux-En-Y gastric bypass surgeries as described in earlier papers from the laboratory (23, 61). The Institutional Review Board of the Ohio State University Office of Research Risks Protection approved the human protocols (Protocol 02H0208). Preparations from the small intestine were obtained by micro dissection for immunohistochemistry, enzyme-linked immuno assay (ELISA), organ bath pharmacology and neuronal electrophysiology.

Immunohistochemistry

Whole-mount immunohistochemistry was done in the same manner as elsewhere (23,61). Therefore, the current presentation of methods summarizes and quotes from these papers. Whole-mount preparations were obtained by dissection from segments of guinea pig and human small intestine and transferred to disposable chambers filled with fixative solution containing 2% formaldehyde and 0.2% of a saturated picric acid solution in 0.1 M phosphate buffered saline (PBS). Nonspecific immunological binding was blocked with 10% normal donkey serum in 0.01 M PBS, pH 7.4 for 1 hr at room temperature. The tissues were incubated with the primary antibodies (Table 1) diluted in 0.01 M PBS containing 10% normal donkey serum, 0.3% Triton
X-100 and 0.05% sodium azide overnight at 4°C after incubation with the primary antibodies (Table 1). The tissues then were washed (3 × 10 min) in PBS pH 7.4 and then incubated with appropriate secondary antibodies conjugated with fluorescence isothiocyanate (FITC) or indocarbocyanin (Cy3) diluted in 0.01 M PBS. The tissues were then rinsed in PBS and cover slipped with Vectorshield (Vector, Burlingame, CA). Pre-absorption of the antibodies with 10 µg receptor protein was done as controls. Specificity of immunostaining was evaluated further by omission of either the primary or the secondary antibody.

Immunohistochemistry was done also with cryostat sections obtained from guinea pig and human intestine in the same manner as we described earlier (77). The guinea pigs were anesthetized with 20% urethane and infused transcardially with chilled 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Segments of human jejunum were fixed with 4% paraformaldehyde in 0.1M phosphate buffer overnight at 4°C. The fixed tissues were washed in 0.2 M PBS and then dehydrated in 30% sucrose overnight at 4 °C. Thereafter, the tissues were frozen and embedded in Tissue-Tek® O.C.T. (Optimal Cutting Temperature) Compound (Ted Pella, Inc., Redding, CA). Sequential 4 µm sections were cut on a cryostat microtome. In order to block endogenous peroxidase activity, the sections were treated with 0.3% hydrogen peroxide solution for 30 min followed by normal horse and goat serum at room temperature for 2 hr to block non-specific protein binding. Pretreated sections were incubated with one or the other of the primary antibodies (Table 1) overnight at 4°C. After incubation, the sections were washed with 0.01 M PBS (pH 7.4) and incubated with biotin-labeled secondary antibody for 60 min. At the end of secondary antibody incubation, the sections were rinsed with PBS and incubated in VECTASTAIN Elite ABC system reagents (Vector Laboratories, Burlingame, CA) for 30 min.
All experiments were done in humidified chambers. Parallel sections incubated with non-immune serum were negative controls.

Whole-mount preparations were examined with an epifluorescence microscope (Nikon Eclipse-1000, Nikon Inc., Melville, NY) and analyzed with filter combinations that enabled separate visualization of multiple fluorophores. Tissue sections were examined with general light microscopy. Digital images were obtained with a SPOT RT cooled CCD digital camera (Diagnostic Instruments, Sterling Hts., MI) and analyzed with SPOT III software. Contrast in the digital images was sometimes enhanced before either converting to JPEG file interchange format (*.jpg) for electronic transfer or printing as photomicrographs.

**Electrophysiology**

Effects of electrically stimulating perivascular nerve bundles in the mesentery were recorded with intracellular “sharp” microelectrodes in ENS neurons. Electrophysiological and synaptic behavior was studied in the same manner as described elsewhere (38, 82). Therefore, the following presentation of methods summarizes and quotes from these papers.

Conventional “sharp” intracellular microelectrodes, filled with 4% biocytin (Sigma, St. Louis, MO) in 2M KCl and containing 0.05M tris-(hydroxy-methyl)-aminomethane buffer (pH 7.4), had resistances of 80-200 MΩ. The preamplifiers (M-767, World Precision Instruments, Sarasota, FL) had bridge circuitry for intraneuronal injection of electrical current. Constant current, rectangular pulses were driven by a Grass SD9 stimulator (Grass instrument Division, Astro-Med Inc., W. Warwick, RI). Data were digitized and stored for analysis.

At the end of each electrophysiological study, the anal end of the preparation was marked and the tissue was washed three times in cold Krebs solution, followed by placement in cold fixative in a disposable recording chamber, and left overnight at 4° C. The fixative
contained 2% formaldehyde in a 15% solution of picric acid. The preparations were cleared in three changes of dimethylsulfoxide and three 10 min washes with phosphate-buffered saline (PBS; 0.9 % NaCl in 0.01M sodium phosphate buffer, pH 7.0). They were then reacted with FITC-conjugated streptavidin. After a thorough wash in PBS, the preparations were covered with Vectorshield (Vector Laboratories, Burlingame, CA) and examined with a Nikon Eclipse E600 microscope. Images were digitized with a SPOT-2 chilled color and B/W camera (Diagnostic Instruments, Sterling Heights, MI) and analyzed with SPOT III software.

**ELISA**

ELISAs were done in the same manner as we described elsewhere (77). Therefore, the current presentation of methods summarizes and quotes from these papers. Protocols for study of SP or CGRP release after 40 min equilibrium were: 1) allow 10 min for basal release to occur followed by withdrawal of 3 samples for analysis of SP and CGRP content; 2) electrically stimulate perivascular nerve bundles in the mesentery of intestinal segments or apply capsaicin for 15-20s followed by withdrawal of 3 samples for SP and CGRP assay. The samples were centrifuged at 10,000 rpm for 15 min and the supernatants stored at -20°C until assayed.

