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Functions and Imaging of Mast Cell and Neural Axis of the Gut

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Abstract

Close association between nerves and mast cells in the gut wall provides the microanatomic basis for functional interactions between these elements, supporting the hypothesis that a mast cell–nerve axis influences gut functions in health and disease. Advanced morphology and imaging techniques are now available to assess structural and functional relationships of the mast cell–nerve axis in human gut tissues. Morphologic techniques including co-labeling of mast cells and nerves serve to evaluate changes in their densities and anatomic proximity. Calcium (Ca⁺⁺) and potentiometric dye imaging provide novel insights into functions such as mast cell–nerve signaling in the human gut tissues. Such imaging promises to reveal new ionic or molecular targets to normalize nerve sensitization induced by mast cell hyperactivity or mast cell sensitization by neurogenic inflammatory pathways. These targets include proteinase-activated receptor (PAR) 1 or histamine receptors. In patients, optical imaging in the gut in vivo has the potential to identify neural structures and inflammation in vivo. The latter has some risks and potential of sampling error with a single biopsy. Techniques that image nerve fibers in the retina without the need for contrast agents (optical coherence tomography and full-field optical coherence microscopy) may be applied to study submucous neural plexus. Moreover, the combination of submucosal dissection, use of a fluorescent marker, and endoscopic confocal microscopy provides detailed imaging of myenteric neurons and smooth muscle cells in the muscularis propria. Studies of motility and functional gastrointestinal disorders would be feasible without the need for full-thickness biopsy.

Keywords

Mast Cells; Enteric Nervous System; Visceral Afferents; Confocal Microscopy; Tomography

The gut is unique because it carries its own nervous system and is the largest immunocompetent organ. The enteric nervous system (ENS) regulates vital gut functions such as motility, epithelial transport, microcirculation, and barrier function. In addition to the ENS, the gut is innervated by terminals of visceral afferent and efferent neurons that

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Supplementary Material

Note: The first 5 references associated with this article are available below in print. The remaining references accompanying this article are available online only with the electronic version of the article. To access the remaining references, as well as additional online-only data, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://dx.doi.org/10.1053/j.gastro.2013.01.040>.

Conflicts of interest

The authors disclose no conflicts.

project to or originate in the brain stem and spinal cord. The ENS serves as a relay station where a relatively small number of vagal fibers (estimated ~40,000 at the level of the diaphragm) can control the integrated circuits of an estimated 100 million neurons in the ENS resulting in a divergence of output signals from the central nervous system to the gastrointestinal tract. The majority of nerve fibers that connect the brain to the gut are sensory; these nerves also exert efferent functions through axon collaterals that ramify in the gut wall. ENS control of gut functions is initiated by neural sensory circuits responding to different intraluminal stimuli. Both sensory circuits and motor pathways in the ENS are constantly exposed to excitatory or inhibitory mediators released from neurons and non-neuronal structures. Immune cells contribute to changes in neuronal excitability; thus, neuroimmune interactions influence motor output or adjust the sensitivity of sensory circuits.

Mast cells are one of the types of immune cells that communicate with nerves.¹ There are other neuroimmune interactions involving brain structures, such as the cholinergic anti-inflammatory reflex, which involves macrophages rather than mast cells. The present article focuses on the interaction of mast cells and nerves in the gut; further detail is provided in the supplementary material. There is a marked gradient in mast cell density across the human gut wall with the highest density in the lamina propria; there are nonrandom spatial associations and membrane-to-membrane contacts, as well as bidirectional communication between mast cells, enteric neurons, and visceral afferents expressing transient receptor potential vanilloid 1 (TRPV1) receptors in the human gut (Figures 1 and 2).

The first 2 sections of this article summarize approaches to image the mast cell–nerve axis in fixed tissues or in vital intestinal preparations. In the third section, we review the potential of novel gastrointestinal imaging to enhance understanding of the pathophysiologic mechanisms that result in motility or functional gastrointestinal disorders. Advances in basic science research illustrated in the first 2 sections should lead to the application of these imaging techniques in tissues from patients.

