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Distribution of Serotonin-immunoreactive Enterochromaffin Cells in the Gastrointestinal Tract of the Least Shrew (*Cryptotis parva*)

Distribución de las Células Enterocromafines Serotonina-inmunorreactiva del Tracto Gastrointestinal de la Musaraña Enana (*Cryptotis parva*)

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SUMMARY: Serotonin is an important neurotransmitter in the central (CNS) and peripheral (PNS) nervous systems. It is involved in a variety of physiological processes both in the gut and in the CNS. The present study examined the distribution of serotonin containing enterochromaffin cells in the gastrointestinal tract (GIT) of a vomit competent species, the least shrew. These cells were easily recognized by their globular granules stained with the H&E and serotonin immune-positive stain. The immunoreactive enterochromaffin cells (IERCs) were mainly confined to the basal portion of the glandular epithelium and were distributed throughout the shrew stomach, small and large intestine. None was found to be associated with the mucosal epithelial lining. Moreover, their distribution and count varied in different regions of the GIT suggesting specific functions for these regions. The highest concentration of IERCs was found in the colon followed by the Jejunum. Appreciable numbers of IERCs were found in the stomach especially at the esophageo-gastric junction. The gastric location of the IERCs was mainly in the basal portion of the gland. However, some IERCs were associated with the parietal cells of the stomach. Two types of IERCs were observed: One with globular secretory granules in their apical portion of the cytoplasm which were located within the glandular epithelial cells facing the glandular lumen which release their secretions into the lumen; and the second were basally located, facing the lamina propria of the mucosa. Their secretory granules were not distinct in shape, and are most probably paracrine in their mode of secretions.

KEY WORDS: Least shrew; Enterochromaffin cells; Serotonin; Immunohistochemical; Stomach; Duodenum; Jejunum; Ileum; Colon.

INTRODUCTION

5-hydroxytryptamine (3-(b-aminoethyl)-5-hydroxyindole = 5-HT, or serotonin) is an important signaling molecule in both the CNS and the periphery. Over 95% of serotonin in the body is located in the periphery especially in the gastrointestinal tract (GIT). Of the peripheral 5-HT, approximately 90% is produced by the gastrointestinal enterochromaffin cells (EC), while the remaining 10% is present in the GIT enteric nervous system (Branchek & Gershon, 1987; Darmani & Ray, 2009). Serotonin is involved in the control and modulation of numerous physiological and psychological processes. In the CNS, 5-HT regulates mood, appetite, emesis and migraine. In the GIT serotonin generally plays a prokinetic role, and is an important mediator of sensation (e.g. nausea and emesis, satiety) between the intestine and the brain. Serotonin produces its diverse effects via seven different families of serotonergic receptors (5-HT1 – 5-HT7), including multiple subtypes within the 5-HT1 (5-HT1A, 5-HT1B, 5-HT1D , 5-HT1E , 5-HT1F and 5-HT1P), 5-HT2 (5-HT2A, 5-HT2B and 5-HT2C), 5-HT3 (5-HT3A, 5-HT3B and 5-HT3C), and 5-HT5 (5-HT5A and 5-HT5B) families. Indeed, serotonin is involved in the mediation of both immediate and delayed phases of chemotherapy-induced vomiting as well as regulating the GIT motor activity and its secretory function via the enteric nervous system (ENS) (Darmani & Ray). Moreover, antagonists of serotonin 5-HT3 receptors attenuate vomiting induced by chemotherapeutics in patients with cancer (Darmani & Ray). At least 5 different 5-HT3

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receptor subunits are known in humans where 5-HT3A, 5-HT3B and 5-HT3C are almost ubiquitously expressed in the CNS and periphery, while the 5-HT3D is predominantly, and 5-HT3E subunit exclusively, expressed in the GIT (Niesler et al., 2008). Tryptophan hydroxylase (TPH) is the rate limiting enzyme in 5-HT synthesis and its distribution is limited to the cytoplasm of those tissues containing serotonin. Further, TPH exists in two isoforms, TPH1 which is primarily expressed in ECs, and TPH2 expressed exclusively in neuronal cells such as the dorsal raphe and myenteric plexus (Liu *et al*., 2008).