Amounts of CGRP and SP in the supernatants were determined with ELISA kits designed for the purpose. Assay kits for substance P (Cat. #583751) and CGRP (Cat. A05482) were obtained from Cayman Chemical Co., Ann Arbor, Michigan, USA. Each experiment was terminated by removing the segments from the bathing medium, blotting on filter paper and recording the weight. Data are expressed as ng or pg/g tissue weight.

**Reagents and Antibodies**

Substance P, CGRP, CGRP<sub>8-37</sub>, RP 67580, capsaicin, GR15897, SB218795, capsazepine, tetrodotoxin and SB366791 were purchased from Tocris, Ellisville, MO. Capsaicin was
purchased from Sigma Biochemicals, St. Louis, MO, USA. The antibodies and antisera for immunochemical staining are listed in Table 1. Histamine ELISA kits were from SPI-BIO, Massy Cedex, France (Cat. A05890) and Oxford Biomedical Research, Inc. Oxford, MI, USA (Cat. EA31). Substance P ELISA kits were purchased from Cayman Chemical Co., Ann Arbor, MI, USA (Cat. 583751). Calcitonin gene-related peptide (CGRP) ELISA kits were purchased from Cayman Chemical Co., Ann Arbor, MI, USA (Cat. A05482). Mast cell protease II ELISA kits were purchased from Moredun Scientific, Penicuik, Midlothian, Scotland, UK.

Data Analysis

Results are expressed as mean ± SEM. Student's t test and paired t test were used for statistical analysis of significance with P<0.05 accepted as significant. Concentration-response relationships were determined using the following least-squares fitting routine: 

\[ V = \frac{V_{max}}{1 + (EC_{50}/C)^{nH}} \]

where V is the observed response, EC_{50} is the concentration that induces the half-maximal response, and nH is the apparent Hill coefficient. Plots were drawn by averaging results from all experiments and fitting to single concentration-response curves with Sigma Plot software (SPSS, Chicago, IL).

Results

Immunohistochemistry

We confirmed that co-expression of immunoreactivity (IR) for the transient receptor potential cation channel subfamily V member 1 (VR1), substance P and CGRP is a reliable marker for intramural spinal afferents in our preparations, as described in recent reviews by others (Fig. 1) (5, 10). VR1-IR was expressed in 99.2% of SP-IR fibers (2370/2390) and 98.4% of CGRP-IR fibers (1810/1840) in 19 small intestinal whole mount preparations. CGRP-IR and SP-IR were co-localized to every observable small diameter nerve fiber in 14 whole mount
preparations. VR1-IR was not found in the neuronal cell bodies in the myenteric plexus or submucosal plexus of the jejunum or ileum from 21 guinea pigs. Small diameter VR1-IR fibers ran parallel to blood vessels and diverged to enter ganglia (Fig. 1A). Fibers with substance P- and CGRP-IR likewise entered the same ganglia (Fig. 1B).

**Enteric mast cells**

We used IR for mast cell tryptase and chymase as markers for identification of intramural enteric mast cells (Figs. 2, 3). Primary antibodies to chymase or tryptase (Table 1) labeled single cells that had diameters in a range of 8-10 μm and characteristics common for enteric mast cells (63, 77). Preabsorption of the antibodies with 10 μg chymase or tryptase always abolished the immunostaining. Chymase- and tryptase-IR mast cells were widely distributed with one or more in close apposition to ganglia in the myenteric or submucosal plexus (Fig. 2). Double immunolabeling revealed expression by mast cells of SP and CGRP receptor protein in guinea pig and human small intestine (Figs. 2, 3). Expression of tryptase- or chymase-IR was never found to be associated with glial cells that were co-labeled for their S-100 protein marker (Fig. 2C).

Expression of the neurokinin NK1 receptor for SP was found for 58% (87/149) of tryptase-IR cells and for 47% of (63/132) of chymase-IR cells in guinea pigs. Expression of CGRP receptor protein occurred for 20% (37/182) of tryptase-IR cells and 13% (32/235) chymase-IR cells in guinea pigs. In human small intestine, expression of CGRP receptor-IR was found for 15% (47/298) of tryptase-IR cells and for 12% (52/423) of chymase-IR cells. Expression of NK1-IR was found for 17% (79/465) of tryptase-IR cells and for 11% (42/343) of chymase-IR cells.

*Electrophysiology*
Antidromic electrical stimulation of perivascular nerve bundles in the mesentery evoked synaptic responses consisting of a canonical noradrenergic slow inhibitory postsynaptic potential followed by a characteristic slow excitatory postsynaptic potential (slow EPSP) in submucosal neurons (Fig. 4) (62, 83, 84, 86). The slow EPSPs in each of 11 S- and 2 AH-type ENS neurons were suppressed in the presence of the CGRP receptor antagonist, CGRP8-37 (Fig. 4A). Slow EPSPs in 8 S- (i.e., synaptic type) and 2 AH-type (i.e., post spike after-hyperpolarization) neurons were suppressed by the neurokinin1 (NK1) receptor antagonist, GR82334, in the same neurons in which the CGRP receptor antagonist suppressed stimulus-evoked EPSPs (Fig. 4B). Pretreatment with the non-peptide NK2 receptor antagonist, GR15897, or the non-peptide NK3 receptor antagonist, SB218795, did not change slow EPSP responses evoked by mesenteric nerve stimulation (data not shown). Cromolyn (5 µM) was present throughout to prevent release of excitatory mast cell mediators whose actions mimic slow synaptic excitation.

**Compound 48/80**

Application of the mast cell secretogogue, compound 48/80 (80 µg/ml), in the bathing medium elevated the excitability of AH-type neurons in the myenteric and submucosal plexuses (Fig. 5A). Elevated excitability was reflected by depolarization of the membrane potential, increased input resistance, suppression of the AH and augmented excitability recorded as increased frequency of action potential discharge during intraneuronal injection of rectangular, constant-current depolarizing pulses. Presence of the mast cell stabilizer, cromolyn (5-µM), in the bathing medium suppressed the responses to compound 48/80 (Fig. 5A).