Imaging Mast Cell–Nerve Axis in Fixed Human Intestinal Samples

Toluidine blue or Alcian blue staining and immunoreactivity for mast cell tryptase or c-Kit are suitable to assess mast cells in human intestinal tissues. When this staining is combined with a pan-neuronal marker, such as protein gene product 9.5, it is possible to analyze the association between mast cells and neural somas or processes (Figures 1 and 2). Some of the novel techniques employ confocal 3-dimensional (3D) microscopy and panoramic view of the gut wall architecture which allow morphometric assessment of different cell types (Table 1). Although tryptase staining seems to be specific for mast cells, it is important to note that c-Kit will also label interstitial cells of Cajal. However, morphometry will readily distinguish the smooth mast cells from the more spindle-shaped interstitial cells of Cajal. Co-labeling of tryptase and c-Kit together with protein gene product 9.5 facilitates identification of the ratio of degranulated to nondegranulated mast cells and the proximity of each type of mast cell to nerves. Under normal conditions, there is an almost total overlap between tryptase and c-Kit labeled mast cells (Figure 2D).

Chymase may also be used to label a particular mast cell population, although this has not been systematically evaluated in gut diseases. The 2 main types of human mast cells in the intestinal wall contain either both tryptase and chymase (minority) or tryptase alone (majority).² Immunoreactivity for chymase depends on the staining protocol including tissue handling, and the functional and maturation state of mast cells. For example, stem cell factor stimulates a predominance of the tryptase/chymase double-positive mast cells, whereas the addition of interleukin-4 supports the chymase negative (exclusively tryptase positive)

subtype of mast cells.³ The proportion of the latter mast cells (tryptase positive, chymase negative) varies considerably in human colonic mucosa (7%–67%) with lower variability in small intestinal mucosa (5%–26%).⁴

Have These Methodologic Advances in Fixed Tissues Impacted Our Understanding of the Pathophysiology or Mechanisms of Irritable Bowel Syndrome?

There is considerable variability in mast cell counts in mucosal biopsies from irritable bowel syndrome (IBS) patients, as summarized by Chang et al.⁵ There are several possible reasons for the discrepant findings in different studies. First, fixation protocol and section orientation are not standardized and insufficiently described in the literature, making comparisons between different studies difficult. Future studies can be standardized by adoption of recently published guidelines for histologic techniques, including the staining methods and consultation with experienced anatomist or pathologist.⁶ Second, tissue handling before fixation and sectioning may have exposed tissue to mechanical forces that cause activation and degranulation of mast cells,⁷ compromising mast cell counts based on stains for preformed mediators, such as tryptase or histamine. On the other hand, c-Kit staining is less likely to be affected by mast cell degranulation. Third, mast cell reactivity and biological effects may not be reflected in the number or density of mast cells.⁸ Fourth, in view of the pronounced mast cell gradient in different layers of the gut (Figure 1), precision in orientation and sectioning is key to reliable mast cell counts and their comparisons, for example, between health and disease. Thus, sections containing the subepithelial layer may reveal less mast cells compared with counts in close vicinity to epithelial cells. Ideally, analysis should separately quantitate both epithelial and subepithelial mast cell density. A fifth potential pitfall in assessing mast cell density is proximity of the analyzed section to blood vessels, because mast cells are found in higher numbers close to blood vessels compared with nonvascular regions (Figure 2E). Finally, differences in mast cell counts may conceivably reflect true differences in IBS subpopulations or overlap of a subgroup of IBS with an enteric mast cell activation syndromes in the absence of mastocytosis or defined allergic or inflammatory reactions.⁹ Further studies to test this hypothesis are eagerly awaited.

Functional Imaging of Mast Cell and Nerves in Vital Tissue Samples

Although co-labeling of mast cell and nerves in fixed human specimens has become routine in many experimental clinical studies, assessing functions of these cells in living human tissue is more challenging (Table 1). This is, however, a crucial step in evaluating the biological significance of the cells; altered cell density, reactivity, or protein expression do not necessarily reflect changes in function of the mast cell–nerve axis. In addition, animal models or isolated cells may not reflect the biology at the functional and molecular levels in humans, because mast cells and enteric neurons exhibit species specificity in release mechanisms, mediator profile, and neuropharmacology.¹

To date, imaging of mast cell activity in vital tissues has been mostly restricted to isolated mast cells or mast cell lines. However, application of advanced imaging techniques based on the use of dyes that detect changes in membrane potential (eg, derivatives of naphthylstyryl-pyridium) or intracellular calcium (Ca^{++}) have enabled the recording in vitro of nerve activity in submucous or myenteric plexus layers of human tissues.^{10–13} It is important to note that these imaging techniques are applicable to record nerve activity in routine biopsies.¹² Calcium imaging has some advantages over potentiometric dye recordings. First, the technique is easier, the equipment is available in many laboratories, and the technique less vulnerable to dye bleaching because of the low sampling rate necessary to reveal Ca^{++} signals. Second, Ca^{++} signals reflect activation of a variety of cells allowing direct recordings of the interactions between different cell populations. Ca^{++} imaging is usually

performed with slow sampling rates to record intracellular Ca^{++} changes over minutes. In addition, ultrafast Ca^{++} imaging revealed spike discharge of enteric neurons and thus the ability to study excitatory synaptic transmission.¹⁴