The least shrew of North America (*Cryptotis parva*), is one of the smallest mammals, that is being used in research as an emesis model for both screening antiemetics as well as investigation of emetic circuits between the brainstem and the GIT (Darmani & Ray; Darmani, 1998). Indeed, use of least shrews has allowed us to decipher for the first time the receptormechanisms of the: i) cannabinoid CB1 receptor-mediated central and peripheral components of antiemetic actions of delta-9-tetrahydrocannabinol (THC) against the immediate and delayed phases of chemotherapy, and radiation-induced vomiting (Darmani & Ray; Darmani, 2001; Darmani *et al*., 2007), ii) central and peripheral components of emetic actions of some (LTC4, LTD4 and LTE4) but not other leukotrienes (LTA4, LTB4, LTF4) (Chebolu *et al*., 2010), iii) brain penetration of substance P and tachykinin NK1 receptor antagonists and their emetic/antiemetic potential via the use of a single species (Darmani *et al.*, 2008), and iv) synergistic antiemetic interactions between serotonergic 5-HT3 and tachykininergic NK1-receptor antagonists (Darmani *et al*., 2011).

Although the regional biosynthesis, release, metabolism and distribution of serotonin and localization of its diverse receptors and uptake sites are well studied in the CNS (Chojnacka-Wojcik, 1995; Kilpatrick *et al*., 1986; Martin & Sibson, 21008), relatively fewer studies have attempted to investigate the regional distribution of such serotonergic parameters in either the gut or the ENS of commonly laboratory animals (Gershon, 1999; Cho *et al*., 2006; Martel, 2006), and only scant data in limited regions of the gut is available in vomit competent species (Fukui *et al*., 1993; Pettersson, 1979; Endo *et al*., 1998). Since the premise of the revised multi-neurotransmitter theory of chemotherapy-induced immediate and delayed vomiting heavily depends on the release of serotonin from ECs, the distribution of these cells along the GIT, especially of an emetic species, is of paramount importance. Therefore, the present study was conducted to obtain needed information regarding the localization of serotonin secreting enterochromaffin cells throughout the entire length of the gastrointestinal tract of the least shrew.

MATERIAL AND METHOD

Tissue preparation and Light Microscopy. Nine least shrews of both sexes (4.0 ± 2.0 g, 45-60 days old) were used in this study. The animals were bred in the animal facilities of Western University of Health Sciences. They were housed and fed food and water ad libitum (Darmani, 1998) according to the animal use and care protocol (Western University IACUC standards). The animals were euthanized with an overdose of pentobarbital (100 mg/kg). The GIT were removed completely without the esophagus (mean WT 5 ± 0.04 g; mean length 9.9 ± 0.07 cm). Buffered neutral formalin solution (10%) was flushed slowly into the lumen of the gut over a 5 min period; samples were then collected from different parts of the GIT tract and labeled in accord with (Fig. 1). The tract was divided into equal segment of 10 mm each. These samples represent different sections of the least shrew stomach, small intestine and large intestine. Further division of the small and large intestine into (duodenum, jejunum, ileum, colon and rectum) was histologically recognized according to the characteristic features of each region, though there were no clear demarcations between these segments. Specimens were processed using standard histological procedures for preparation of 5 micrometers thick sections collected on glass slides. Sections were stained with hematoxylin and eosin (H&E) for routine histological evaluations and Periodic Acid Schiff (PAS) (Bancroft & Gamble, 2005).

Immunocytochemistry: Immunocytochemistry was performed on 5 micron thick paraffin slide mounted sections. After deparaffinization in xylene and rehydration through downgraded series of ethanol, the sections were placed flat on a plastic grid, preblocked in a 0.1M PB solution with 1 % triton, containing 5% normal horse serum and 3% peroxide for 20-30 min at room temperature. The sections were then incubated overnight (18 h) at 4 OC with the primary antibody {1:1500 Immunostar RbX5-HT in 0.1M PB+ 5% serum}. The sections were then rinsed thrice with PB followed by incubation in secondary antibodies HRP MsX Rb [1:2000 in 0.1M PB + 5% serum] (Jackson Immunoresearch, West Grove, PA, USA)for 90 minutes at room temperature, then washed thrice with PB. The sections were then incubated in fluorescent dye- conjugated 488 tyramide solution [1:300 +3% peroxide1:500] for 20 minutes (Ray *et al*., 2009) (Alexa Fluor™ dyes from Invitrogen and tyramine from Sigma-Aldrich.). Sections were washed thrice with PB. Finally, they were dehydrated through graded ethanol series and cleared in xylene and cover slip mounted. Following immunocytochemical processes for serotonin, optical sections were imaged with Nikon E400 eclipse light and fluorescent microscope using appropriate filter (488 nm excitation filter) to visualized immunoreactive enterochromaffin cells in the GIT of the least shrew.