**Capsaicin**

Bath application of the VR1 receptor agonist, capsaicin (0.1-1.0 µM), mimicked the action of compound 48/80 to elevate the excitability of AH-type neurons in the myenteric plexus.
Responses to capsaicin were reduced when it was applied in the presence of PAR3888, a selective antagonist for the PAR2 protease-activated receptor (Fig. 5B2) (1).

Mesentery Afferents

Slow EPSP-like responses, evoked by antidromic electrical stimulation of perivascular nerve trunks in the mesentery, were suppressed after adding 30 µM doxantrazole, a mast cell stabilizer, in the bathing medium (Figs. 6 A1,2, 6 C1-2). Bath application of 0.1 µM capsaicin mimicked the electrophysiological changes taking place during stimulus-evoked slow EPSPs (Figs. 6 B1-2). Capsaicin-evoked slow EPSP-like responses were suppressed when the VR1 receptor antagonists, capsazepine (10-20µM) or SB366791 (10 µM), were present in the bathing media (Fig. 6, B1-2, C1-2). This action of capsaicin likely was due to stimulation of VR1 receptors expressed by afferents, because neural blockade with tetrodotoxin suppressed capsaicin-evoked release of mast cell protease II (see Fig. 9).

Antidromic electrical stimulation of mesenteric nerves elevated excitability of AH- and S-type neurons in the myenteric or submucosal plexus. Elevated excitability occurred in 22 of 25 AH-type neurons in the myenteric plexus (Fig. 7A, B). Elevated excitability, in AH-neurons, was reflected by depolarization of the membrane potential, increased input resistance, suppression of the AH and augmented excitability recorded as increased frequency of discharge of action potentials during intraneuronal injection of rectangular, constant-current depolarizing pulses. Mesenteric nerve stimulation did not elevate excitability in the AH-type neurons when applied with the mast cell stabilizing drugs 5-µM cromolyn or 20-30 µM doxantrazole present in the bathing medium (data for doxantrazole not shown) (Fig. 7C).

Elevated excitability in response to mesenteric nerve stimulation occurred also in 11 of 12 S-type neurons in the myenteric plexus (see Fig. 4A, B). It was reflected by depolarization of
the membrane potential, decreased input resistance, and augmented excitability recorded as increased frequency of discharge of action potentials during intraneuronal injection of rectangular, constant-current depolarizing pulses and spontaneous action potential discharge.

Mesenteric nerve stimulation did not elevate excitability in S-type neurons when applied with cromolyn (5 µM) or doxantrazole (20-30 µM) in the tissue bath (data not shown). Ketotifen, another mast cell stabilizing drug in concentrations up to 100 µM, did not suppress excitatory responses to mesenteric nerve stimulation or to capsaicin in either AH- or S-type neurons.

Focal electrical stimulation of interganglionic fiber tracts in either the myenteric or submucosal plexus evoked characteristic slow synaptic excitation (Fig. 8A1) (38, 82-84).

Simultaneous electrical stimulation of mesenteric nerves enhanced the slow EPSPs evoked by focal ENS stimulation (Fig. 8A2). Bath application of the mast cell stabilizer, cromolyn, counteracted the augmenting effects of mesenteric nerve stimulation on slow EPSPs evoked by stimulation in the ENS (Figs. 8A3, 8B, 8C).

ELISA

We used ELISA to study effects of antidromic electrical stimulation of mesentery nerves and capsaicin activation of spinal afferents on release of the mast cell degranulation products, histamine and mast cell protease II, from intact intestinal segments. Application of 0.5-20 µM capsaicin stimulated release of mast cell protease II and histamine into the media bathing small and large intestinal preparations from guinea pigs and segments of jejunum from human small intestine (Figs. 9, 10, 11).

Mast cell protease II

Bath application of compound 48/80 (80 µg/ml) evoked release of mast cell protease II in concentrations greater than basal release in the small and large intestine of guinea pigs (Fig. 9).
Pre-application of 20 µM cromolyn suppressed the release of mast cell protease II evoked by compound 48/80 (Fig. 9). Bath application of the Ca^{2+} ionophore, A23187 (20 µM), likewise stimulated release of mast cell protease II relative to basal release and this effect was suppressed by the presence of 20 µM cromolyn (Fig. 9).

We used release of mast cell protease II as a marker in investigation of afferent input to intramural mast cells. Application of 20 nM capsaicin, to stimulate intramural afferents, elevated release of mast cell protease II to significant levels above basal release (Fig. 9). Electrical stimulation of mesenteric nerves, to antidromically activate intramural afferents, elevated release of mast cell protease II in similar manner to the action of capsaicin (Fig. 9). Blockade of action potential conduction in intramural afferents by tetrodotoxin prevented elevation of mast cell protease II release during electrical stimulation of mesenteric afferents (Fig. 9). Placement of SP into the organ bath, as a putative spinal afferent neurotransmitter, evoked release of mast cell protease II (Fig. 9). On the other hand application of CGRP, in the same manner as for SP, did not elevate the release of mast cell protease II to levels significantly greater than basal release (Fig. 9).

Histamine

We studied release of histamine from intact segments of guinea pig and human small intestine in the same manner as was done for mast cell protease II. Stimulation of intramural afferents, by 0.05-0.5 µM capsaicin, evoked release of histamine beyond basal levels in guinea pig and human intestinal segments (Figs. 10, 11). The action of capsaicin to stimulate histamine release was concentration-dependent with an EC_{50} 0.4±0.1 µM for guinea pig small bowel from 4 animals and an EC_{50} of 0.7±0.1 µM for four human jejunal preps (Figs. 10B, 11B). Both EC_{50}s were calculated for a range of 0.01-1.2 µM due to desensitization at higher concentrations.
Capsaicin-evoked stimulation of histamine release was suppressed by 5 µM cromolyn in guinea pig preparations and by 30 µM doxantrazole in human preps (Figs. 10A, 11A).