Imaging studies in vital tissues provided insights into mast cell–nerve signaling in human intestine, including neural activation by immune cell mediators. Thus, for example, human submucous neurons were activated by a cocktail of mediators released from human intestinal mast cells after stimulation by immunoglobulin E receptor cross-linking.¹⁵ Histamine and proteases are 2 active components in this cocktail. Histamine excited human enteric neurons primarily via activation of H1, H2, H3, and H4 receptors; this involvement of 4 different histamine receptor subtypes in excitation of human enteric neurons emphasizes the advantage of studying human tissues. Thus, in the guinea pig ENS, H3 receptors mediate presynaptic inhibition rather than excitation of enteric neurons,¹⁶ and proteases signal in human submucous plexus primarily via proteinase activated receptor 1 (PAR)1 and to a lesser degree via PAR2 receptors, in contrast to the dominant PAR2-mediated responses in rodent submucous plexus.¹⁷

Reports on direct recordings of the bidirectional interactions between mast cells and nerves are rare, but they have been accomplished in murine superior cervical ganglia,¹⁸ where direct neurite–mast cell communications demonstrated that Ca^{++} transients in mast-like cells lead to degranulation and release of histamine and other mediators, and addition of bradykinin-induced, nerve-triggered Ca^{++} transients in those cells. Although similar recordings in intact human preparations are not available, the submucous plexus would likely be most suitable to image activity of the mast cell–nerve axis using this technique.

In principle, Ca^{++} imaging techniques could also be applied to record cell activity in vivo. This has been successfully accomplished in the mouse brain, in which fiber optic measurements in combination with 2-photon Ca^{++} imaging revealed neuronal Ca^{++} signals.¹⁹ However, several obstacles have to be overcome before applying such techniques to the gut. First, unlike the relatively immobile brain, nerve cells in the gut change location with muscle movements, requiring flexible sensors to compensate for muscle activity. Second, stereotactic guidance of the optical fiber to specifically target the ganglionated plexuses has not yet been achieved in the gut wall and would require higher spatial resolution of optical equipment to position the sensors onto a ganglion. Some of the in vivo imaging approaches discussed may be valuable tools to realize such experiments on vital tissues in the future.

Have These Methodologic Advances in Vital Tissues Impacted Our Understanding of the Pathophysiology or Mechanisms of IBS?

The functional relevance of mast cell to nerve signaling was demonstrated by the excitatory action of supernatants of mucosal biopsies from IBS patients on human enteric neurons²⁰ or rat visceral afferents.²¹ These excitations were inhibited by histamine receptor antagonists and inhibition of serine proteases. These observations suggest that the nerve-activating effect of the mucosal supernatants may be pathologically relevant in IBS, and that mast cell mediators activate enteric as well as visceral afferent nerves. This concept is supported by the observation that the mast cell stabilizer–H₁ receptor antagonist ketotifen reduced visceral hypersensitivity and improved IBS symptoms in hypersensitive patients.²²

Why Is Advanced Imaging of Potential Relevance in Gut Neuromuscular Disease?

At present, pathologic diagnosis of gut neuromuscular disease^{6,23–25} is mainly based on full-thickness biopsies of tissues acquired at laparotomy or laparoscopy. Therefore, the tissue

diagnosis of neuromuscular diseases is seldom available, except in patients with the most severe conditions for which laparotomy or laparoscopy is performed for another clinical indication, such as placement of enteral feeding or decompression tubes, or gastric electrical stimulators in patients with gastroparesis.^{26,27} Other approaches, such as endoscopic full-thickness or deep muscle biopsies, provide different levels of resolution and are reviewed in the supplemental material.^{28–33}

Among the imaging methods, some require contrast to be taken up by the cells of interest (eg, epithelia). The neuronal elements cannot be imaged in vivo with vital dyes that facilitate imaging in vitro. However, 3 novel imaging modalities have the potential to image neuronal structures without need for administration of contrast materials.