Quantification of immunoreactive enterochromaffin cells (IERCs) was performed by random selection of three fields of view of each region of the GIT at an objective magnification (X10). Region of interest (ROI) was drawn on each field of view with an area of (0.43 mm^2). The relative frequency of occurrence, mean number and standard deviation of enterochromaffin cells per (0.43 mm^2 area) of microscopic field were determined using image J-NIH (Collins, 2007).

RESULTS

The IERCs were scattered throughout the GIT and their density varied along its different regions. The number and distribution of IERCs along the shrew GIT is depicted in Figure 1.

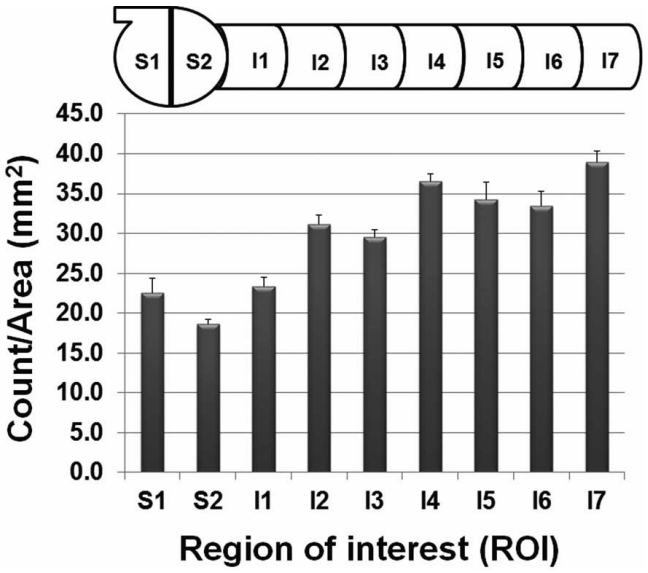

Fig. 1. Graphical analysis of the distribution of enterochromaffin cells in different regions of the GIT of the least shrew- Stomach (S1, S2), duodenum (I1, I2), jejunum (I3, I4), ileum (I5), colon (I6, I7). The data represent IRC count expressed as mean ± STD per mm2 area of visual field.

The immunocytochemical examination of the samples from gastric mucosa revealed that the serotonin containing IERCs are present in all regions of the gastric mucosa (cardiac, fundic and pyloric). The IERCs were scattered in the lamina propria of the esophageo-gastric junction (Fig. 2 A-S1) and none were associated with the cardiac gland (Fig. 2 A). The frequency of IERCs tended to peak towards the fundic region of the stomach