Antidromic electrical stimulation of mesenteric nerves mimicked the action of capsaicin to stimulate release of histamine (Fig. 10A). Substance P (0.2 µM) or CGRP (0.05 µM), placed in the organ bath as putative afferent neurotransmitters, evoked increases in release of histamine relative to basal release in guinea pig and human intestinal segments (Figs. 10A, 11A). Pre-treatment with 5 µM cromolyn suppressed this action of SP and CGRP in guinea pig preps (Fig. 10A). Pre-treatment with 30 µM doxantrazole suppressed SP- or CGRP-evoked release of histamine in human jejunal segments (Fig. 11A).

**Discussion**

**Immunohistochemistry**

Co-expression of VR1-IR, SP-IR and CGRP-IR in small diameter intramural fibers identified the fibers, in our preparations, as primary spinal afferents and was consistent with many reports in the literature, which establish the co-expression as a marker for spinal afferents in the gut (5, 6, 10, 32, 37, 70). Projection of the afferents into enteric ganglia (see Fig. 1) was reminiscent of retrograde tracing studies of others showing that spinal afferents in mesenteric nerve trunks enter the intestinal wall and branch to send projections into ENS ganglia. Inside the ganglia, some of the afferents appear to form synapses with ganglion cell somas, while others have specialized terminals that discharge action potentials in response to mechanical stimulation in like manner to mechanoreceptors elsewhere (4, 44, 71). Connections of this nature might be indicative of local axon reflex connections between afferent collaterals and enteric neurons, as well as other cell types such as occurs in the classical “triple response of Lewis”, which involves afferent-evoked release of histamine from cutaneous mast cells.

**Afferent Stimulation**
Release of SP and CGRP evoked by firing of intramural afferents, either by antidromic electrical stimulation of the afferents as they entered the intestine from the mesentery or by exposure to capsaicin, was consistent with our immunohistochemical results showing co-expression of SP-, CGRP- and VR1-IR by small diameter intramural fibers. Capsaicin evoked release of SP and CGRP into the bathing media of the intestinal segments was most likely due to its well-known action to open VR1 channels, expressed by primary afferents (3, 36, 37). Opening of the VR1 channels is expected to evoke action potential discharge, which in turn would release SP and CGRP simultaneously from intramural terminals of afferent collaterals at junctions with mast cells and at synapses with ENS neurons, as we found (19).

**Electrophysiology**

Our results obtained with electrical stimulation of afferents, as they entered the intestine via the mesentery, closely resembled what was first reported by Takaki and Nakayama, who found that afferent stimulation evoked capsaicin-sensitive fast and slow synaptic responses mediated by acetylcholine and SP respectively in AH- and S-type ENS neurons (66, 67). Entry of the afferents into ENS ganglia (see Fig. 1) and finding that SP and CGRP receptor antagonists suppressed the slow EPSPs, evoked by mesenteric nerve stimulation in the present study, is consistent with the conclusion of Takaki and Nakayama that spinal afferents enter ENS ganglia where they release SP and CGRP at synapses with ENS neuronal cell bodies (67, 68).

Sympathetic postganglionic nerve fibers, known to release norepinephrine and ATP at synaptic connections with ENS neurons, also enter the intestine in mesenteric nerves alongside spinal afferents (38, 86). Stimulus-evoked slow IPSPs, which preceded the stimulus-evoked slow EPSPs (see Fig. 4) are known to be noradrenergic and are a marker for S-type non-cholinergic
submucosal secretomotor/vasodilator neurons that release vasoactive intestinal peptide as a neurotransmitter at neuro-glandular junctions (7, 22, 24, 62).

Electrical stimulation of mesenteric nerves can also backfire intestinofugal axons, known to be projections from S-type ENS neuronal cell bodies to prevertebral sympathetic ganglia (29, 35, 60, 65). Antidromically propagating action potentials in these fibers can be recorded, with intracellular microelectrodes in the S-type neuronal cell bodies, as they invade the cell body electrotonically (66, 67, 84). Nevertheless, these are rare events in AH- or S-type neurons that usually do not reach somal spike threshold because the electronic spread of current from the small diameter unmyelinated C fibers is into the large volume and low input resistance of the cell body (66, 67). Although antidromic stimulation of intestinofugal fibers, firing of their cell bodies and release SP or CGRP is unlikely, it cannot be fully ruled out in our study. On the other hand, most intestinofugal fibers in the mesenteric nerve trunks are projections from single-axonal S-type neurons that would not be expected to have terminal release sites inside the ganglia or at motor effector junctions (35, 60).

Slow EPSPs, evoked by focal electrical stimulation applied to ganglia or interganglionic fiber tracts in the ENS have been investigated for over three decades in terms of electrical and synaptic behavior with SP and serotonin being among the first putative neurotransmitters for slow synaptic excitation (83, 84).

Compound 48/80.

Compound 48/80 is a secretagogue commonly used experimentally to evoke degranulation of mast cells and release of preformed mediators, such as histamine and mast cell proteases (55). Our observations of elevated excitability in AH-type ENS neurons, following application of compound 48/80, most likely reflected mast cell degranulation and a diffuse
paracrine action of released mast cell mediators, because cromolyn-stabilization of the mast cells
suppressed this action of compound 48/80. Cromolyn, itself, is a common mast cell stabilizing
drug, used in asthma therapy and other allergies. Like doxantrazole, another mast cell stabilizing
agent used in our studies, cromolyn acts to prevent opening of Ca\(^{2+}\) channels necessary for
degranulation of mast cells (15, 56, 59). Histamine and mast cell proteases are known to be
excitatory neuronal mediators that can reach the ENS in paracrine fashion following antigen-
evoked release from sensitized enteric mast cells (27, 28, 30, 43).

Capsaicin

We applied capsaicin to guinea pig preparations in view of its much reported action to
stimulate VR1 receptors, fire afferent fibers and thereby evoke release of SP and CGRP from
intramural afferent nerve terminals (36, 37, 64). The action of capsaicin to elevate excitability of
AH-type neurons in these preparations, like the action of compound 48/80 on the mast cells \textit{per}
\textit{se}, appeared to result from mast cell degranulation and release of mast cell proteases because the
excitatory action was suppressed by antagonists for PAR2 protease-activated receptors. Mast cell
proteases are known to act at G protein-coupled PARs receptors, expressed by AH-type neurons,
and thereby to evoke slow EPSP-like excitation (30). We cannot exclude the possibility that the
slow EPSP-like actions of capsaicin were due entirely to stimulation of afferents, because TTX
suppressed only a fraction of the capsaicin-evoked release of mast cell protease II.

