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The presence of 5-HT in myenteric varicosities is not due to uptake of 5-HT released from the mucosa during dissection: use of a novel method for quantifying 5-HT immunoreactivity in myenteric ganglia.

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Abstract

Background

Quantifying the relative abundance of different neurotransmitters in the myenteric plexus has proved challenging using conventional immunocytochemical approaches. Here, we present a new method of quantifying neurotransmitter content of an important enteric signalling molecule, serotonin (5-HT), in the myenteric plexus of guinea pig colon under different experimental conditions.

Methods

Sections of guinea pig distal colon were exposed to different conditions including changes in temperature, dissection protocol, stimulation with fecal pellet distension and exogenous 5-HT. Sections were fixed and immuno-labelled for 5-HT. 5-HT staining density was quantified within myenteric plexus ganglia using defined settings and an analysis approach that uses threshold settings allowing for variances in background and tissue staining intensities and which calculates the area of tissue containing 5-HT above these thresholds.

Key Results

No differences were found in 5-HT immunoreactivity in the myenteric plexus when compared between tissues that were freshly fixed, undissected, or with mucosa and submucous plexus dissected away at either 4°C or 37°C. Increased myenteric plexus 5-HT density was observed in preparations repeatedly stimulated using fecal pellet stimulation prior to fixation. Furthermore, exogenous 5-HT also increased 5-HT density.

Conclusions & Inferences

We demonstrate that quantitative differences in 5-HT immunoreactivity can be characterized using immunohistochemistry. This approach may be applied to measuring other neurotransmitter(s) within the enteric nervous system. While 5-HT is present in the guinea-pig enteric ganglia, this is not due to accumulation via *in vitro* handling and release from the mucosa, and furthermore, repeated colonic stimulation via distension increases 5-HT in the myenteric plexus.

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a hormone and neurotransmitter with a broad range of physiological functions. In the gastrointestinal (GI) tract, 5-HT is contained in both serotonergic neurons and enteroendocrine cells known as enterochromaffin (EC) cells. 5-HT plays important roles in the gut and this is supported by the multitude of effects on GI function of various 5-HT receptor agonists and antagonists (1). While EC cell-derived 5-HT was long thought to trigger peristalsis in the gut, dissection studies demonstrated that EC cell derived 5-HT is not responsible for initiating gut peristalsis (2) or motility (3) but may modulate the frequency of such events. We sought to determine whether an increased level of 5-HT in the myenteric plexus may occur during these types of experiments to potentially explain any such changes in contractile frequency.

This present study therefore focused on quantifying 5-HT in the myenteric plexus under a variety of conditions. To do this we developed a novel but straight forward protocol that facilitated quantification of 5-HT in the myenteric plexus using freely available software. This protocol should be applicable to the quantification of any transmitter or synaptic protein and takes into consideration variations in background and staining intensities across different images and experiments. Our results confirm that while the *in vitro* application of exogenous 5-HT results in a higher density of 5-HT in the myenteric ganglia (4), we observe no changes in 5-HT in relation to mucosal dissection, temperature or variations in time to allow accumulation of 5-HT. Most interestingly we observe a significant increase in 5-HT density in the myenteric ganglia after a period of colonic activity. Thus we illustrate a novel method for quantifying 5-HT in the myenteric plexus and demonstrate that 5-HT in the myenteric plexus is dynamically regulated by physiological stimulation.

Methods

Tissue preparation

Guinea pigs (350–500 grams) of either sex were killed humanely by stunning with a blow to the head followed by severing of the carotid arteries, approved by the Flinders University Animal Welfare Committee. An 8-cm-long segment of distal colon was excised from guinea pigs (taken 4–6 cm from the anus) and placed in Krebs solution ((in mM): 118 NaCl, 4.7 KCl, 1.0 NaHPO₄·2H₂O, 25 NaHCO₃, 1.2 MgCl₂·6H₂O, 11 D-glucose, and 2.5 CaCl₂·2H₂O), which was constantly bubbled with carbogen gas (95% O₂-5% CO₂) and maintained at the required temperatures. Tissue sections were taken from this and different treatments undertaken as outlined below. We included 7 different groups of tissue; freshly fixed directly after dissection to evaluate endogenous 5-HT in the myenteric plexus, intact colon maintained at

either 4°C or 37°C to assess the effect of temperature on 5-HT content, colon maintained at either 4°C or 37°C that had the mucosal layer and submucous plexus dissected away (2) to assess the effect of EC cell 5-HT on 5-HT density in the myenteric ganglia, colon in which a pellet was repeatedly passed through it or colon bathed in 100µM 5-HT in the bath solution. All groups (except freshly fixed) were maintained in these conditions for 2 hours.