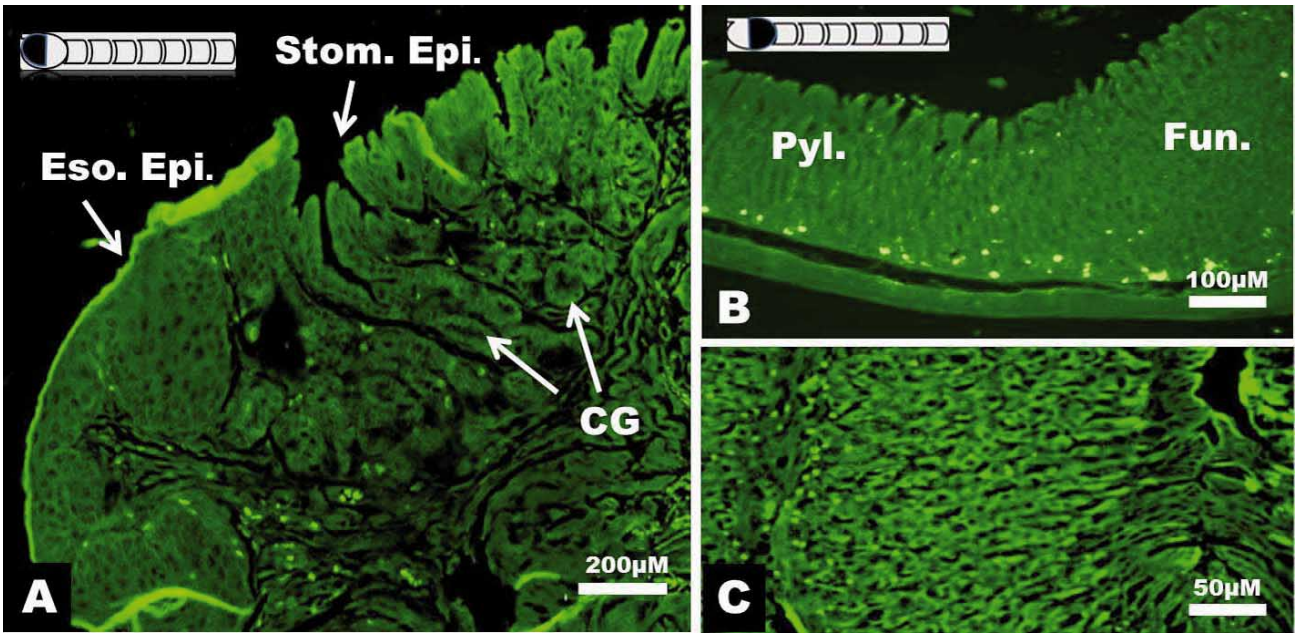

Fig. 2. Immunohistochemical localization of 5-HT immunoreactive cells in sections taken from the stomach region of the least shrew. Inset A in the stomach S1 region shows the concentration of IERCs in the lamina propria of the cardiac region in the esophageo-gastric junction. Inset B in the stomach S2 region shows the transition zone from the fundic (Fun) to pyloric (Pyl) regions of the stomach. Inset C in the stomach S2 region represents the fundic region of the stomach, showing IERCs concentrated in the basal portion of the fundic glands. Eso-Epi. = Esophageal epithelium, Stom-Epi.= Stomach epithelium, and CG = Cardiac glands.

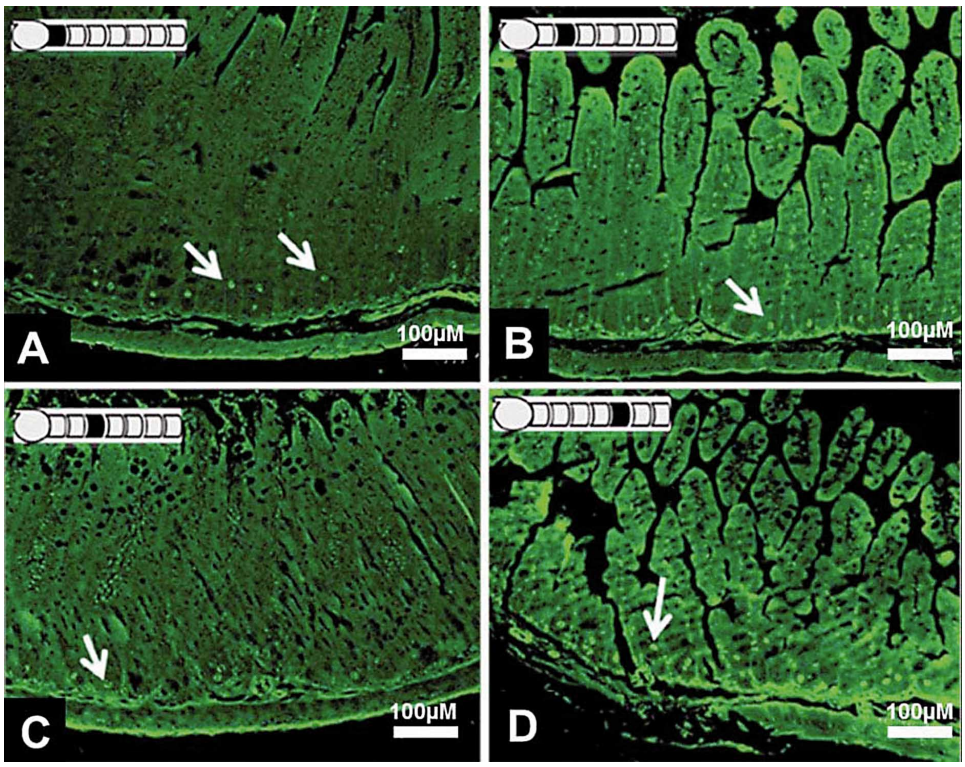

Fig. 3. Immunohistochemical localization of IERCs in the small intestine of the least shrew GIT. Diagrammatic intestinal inserts demonstrate the sites of sections collected from the GIT (A-B) duodenum; (C) Jejunum; (D) Ileum. The IERCs are preferentially localized in the crypt region of the intestinal glands (arrows), a few are found along the mucosal villi.