Mesentery Afferents

Excitation of AH- and S-type neurons during electrical stimulation of mesenteric
afferents was, not only sensitive to blockade of receptors for SP and CGRP, but was reduced by
prevention of mast cell degranulation by the mast cell stabilizing drugs, cromolyn or
doxantrazole, but not ketotifen. This suggests that part of the excitatory effects of antidromic
firing of afferents, in mesenteric nerves, resulted from evoked release and diffusion of mast cell mediators to bind with excitatory receptors expressed by ENS neurons in the neighborhood of the mast cells. Failure of ketotifen to suppress afferent-evoked excitation of ENS neurons in the present study differed from its suppression of membrane depolarizing responses evoked by application of sensitizing antigen in one of our earlier studies in guinea pigs (43). How degranulation evoked by afferent nerve input might differ mechanistically from degranulation evoked by antigen binding to Fc receptors on sensitized cells remains unresolved.

Slow and fast synaptic excitation in the intrinsic microcircuitry have important significance in the integrative functioning of ENS neural networks. Several paracrine mediators, as well as pharmacotherapies for GI disorders, produce their effects by influencing synaptic transmission. For example, histamine release from enteric mast cells acts at presynaptic receptors to suppress slow EPSPs and fast nicotinic transmission; whereas, the promotor drug, tegaserod (HTF919), facilitates fast nicotinic neurotransmission in the ENS (23, 43, 68). Our results suggest that firing of intramural afferents releases mast cell degranulation products, which in turn function in paracrine fashion to facilitate slow EPSPs at synapses in the ENS neural networks (see Fig. 8).

**Mast Cell Protease II and Histamine Release**

Our findings suggest that effects of afferent input to enteric mast cells mimics the action of compound 48/80 to degranulate mast cells and release preformed mediators. Five lines of evidence support the hypothesis that spinal afferents innervate enteric mast cells, degranulate enteric mast cells and release mast cell proteases and histamine into the extracellular milieu in the small intestine of guinea pigs and humans: 1) enteric mast cells expressed receptors for the sensory neurotransmitters, SP and CGRP; 2) exposure to the putative sensory neurotransmitters,
SP and CGRP, released mast cell protease II and histamine in cromolyn/doxantrazole-sensitive manner; 3) back firing of afferents, entering the gut in the mesentery, released mast cell protease II and histamine; 4) afferent stimulation, by capsaicin, mimicked the effects of backfiring afferents; 5) mast cell stabilizing drugs or neural blockade with tetrodotoxin suppressed afferent-evoked release of mast cell protease II and histamine.

Translational Implications

Figure 12 is a heuristic model for interpretation of our findings, in terms of how spinal afferents, mast cells and the ENS might interact in signaling involving mast cell proteases and histamine as key paracrine mediators in normal and disordered intestinal sensitivity. Elevated levels of the proteases and histamine are expected when mastocytosis occurs in the esophagus, stomach or intestinal tract. In fact, mast cell hyperplasia is reported to be associated with the diarrhea-predominant form of the irritable bowel syndrome (IBS-D), where exaggerated release of histamine and mast cell proteases, acting at PAR 2 receptors, is associated with the symptoms of cramping abdominal pain, acute watery diarrhea and fecal urgency (2, 52, 76).

Stress

Stressful life events are associated with onset and exacerbation of the symptoms in the diarrhea predominate form of the irritable bowel syndrome (39, 47, 72, 73). Release if mast cell proteases occurs during responses to stress in human and rodent intestine (45, 58). Moreover, exposure to the stress hormone, corticotropin releasing factor, stimulates mast cells to release histamine in animals (42, 43). Restraint stress in rats intensifies nociceptive sensory responses to colonic distension that likely reflects sensitization of afferents by mast cell products, such as histamine and proteases. (8, 34, 49).

Food allergy and infectious enteritis
Secondary exposure to the offending antigen in animals with small intestinal parasitic infection (e.g., *Trichinella spiralis*) or food allergy (e.g., milk protein) evokes histaminergic excitatory responses in AH- and S-type ENS neurons, which mimic the effects of afferent-induced degranulation of mast cells in the present study (27, 28). Earlier work that described actions of a variety of mast cell degranulation products, such as histamine and mast cell protease II on electrical and synaptic behavior of ENS neurons, provided a foundation for the work on antigen-sensitized animal models and the present study (28, 43, 50, 68, 69). Work of this nature, at the cellular level, translates to the whole organ *in vitro* where bath application of histamine or histamine release evoked by application of sensitizing antigens, in food allergy or parasitic infection, activates a central pattern generator in the ENS that drives precisely timed recurrent cycles of mucosal secretion of H$_2$O and electrolytes linked with propulsive musculomotor activity (16, 17, 18, 78, 89).