Immunocytochemistry

Isolated segments of guinea-pig distal colon were fixed by pinning sheet preparations of colon under constant tension in a Sylgard lined Petri dish (Dow Corning Corp, USA) and immersing overnight in Zamboni's fixative (5% Formaldehyde and 15% saturated picric acid in 0.1M phosphate buffer; pH 7.2) at 4°C. Preparations were then cleared in dimethyl sulfoxide (10 min x3), tissue was washed in phosphate buffered saline (PBS); (0.2M sodium phosphate buffer, pH 7.2) and a whole mount of the myenteric plexus and longitudinal muscle was prepared by removing the mucosa, submucosa and circular muscle with the aid of a dissecting microscope. Goat 5-HT antisera (Immunostar, USA) was applied at a dilution of 1:1500 overnight at room temperature then washed in PBS (10 mins x 3). Tissue was then incubated for a further 2 hours in secondary antisera (Donkey anti Goat Cy3; Jackson Immunoresearch, USA) at a dilution of 1:400 then washed in PBS (10 mins x 3) and mounted in bicarbonate- buffered glycerol (pH 8.6).

Image Capture & Analysis

Images were captured on an IX-71 fluorescence microscope (Olympus, Australia) utilising the Analysis-FIVE imaging software (Soft Imaging System Corporation, USA) at 40x magnification and 200ms exposure time. Images were analysed using the Image J digital image analysis software (National Institute of Health, USA). Images were analysed by a three step process. First, the 'starting threshold' was set for each image. This was defined as a threshold value which captured all 5-HT staining within the ganglia while eliminating all background staining in the extra-ganglionic spaces. This value varied in each image based on staining and background intensity. The average starting threshold for all images was then calculated and used as the starting threshold for analysis of images across all groups. We took this value into consideration as it is a constant threshold that represents 5-HT staining across all experimental groups including optimally-labelled and poorly labelled images. Secondly, background intensity was measured in three regions within the extra-ganglionic spaces and averaged for each image; this was defined as the 'background intensity'. We obtained this background intensity value to take into account the inherent background labelling that occurs for a specific fixation protocol and to consider for the variability in background intensity across different

images and experimental conditions. Finally, the mean starting threshold value (constant for all images) was added to the background intensity (variable for each image) and defined as the 'working threshold' for each image. The working threshold value is a measure of the region(s) of positive 5-HT staining within the myenteric plexus which takes into consideration the variations in background intensity between images and/or samples. Once the working threshold was set for each image the percentage of 5-HT staining within a defined region of the ganglia was measured. Our protocol therefore does not measure staining intensity but rather the area that contains 5-HT that can be detected above the thresholds we have set. Thus it may be considered that we are measuring the density of 5-HT staining above a threshold rather than the level of 5-HT present in the tissue. For the measurements of fluorescence within ganglia, the region of interest for analysis was located at the vertical mid-point of the right-side border within a single ganglion, as demonstrated in (Figure 1D). Only on rare occasions was 5-HT immunoreactivity detected in cell bodies; and this only occurred in the samples that were incubated in exogenous 5-HT. In such instances we did not use these areas for our analysis. Percentages of 5-HT staining within the region of interest was measured from a single ganglion and in total 22 images were obtained from 22 different ganglia and averaged to provide a single data point for each sample. The data represents the percentage of the region of interest with 5-HT staining above the set threshold. This value was averaged across all samples for each treatment and the variation was represented in the standard error of the mean. A step-by-step method for undertaking this analysis within ImageJ is provided in the Supporting Information. The mean 5-HT staining in each experimental group was compared using a one-way analysis of variance (ANOVA) and post-hoc analysis undertaken using a Tukey's test. Statistical differences were taken at a minimum $p < 0.05$.