Three Novel Imaging Modalities With the Potential to Image Gut Neuronal Elements In Vivo

Optical Coherence Tomography

In optical coherence tomography (OCT), optical probes are placed in apposition with the intestinal mucosa. By using somewhat long wavelength light, OCT can penetrate the tissue so that the mucosa and submucosa are normally displayed (penetration depth, 1–2 mm). OCT is an interferometric technique, typically using near-infrared light. Interferometry measures the interference of 2 light beams that are derived from a single source. One beam is directed at the sample and the other to a reference mirror (of known location). Light reflected from the sample and the reference beam is then recombined at a detector. The interference between the 2 beams is measured and an image is created by analyzing single points from different depths within 1 axis.³⁴ There are no reports to date of this technique in the evaluation of enteric neurons, but it has been used extensively to evaluate nerve fibers in the retina or to identify early signs of neurodegeneration of the retina in patients with type 2 diabetes mellitus.³⁵ Combination of this technique with Doppler ultrasonography has been applied in the gastrointestinal tract to examine normal and pathologic structures, and with the possibility to investigate angiogenesis in the gastrointestinal tract.³⁶

Full-Field Optical Coherence Microscopy

Full-field optical coherence microscopy (FFOCM)^{37,38} is a wide-field, high-resolution form of OCT. It utilizes a relatively inexpensive, spatially incoherent illumination source and an array detector to produce en face images. In contrast with other types of tissue microscopic imaging modalities, like confocal microscopy, FFOCM can achieve resolution in all orientations of <1 μm in 3D.³⁹ FFOCM allows the study of microstructural morphology and subcellular structures in biological samples⁴⁰ deep within tissues.⁴¹

FFOCM can provide structural information without administration of any contrast dye.⁴² Fluorescence microscopy can record images with enhanced contrast of specific molecules, if an appropriate fluorescent label is used. Combining the 2 techniques increases the versatility of each modality⁴³ and has been demonstrated to allow intra-arterial imaging in vivo.⁴⁴ Furthermore, standard FFOCM is suitable to perform conventional wide-field fluorescence imaging with the addition of a set of appropriate filters and a more sensitive camera. FFOCM permits thicker tissues to be imaged using a grid and mathematical processing to extract an optically sectioned fluorescence image.⁴⁵ This technique has been applied to examine mouse retina,⁴² and it has very recently also been applied to ex vivo study of submucosal as well as myenteric neurons.⁴⁶

Endoscopic Confocal Microscopy

Confocal microscopy provides microscopic images at subcellular resolution; fluorescent dyes are applied locally or systemically to retrieve fluorescence energy from a single point within a defined field and depth of a tissue sample. Fluorescence is then excited by a low-power laser, and the point of illumination coincides with the point of detection within the specimen, leading to a high spatial resolution. In contrast, light emitted from outside the focal point is not captured. Multiple points are analyzed and the intensity of fluorescence energy is displayed as a gray-scale image providing an optical section representing 1 focal plane within the specimen. By imaging multiple depths and horizontal sections, 3D images of the structures of interest can be obtained, although the slow speed of image acquisition and motion artifact in the gut in vivo present significant challenges for proper 3D reconstruction (reviewed in Kiesslich et al³³). Recently, 3D confocal microscopy with optical clearing has been combined with c-kit and nuclear fluorescent staining to identify the processes and cell bodies of interstitial cells of Cajal in resected specimens.⁴⁷ It remains to be seen whether this can be accomplished in vivo.

Sumimaya et al have demonstrated the potential application of probe-based confocal laser endomicroscopy to image muscularis propria and myenteric neurons in porcine tissues.^{48,49} This technique is made possible through access to the submucosa, a technique that is widely used in endoscopic mucosal resection or in resection of precancerous or cancerous lesions confined to the mucosa.⁵⁰ The marker used is a fluorescent neuronal molecular probe, which binds to the Nissl substance in neurons. Spindle-shaped smooth muscle cells were also visualized in vivo, and confocal neuronal elements and smooth muscle can be imaged although peristaltic movements, contact bleeding, and entrapped air bubbles under the probe tip occasionally hamper the imaging.

In summary, these 3 methods have the potential to visualize gut neural elements in vivo without the need for administration of contrast materials. To image the mast cell–neural axis in vivo appeared impossible until recently when a method was developed to image mast cells in vivo.⁵¹ This involves intravenous injection of VM249, a fluorescent probe that becomes optically active in the presence of cathepsin proteases which are present in mast cells. A hand-held imaging device is then used intraoperatively to identify fluorescence arising in mast cells.