*Visceral Sensitization*

Frequent overlap of morbidity in one or more of the visceral pain syndromes was mentioned earlier (12, 13). How hypersensitivity in one visceral organ can be transferred to a nearby neighbor or be transferred from a visceral organ to expression as tactile allodynia in the skin, in the functional pain syndromes, has not been resolved satisfactorily. Nevertheless, it is apparent, in animal models, that mast cells, spinal afferents and neurogenic inflammation form a common denominator in the sharing of hypersensitivity between abdominal viscera (88). Uterine inflammation induced by either chemical irritation or endometriosis in rat models induces inflammation and hypersensitivity in the urinary bladder and colon and the inflammatory crosstalk is interrupted by resection of sensory afferents in the hypogastric nerve (80). Likewise, colonic irritation by either chemical mucosal irritants (e.g., 2,4,6-trinitrobenzenesulfonic acid) or
by mechanical distension induces inflammation associated with sensitization of spinal afferents and mast cell activation in the urinary bladder of rats (46, 54, 74, 75). Firing of urinary bladder afferents, by instillation of capsaicin, evokes hypersensitivity to distention in the colon (56, 57). Blockade of afferents by instillation of a local anesthetic (e.g., lidocaine), together with capsaicin, prevents the sensitizing cross talk from the bladder to the colon. In similar manner, experimentally-induced inflammation in the urinary bladder evokes tactile cutaneous allodynia in mouse models. Release of mast cell mediators is implicated as the primary factor in the sensitization of bladder afferent innervation to distension in these studies (56, 57). Receptors for histamine and mast cell proteases are expressed by spinal afferent terminals in the intestine (10, 40). These mast cell degranulation products fire the afferent terminal, when applied experimentally, and have potential for elevating the sensitivity of the terminal to its preferred stimulus modality, especially in disordered conditions of mastocytosis and associated inflammation. This possibility is reinforced by findings that a reduced threshold for painful responses to balloon distension in the large bowel is associated with degranulation of mast cells in animal models. Treatment with mast cell stabilizing drugs prevents lowering of the pain threshold to distension, which occurs during mucosal inflammation in the animal models (14).

A reasonable explanation for the cross talk between the intestine and neighboring pelvic organs is that information transmitted by sensitized intestinal afferents project to the same spinal interneurons that receive afferent input from visceral neighbors. For example, the majority of spinal neurons that respond to bladder stimulation also respond to colon stimulation, and vice versa (48). Sensitization of intestinal afferents by mediators released from enteric mast cells is likely to underlie both the well documented hypersensitivity to colonic distension associated with
inflammation or stress in rodent models and the cross talk now known to take place between the colon and urinary bladder.

**Heuristic Model (Figure 12)**

The interactive connections between spinal afferents, enteric mast cells and the ENS, identified in the present and earlier studies and illustrated by the model in Fig. 12, suggest how elevation of intestinal hypersensitivity for a specific stimulus modality, such as derived from a nociceptor or a mechanoreceptor, might occur. Connectivity is arranged in a positive feed-forward configuration of mast cell $\rightarrow$ afferent $\rightarrow$ mast cell. A consequence of a feed-forward interaction is that hypersensitivity becomes amplified and continues to escalate if unchecked.

Enteric mast cells are at the vortex of signaling in the induction of afferent hypersensitivity (88). Mast cells emit multiple kinds of paracrine signals when the antigen-sensitized IgE antibodies, which are bound to their surface, detect antigens associated with infectious organisms, food allergens and toxins that might breach mucosal barrier function.

Afferent nerve terminals express receptors for many of the mast cell signals (Fig. 12). Histamine and serine proteases are prominent signals for sensitization of the terminals. Sensitization can be reflected as activation of a silent afferent, such as the case for nociceptors or as activation by release from mast cells associated with IBS (2, 31).

Mast cell function in immuno-afferent communication can be viewed as a neuroimmune counterpart of sensory neurophysiology. Sensory neurons are genetically programed to express selective detection for specific stimuli (e.g., touch, temperature, pH or pain) that are fixed throughout an individual’s lifetime. Mast cells, instead, acquire specific detection specificities by virtue of flexibility of recognition functions inherent in production by the immune system of new antibodies that become attached to mast cell surfaces. Detection specificity for foreign
antigens is thus acquired and reinforced throughout life due to formation of new antibodies that bind to and remain attached to immunoglobulin receptors on mast cells. Output signals from mast cells, which are triggered by antigen cross-linking with the attached antibodies, are chemical entities and analogous to chemical output signals (i.e., neurotransmitters) from primary afferents to second order neurons in the ENS and central nervous system. Hence, mast cells and sensory neurons ultimately code information on a sensed parameter by releasing a chemical message that is decoded by information processing circuits in the nervous system.

Conclusion

Co-expression of immunoreactivity for VR1, SP and CGRP is a marker for intramural spinal afferent fibers that project into ENS ganglia. Firing of these intramural afferents by antidromic stimulation of perivascular nerve bundles in the mesentery or by exposure to capsaicin released SP and CGRP at excitatory synapses in the ENS and at afferent junctions with intramural mast cells. Intramural mast cells, which express SP and CGRP receptors, are in close proximity to the ENS and intramural afferent terminals. Afferent input, mediated by SP and CGRP, releases histamine and mast cell protease II. Histamine and mast cell protease II act, in diffuse paracrine manner, to influence the behavior of ENS neurons and to elevate the sensitivity of spinal afferent terminals to their preferred modality. A heuristic model suggests that sensitization and elevated firing frequencies of the afferent terminals feed forward to amplify mast cell release, which further sensitizes the terminal and continues a forward feed that intensifies actions in the ENS and sensory input to the spinal cord. Intensified input to the spinal cord, if from nociceptors, might be interpreted as pain and/or discomfort at the level of consciousness (see Fig. 12).
GRANTS

This work was supported by National Institutes of Health grant RO1 DK37238 and KO8 DK060468.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

Grants

This work was supported by National Institutes of Health grant RO1 DK37238 and KO8 DK060468 to YX.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors.

Author Contributions

G.-D.W., S.L., Y.X., B.J.N., D.J.M., and J.D.W. are responsible for conception and design of the research; G.-D.W., X.-Y.W., F.Z., M.Q., S.L. J.D.W. performed the experiments; G.-D.W., X.-Y.W., M.Q., S.L., Y.X., B.J.N., D.J.M., and J.D.W. analyzed the data; G.-D.W., M.Q., S.L., Y.X., B.J.N., D.J.M. and J.D.W. interpreted the results of the experiments; G.-D.W., X.-Y.W., M.Q., S.L., Y.X., B.J.N., D.J.M. and J.D.W. approved the final version of the manuscript; S.L. and J.D.W. edited and revised the manuscript; J.D.W. prepared the figures; J.D.W. drafted the manuscript.