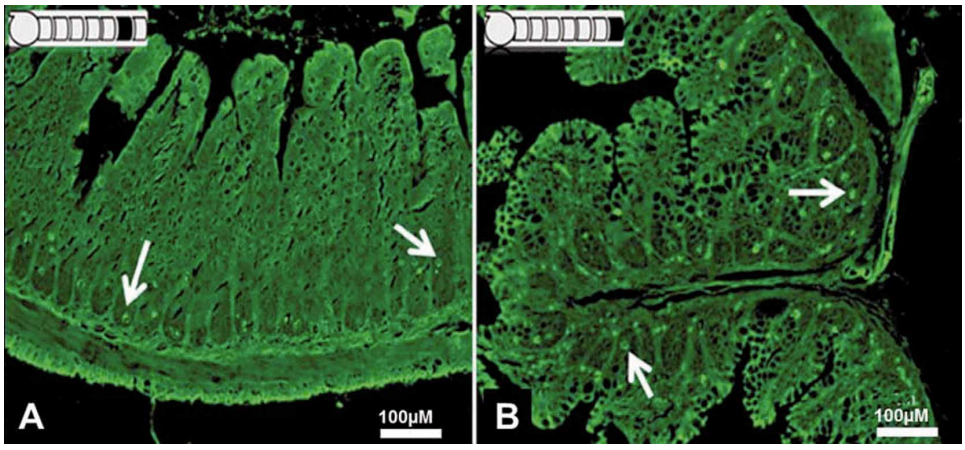

Fig. 4. Immunohistochemical localization of IERCs in the large intestine of the least shrew. Diagrammatic intestinal inserts show the sites of sections collected from the GIT. A-B; are colorectal regions of the GIT. The IERCs are localized in the crypts of intestinal glands (Lieberkuhn) (arrows).

(22.6 ± 3.1) (Fig. 2 B-S2), whereas the pyloric region, had fewer IERCs (18.7 ± 1). The location of IERCs in the gastric mucosa varied regardless of the gastric regions. Indeed, most of the IERCs were associated with the basal portion of the fundic glands; a few were located in the neck region of the fundic glands, where they were in close proximity to parietal cells (Fig. 2 B, C-S2). None of the IERCs were seen to be located within the gastric mucosal epithelial cells (Fig. 2).

Based on the histological changes, shape and size of the mucosal villi, in the mucosa along the GIT, it is easy to identify the least shrew small intestine encompassing regions I1 to I5 (see figure 1). However, this change was gradual with no clear delineation of duodenum (I1, I2), jejunum (I3, I4) and ileum (I5). Collectively, the small intestine contains a relatively large number of IERCs (31.0 ± 1.5) compared to the stomach (22.6 ± 3.1). Among the three regions of the small intestine, the Jejunum contained the highest number of IERCs (36.6 ± 1.5) followed by the ileum (34.3 ± 3.8) and duodenum (23.4 ± 2). Regardless of their count, the IERCs were mainly located in the crypts of Lieberkühn of the intestinal glands (Fig. 3 A-D).

The Least shrew large intestine (mean IERCs = 33.7 ± 2.8) consists of regions I6 and I7 representing the proximal (33.5 ± 3.2) and distal (38.9 ± 2.5) portions of the colon. The IERCs were located in the crypts of Lieberkuhn (Fig. 4 A-B).

Histological examination of serotonin immunoreactive cell revealed certain morphological features, regardless of their regional distribution in the whole GIT. In fact the cytoplasmic granules are clearly recognized when they were stained red with H&E, and negatively stained with PAS

stain (Fig. 5 A-B). Their cytoplasm contained variable density of serotonin immunoreactive globular granules (Fig. 6A). Some IERCs were in close association with the glandular epithelium, their granules located in the apical portion underneath the surface membrane reaching the glandular lumen. In other areas, the granules were not distinct in shape and basally located alongside the basement membrane of the glandular epithelium facing the lamina propria of the mucosa (Fig. 6B).