Remaining Challenges of In Vivo Imaging of Gut Neural Elements

The methods described have the potential to quantify enteric nerves without the need for full-thickness biopsies and their attendant risks. Remaining challenges are the absence of neurotransmitter specificity and the inability to identify inflammatory cells or increased collagen deposition or fibrosis, a common histopathologic feature of myopathic disorders like progressive systemic sclerosis. However, the advantage of reviewing several regions within an organ, and thereby avoid sampling error associated with random biopsies, is a particular strength of these imaging techniques, and it augurs well for the future application of enteric neuronal imaging in clinical diagnosis. Clearly, further validation and experience are required before this discipline can be launched in clinical practice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this paper

ENS	enteric nervous system
FFOCM	Full-field optical coherence microscopy
IBS	irritable bowel syndrome
OCT	optical coherence tomography
PAR	proteinase activated receptor
TRPV1	transient receptor potential vanilloid 1.

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Figure 1.

Close association between nerves and mast cells in human sections. Tissues were stained for the pan-neuronal marker protein gene product 9.5 (PGP9.5), mast cell tryptase (MC tryptase), or c-Kit as mast cell markers and transient receptor potential vanilloid 1 (TRPV1) as a marker for extrinsic sensory nerve terminals. (A) Low-power image of a cross-section of the human colonic wall with the epithelium, submucous plexus, circular muscle, myenteric plexus, and longitudinal muscle layers. Note that the highest density of mast cells is in the epithelial and submucous layers. (B) High-power image of the inner submucous plexus showing a ganglion (marked by *arrow head*) and a mast cell nearby. (C) High-power image of the epithelial layer illustrating numerous mast cells in close vicinity to nerve fibers.

Figure 2.

Close association between nerves and mast cells in human whole mount tissue. (A) Whole-mount preparation with the exposed submucous plexus as an intact network. Note the intimate relation between mast cells, nerve fibers, and enteric ganglia (one is marked by *arrowhead*). (B) Paucity of mast cells in a whole mount preparation of the myenteric plexus. This image shows only 12 mast cells, most of them far away from the ganglia. (C) Contact between enteric ganglia (one is marked by *arrowhead*), mast cells, and transient receptor potential vanilloid 1 (TRPV1) terminals. Numerous TRPV1 terminals (in light blue because of co-localization with PGP.9.5) are present in submucous plexus ganglia. Note that close proximity between mast cells and TRPV1 fibers only occurs nearby an enteric ganglion. In the extra-ganglionic regions there are hardly any TRPV1 fibers that run close by a mast cell. (D) Almost all mast cells in the submucous plexus layer are co-labeled by MC tryptase and c-kit (*purple*; one ganglion marked by *arrowhead*). (E) Whole-mount preparation of the submucous plexus region containing blood vessels. Note that mast cells are located along blood vessel. In addition, this image shows that submucous plexus ganglia (2 are marked by *arrowheads*) are often close to blood vessels.

Table 1**Novel Imaging Techniques With the Potential to Assess Peripheral Neuroimmune Interactions**

Technique	Application	Resolution	Limitations	Future Potential	References
CCD imaging using Ca ⁺⁺ and voltage-sensitive dyes	Recording of Ca ⁺⁺ transients and action potentials in vital human whole-mount preparations	<2 μ m	Mostly used for in vitro imaging; requires movement compensation and careful assessment of dye toxicity, in particular for in vivo use	In particular, Ca ⁺⁺ imaging may be used to directly record mast cell nerve crosstalk; potential for use in intact gut in combination with 2-photon microscopy and optical fibers	11–14
Full-field optical coherence microscopy	Quantitative assessment of ganglion density in the ENS	1 μ m	Used in ex vivo studies	Assessment of mast cell nerve apposition, improved diagnostic of enteric neuropathies	46
3-D confocal microscopy with optical clearing	In-depth imaging (300 μ m) in human specimen	Subcellular	In vitro application in fixed specimen	Panoramic illustration of mast cell nerve apposition	47
Probe-based confocal laser endomicroscopy	Submucosal tunneling to endoscopically access deeper tissue layers ex vivo and in vivo	Subcellular	Currently only used in animal studies, need to improve reliable identification of neurons	Diagnosis and assessment of neuroenteric dysfunction	49