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Figure 1. Universal coexpression of VR1-IR, SP-IR and CGRP-IR by intramural spinal afferents in the submucosal plexus of guinea pig small intestine. (A1) VR1-IR. (A2) Anti-hu-IR, which is a pan enteric neuronal marker labels neuronal cell bodies in a ganglion. (A3) VR1-IR fibers project along blood vessels and enter the ganglion. (B1-3) Afferent fibers that coexpressed CGRP-IR and SP-IR projected into a ganglion in the submucosal plexus. (B4) B3 at higher magnification. Calibration = 20µm. (C, D) Electrical stimulation of spinal afferents or exposure to capsaicin evoked release of substance P and CGRP into the bathing medium of intact small intestinal segments from guinea pigs. (C) CGRP release. (D) Substance P release. ★P<0.05 for difference from basal release.
Figure 2. Localization of mast cell tryptase, SP NK1 receptor and CGRP receptor in whole mount preparations of the submucosal plexus of guinea pig and human small intestine. (A) Mast cells expressing tryptase-IR (green) are in close proximity to a submucosal ganglion in which the neurons express IR for the pan neuronal marker, anti-Hu (red) in guinea pig. (B) Mast cells expressing trypsin-IR (red) are in close proximity to a submucosal ganglion in human small intestine. (C) Mast cells expressing tryptase-IR are distinct from glial cells that express IR for S-100 protein (red). (D1) Tryptase-IR. (D2) Neurokinin 1 receptor-IR. (D3) Merged images. (E1) Chymase-IR. (E2) Neurokinin 1 receptor-IR. (E3) Merged images. (F1) Chymast-IR. (F2) CGRP receptor-IR. (F3) Merged images. (G1) Tryptase-IR. (G2) CGRP receptor-IR. (G3) Merged images. Calibration = 20 µm.

Figure 3. Enteric mast cells express immunoreactivity for the substance P NK1 receptor and the receptor for CGRP in cryostat sections of human jejunal mucosa and lamina propria. (A1) Tryptase-IR. (A2) Neurokinin 1 receptor-IR. (A3) Merged images. (B1) Chymase-IR. (B2) Neurokinin 1 receptor-IR. (B3) Merged images. (C1) Tryptase-IR. (C2) CGRP receptor-IR. (C3) Merged images. (D1) Chymase-IR. (D2) CGRP receptor-IR. (D3) Merged images. Calibration = 20µm.

Figure 4. Slow inhibitory postsynaptic potentials (IPSPs) and slow excitatory postsynaptic potentials (EPSPs) evoked in a submucosal neuron by electrical stimulation of perivascular nerve bundles in the intestinal mesentery. (A) Electrical stimulation evoked a slow inhibitory postsynaptic potential (IPSP) followed by a slow EPSP, which aborted the IPSP. Suppression of the EPSP by the calcitonin gene-related peptide receptor antagonist, CGRP8-37 identifies the EPSP as CGRP-mediated. (B) Electrical stimulation evoked a slow IPSP followed by a slow EPSP, which aborted the IPSP. Suppression of the EPSP by the selective NK-1 tachykinin
receptor antagonist, GR82334, identifies the EPSP as a substance P-mediated EPSP. Morphology of the uniaxonal neurons from which the recordings were made appears in each inset. Cromolyn (5 µM) was present throughout to prevent release of excitatory mast cell mediators. Both neurons exhibited S-type electrophysiological behavior.

**Figure 5.** Mast cell degranulation, evoked by the secretagogue, compound 48/80, or by capsaicin-induced afferent activation, released mediators that elevated excitability of ENS neurons. (A1) Application of compound 48/80 increased excitability in an AH-type guinea pig myenteric neuron. Elevated excitability is reflected by suppression of post action potential after hyperpolarization (i.e., the AH), membrane depolarization and increased frequency of action potential discharge during repetitive intraneuronal injection of constant-current depolarizing pulses. (A2) Application of compound 48/80 in the presence of the mast cell stabilizing drug, cromolyn, did not increase the excitability of the same AH-type neuron in A1. (B1) Application of capsaicin increased excitability in an AH-type guinea pig myenteric neuron in the same manner as in A1. (B2) Application of capsaicin in the presence of the PAR2 receptor antagonist, PAR3888, did not increase the excitability of the same AH-type neurons in B1, suggesting that capsaicin acted to release a mast cell protease that reached the neuron by paracrine-like diffusion. Repetitive upward deflections on the records are electrotonic potentials evoked by intraneuronal injection of rectangular constant-current pulses.

**Figure 6.** Release of mast cell degranulation products, evoked by antidromic electrical stimulation of mesenteric afferents or by capsaicin-induced activation of intramural afferents, elevated excitability in the same AH-type neuron in guinea pig myenteric plexus. (A1) Elevated excitability evoked by mesenteric nerve stimulation. Elevated excitability was reflected by depolarization of the membrane potential, increased input resistance, and augmented excitability
reflected by increased frequency of action potential discharge. Repetitive downward deflections, on the records, are electrotonic potentials evoked by intraneuronal injection of rectangular constant-current pulses. Increases in the amplitude of the electrotonic potentials are a reflection of increased neuronal input resistance. (A₂) Mast cell stabilization by the mast cell stabilizing drug, doxantrazole, suppressed the excitatory action of mesenteric nerve stimulation, suggesting that stimulation of the afferents released one or more mast cell degranulation product that reached the neuron by paracrine-like diffusion. (B₁) Bath application of capsaicin mimicked the excitatory effects of electrical stimulation of mesentery afferents. (B₂) SB366791, a potent and selective antagonist for VR1 receptors, suppressed the excitatory action of capsaicin for the same neuron, suggesting that stimulation of intramural afferents by capsaicin released one or more mast cell degranulation products that reached the neuron by paracrine-like diffusion. (C₁) Quantitative data for effects of mesenteric nerve stimulation and capsaicin for 24 AH-type myenteric neurons, 8 from the jejunum and 16 from the ileum. (C₂) Quantitative data for effects of mesenteric nerve stimulation and capsaicin for S-type myenteric neurons, 9 from the jejunum and 5 from the ileum.