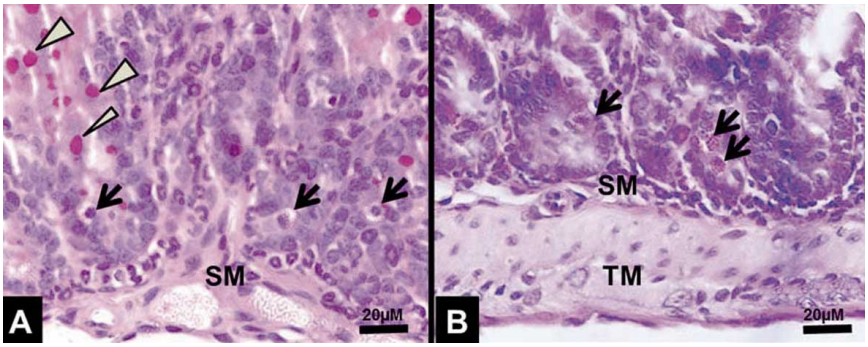

Fig. 5. Light microscopic photograph of colon (I7 region) in inset A stained with PAS, and duodenum (I2 region) in inset B stained with H&E, showing enterochromaffin cells concentrated in the intestinal crypts (arrows) containing secretory granules. Goblet cells pointed by (arrow head). SM = Submucosa. TM = tunica muscularis.

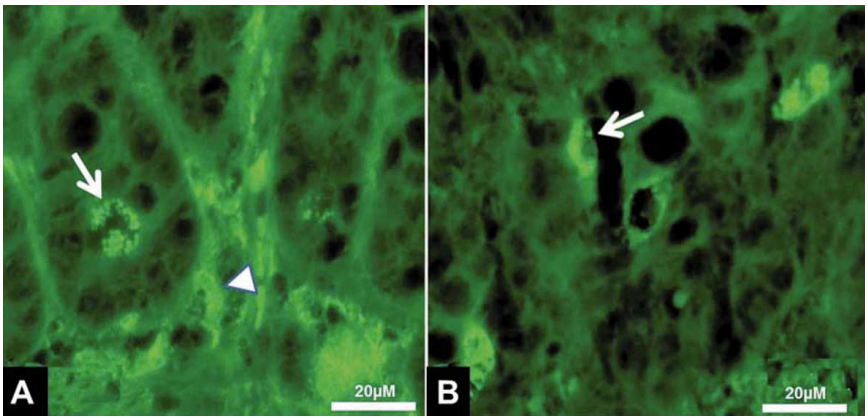

Fig. 6. High magnification of immunoreactive enterochromaffin cells (IERCs), showing the globular nature of the serotonergic secretory granules (arrows). Note that the IERCs are localized either within the glandular epithelial cells in the crypts with their secretory granules beneath the cell surface membrane facing the lumen (inset A), or closed to the basal lamina where it's secretory granules facing the lamina propria of the mucosa (inset B). Arrow head points to mast cell located in the lamina propria of the mucosa.

DISCUSSION

Increasing evidence indicate that the least shrew (*Cryptotis parva*) appears to be an excellent animal model for both screening antiemetic agents as well as investigating emetic circuits between the brain and the GIT (Darmani, 1998; Darmani & Ray). The ECs are part of enteroendocrine system that are responsible for the production and storage of a large pool of peripheral serotonin in the GIT (Gershon &Tack, 2007). The involvement of gastrointestinal serotonin in the mediation of both phases of chemotherapy-induced vomiting as well as regulating the GIT motor activity and its secretory function via the enteric nervous system are well established (Darmani & Ray; Gershon & Tack). Although it is generally thought that duodenal serotonin plays a primary role in the mediation of chemotherapy-induced vomiting (Cubeddu *et al*., 2000; Davison & Andrews, 1993), more recent studies indicate that jejunal serotonin release may be of prime importance since peak jejunal 5-HT tissue concentration corresponds with peak mean vomit frequency following cisplatin-induced early and delayed emesis (Darmani *et al*., 2009). In the current study we have investigated the normal distribution of serotonin producing EC cells in the GIT of the least shrew. We demonstrate the distribution and the relative frequency of serotonin enterochromaffin cells in the least shrew GIT using immunohistochemistry to compare our findings with those published in other species.