Figure 7. Electrical stimulation of perivascular nerve bundles in the intestinal mesentery released mast cell degranulation products that elevated excitability in an AH-type myenteric neuron. (A) Action potentials evoked by intraneuronal injection of rectangular depolarizing current pulses are followed by characteristic after-hyperpolarization (i.e., the AH). (B) Electrical stimulation of mesenteric nerves evoked canonical slow EPSP-like excitation marked by membrane depolarization, suppression of the AH and augmented excitability reflected by spontaneous spike discharge and repetitive discharge during depolarizing current pulses. Application of the mast cell stabilizer, cromolyn, abolished the slow EPSP-like excitation,
suggesting that mesenteric nerve stimulation released one or more excitatory mast cell
degranulation products that reached the neuron by paracrine-like diffusion. (D) Return to control
following washout of cromolyn.

**Figure 8.** Electrical stimulation of perivascular nerve bundles in the intestinal mesentery
released mast cell degranulation products that facilitated excitatory slow synaptic transmission in
guinea pig myenteric plexus networks. (A1) Slow EPSP evoked by focal electrical stimulation of
an interganglionic fiber tract. (A2) Simultaneous electrical stimulation of the same
interganglionic fiber tract and a perivascular nerve bundle in the intestinal mesentery enhanced
the duration of the Slow EPSP and the frequency of action potential discharge evoked by the
EPSP. (A3) Simultaneous electrical stimulation of the same interganglionic fiber tract and a
perivascular nerve bundle in the intestinal mesentery, in the presence of 5 µM cromolyn, did not
result in facilitation of the slow EPSP. (B) Quantitative data for effects of cromolyn on slow
synaptic excitation evoked by antidromic electrical stimulation of perivascular nerve bundles in
the intestinal mesentery. Data for mean number of action potentials during initial 30s of EPSP
were obtained from 26 neurons AH-type neurons, 11 in jejunal myenteric plexus and 15 in ileal
myenteric plexus. (C) Quantitative data for effects of simultaneous electrical stimulation of
interganglionic fiber tracts in the myenteric plexus and mesenteric nerves in the presence and
absence of cromolyn. Data for duration of slow EPSPs were obtained from 12 AH-type neurons,
5 in jejunal myenteric plexus and 7 in jejunal myenteric plexus.

**Figure 9.** Release of protease II was used as a marker for guinea pig mast cell degranulation. (A)
Small intestine. (B) Colon. Data are derived from five methods: 1) exposure to the putative
sensory neurotransmitters, substance P or calcitonin gene-related peptide (CGRP); 2) capsaicin-
evoked firing of afferents; 3) direct degranulation evoked by compound 48/80; 4) elevation of
intracellular Ca\textsuperscript{2+} by the ionophore, A23187; 5) antidromic electrical stimulation of afferents in perivascular nerve bundles in the intestinal mesentery. The mast cell stabilizing drug, cromolyn, suppressed release of protease II when evoked by compound 48/80 or A23187. Neural blockade by tetrodotoxin prevented the mast cell protease II release that occurred in response to electrical stimulation of mesenteric nerves. ★P<0.05 relative to basal release. ★P<0.05 for responses to compound 48/80 and A23187 in the presence of cromolyn relative to application in the absence of cromolyn.

**Figure 10.** Release of histamine was used as a marker for guinea pig mast cell degranulation. (A) Data were obtained with five methods: 1) putative capsaicin-evoked firing of intramural afferents; 2) exposure to the putative sensory neurotransmitters, substance P or calcitonin gene-related peptide (CGRP); 3) antidromic electrical stimulation of afferents in perivascular nerve bundles in the intestinal mesentery. The mast cell stabilizing drug, cromolyn, suppressed release of histamine when evoked by substance P, CGRP, capsaicin or electrical stimulation of afferents. (B) Capsaicin-evoked firing of intramural afferents was concentration-dependent. ★P<0.05 relative to basal release; ★P<0.05 for responses to capsaicin, substance P, CGRP and electrical stimulation of afferents in the presence of cromolyn relative to application in the absence of cromolyn.

**Figure 11.** Release of histamine was used as a marker for mast cell degranulation in human small intestine. (A) Release data compare effects of putative capsaicin-evoked firing of intramural afferents and exposure to the putative sensory neurotransmitters, substance P or calcitonin gene-related peptide (CGRP). The mast cell stabilizing drug, doxantrazole, suppressed release of histamine when evoked by capsaicin, substance P or CGRP. (B) Capsaicin-evoked firing of intramural afferents was concentration-dependent. ★P<0.05 relative to basal release.
P<0.05 for responses to capsaicin, substance P, CGRP and electrical stimulation of afferents in the presence of doxantrazole relative to application in the absence of doxantrazole.

**Figure 12.** Heuristic model for connectivity of spinal afferents, enteric mast cells and the enteric nervous system. (A) Enteric mast cells are a major node in the interactive induction of afferent hypersensitivity. An afferent terminal fires when its receptors and/or channels for a sensory modality respond to the preferred stimulus. Signals in the form of action potential codes release substance P and CGRP at afferent-mast cell junctions and at synapses with neurons in the enteric nervous system and second order neurons in the CNS. Mast cells express receptors for substance P and CGRP, which degranulate the mast cells and release mediators such as mast cell protease II and histamine. The mast cell proteases, histamine and additional mediators diffuse to their receptors on afferent terminals in paracrine fashion and act to enhance the sensitivity for a stimulus to trigger action potentials and augment firing frequency in the terminal. When this occurs, afferent input to the mast cells, enteric neuronal networks and the CNS is enhanced. Mast cells and afferents connected in this manner form a positive feed forward loop that self-perpetuates in the sensitization of afferent terminals. (B) Functional connectivity of an interactive enteric mast cell-afferent-enteric neuronal network that might underlie sensory hypersensitivity in the gut as well as in neighboring viscera.

**Table 1.** Codes and sources of primary antibodies.
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Anti-Hu, anti-human neuronal protein HuC/HuD; anti S-100 low molecular wt protein.