In a semiquantitative study Yamada and co-workers (Yamada *et al*., 1999) have described the regional distribution of serotonin immunoreactive cells along the GIT of the common tree shrew (*Tupaia belangeri*). In this species, similar levels of serotonin immuno-reactivity were reported to be present in its gastric and intestinal mucosa along the entire length of the GIT except in the Brunner's glands in the proximal duodenum. Similar results have been found in several bat species (Marchado-Santos *et al*., 2009). In the present study, we describe the presence of IERCs throughout the GIT in a quantitative manner. The distribution of IERCs was variable in different regions of the least shrew GIT. In the gastric mucosa, IERCs were more concentrated in the lamina propria of the esophageo-gastric junction and

were associated with the esophageal sphincter. Likewise, Voutilainen *et al*. (2002) have found that in human gastric biopsy, serotonin-containing EC cells are located at the cardiac region of the gastric mucosa and regulate the function of the esophageal sphincter (Gershon & Tack). In the gastric body and pyloric regions, the serotonin IRECs were mainly found in the basal portion of the fundic glands, a few in the neck region of the gland and none were associated with the gastric lining epithelium. The latter is in contrast with EC cells scattered among the lining epithelium of human gastric biopsy samples (Penkova *et al*., 2010).

The shape of EC cells and their granules are not only varied among different species (Modlin *et al.*, 2006; Penkova *et al*.), but also affected by physiologic or pathologic status of the subject (Wheeler & Challacombe, 1984; Bian *et al*., 2007). Using thick frozen sections (60-80 μm), Kuramoto and coworkers (Kuramoto *et al*., 2007) have demonstrated that serotonin containing cells in the rat colon possess long cytoplasmic bipolar extension located along the body of the intestinal crypts. However, other investigators using thinner paraffin or frozen sections have failed to report such cytoplasmic processes (Glisic *et al.*, 2006). Kuramoto *et al.*, attributed this controversy to methodological differences in tissue section preparation. Our current data suggest that the serotonin-containing EC cells of the least shrew are rounded / ovoid in shape with a centrally located nucleus, and lack cytoplasmic processes regardless of the method of preparation.

In conclusion, the data presented here indicate that serotonin producing enterochromaffin cells in the least shrew are not different from those reported in human or other animal species. However, some differences were observed in the distribution of these cells which could be attributed to the physiological and dietary conditions of the animal.

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AL-TIKRITI, M. S.; KHAMAS, W.; CHEBOLU, S. & DARMANI, N. A. Distribución de las células enterocromafines serotonina-inmunorreactiva del tracto gastrointestinal de la musaraña enana (*Cryptotis parva*). ***Int. J. Morphol., 30(3)***:916-923, 2012.

RESUMEN: La serotonina es un importante neurotransmisor del sistema nervioso central (SNC) y periférico (SNP). Está implicado en una variedad de procesos fisiológicos, tanto en el intestino y el SNC. El presente estudio examinó la distribución de la serotonina contenida en las células enterocromafines del tracto gastrointestinal (TGI) de una especie competente al vómito, la musaraña enana. Estas células se reconocen fácilmente por sus gránulos globulares teñidas con H-E y la inmuno-tinción positiva para serotonina. Las células enterocromafines inmunorreactivas (CEI) se limitan principalmente a la parte basal del epitelio glandular y se distribuyeron por todo el estómago, intestino delgado e intestino grueso de la musaraña. Ninguna célula se encontró asociada al revestimiento epitelial mucoso. Además, su distribución y el recuento varió en diferentes regiones del TGI sugiriendo funciones específicas de estas regiones. La mayor concentración de CEI se encuentran en el colon seguido por el yeyuno. Números apreciables de CEI se encontraron en el estómago, especialmente en la unión esofago-gástrica. La ubicación de las CEI gástricas fue principalmente en la porción basal de la glándula. Sin embargo, algunas CEI se asociaron con las células parietales del estómago. Dos tipos de CEI se observaron, una con gránulos secretores globulares en su porción apical del citoplasma que se encuentra dentro de las células epiteliales glandulares que enfrenta el lumen glandular que liberan sus secreciones en el lumen, y el segundo se encuentra basalmente, frente a la lámina propia de la mucosa. Sus gránulos secretores no fueron diferentes en forma, y probablemente son más paracrinas en su modo de secreción.

PALABRAS CLAVE: Musaraña enena; Células enterocromafines de serotonina; Inmunohistoquímica; Estómago; Duodeno; Yeyuno; Íleon; Colon.

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