Results

We obtained a series of fluorescent images of 5-HT immunoreactivity from within colonic myenteric ganglia, under various experimental conditions. With each image we followed our image analysis protocol by measuring the image background across all images in order to set a working threshold for each image (Figure 1). We could then quantify the density of 5-HT immunoreactivity within a single ganglion and obtain a mean density for each sample, calculated from 22 single images obtained from each colonic preparation. We imaged 5-HT within the myenteric plexus under all experimental conditions (Figure 2A-F). Quantification using our protocol demonstrates no difference in 5-HT density between tissue that was either freshly fixed, or in which dissected or undissected tissue was bathed for two hours at either 4°C or 37°C (Figure 2G). Interestingly, we did observe significant increases in 5-HT staining in the myenteric ganglia in colon stimulated by intraluminal pellet distension, which triggered peristalsis, compared to the non-stimulated and freshly fixed groups ($p < 0.05$). Furthermore we demonstrate that the *in vitro* application of 5-HT to this

tissue with exogenous 5-HT in the bath results in 5-HT densities higher than the pellet run group ($p < 0.01$) and the unstimulated and freshly fixed groups ($p < 0.001$). We also wished to ascertain whether our quantification method can distinguish between subtle differences in 5-HT staining due to different fixation and permeabilisation methods. Other groups have used a paraformaldehyde fixation and Triton X-100 permeabilisation technique which often provides less background staining compared to the use of Zamboni's fixative as we have done in the present study (4-7). We quantified 5-HT staining in tissue from the same animals under control conditions fixed and permeabilised with these different methods (Supporting Information). We find that these two methods provide different intensities of staining and background (Figure S1). When this was quantified we detected much higher variability in 5-HT staining in the paraformaldehyde fixation group. We also see a moderate, but significant difference in 5-HT staining clearly demonstrating the ability of the quantification method to detect moderate differences in staining and highlighting the need to use identical fixation processes across all images being compared.

Discussion

In this study, we demonstrate a novel approach to quantifying 5-HT in the myenteric plexus using immunocytochemical images. We suggest that the quantification approach presented here is likely to be applicable to other neurotransmitter(s) or synaptic proteins. Our previous work has demonstrated that EC cell-derived 5-HT may act to modulate colonic peristalsis (2) and motility (3) although 5-HT in EC cells is clearly not required for either of these motor patterns (2, 3). Our current results also demonstrate that this is unlikely to be attributed to uptake of 5-HT into the myenteric plexus during dissection, since 5-HT accumulation does not occur in dissected or undissected preparations at either 4°C or 37°C. Our method of quantifying 5-HT in the myenteric ganglia is validated by the pronounced increase in 5-HT observed when tissue is bathed in exogenous 5-HT. While 5-HT uptake can occur with exogenously applied 5-HT, it does not occur in resting, dissected or unstimulated tissue at either ice-cold or physiological temperatures. 5-HT density in the myenteric ganglia does increase in response to pellet movement through the colon. What the source of this 5-HT is remains unclear. It may be derived from EC cell 5-HT being released by colonic peristalsis (2) and transported into the myenteric plexus. Another explanation may be that neuronal 5-HT transcription or synthesis may be controlled in an activity-dependent manner such as the well documented changes in CNS gene expression that occur in response to activity (8).. Our finding that 5-HT increases in an activity-dependent manner is worth considering in light of this as it raises the possibility that 5-HT may play a more important role in controlling gut motility as the level of gut motility increases.

In guinea-pig intestine, there has been some discussion regarding the presence of 5-HT in enteric ganglia. It has been proposed that perhaps 5-HT was only detected in enteric ganglia because it had accumulated 5-HT from other sources, such as the mucosa (9). In this study, we found that immediate fixation of the colon still revealed 5-HT was present in enteric ganglia. Importantly, the density of 5-HT was not found to be any different from those in either intact preparations, or preparations that had the mucosa dissected away and then maintained in either ice cold Krebs solution, or Krebs solution maintained at physiological temperatures. This study demonstrates that 5-HT is present in enteric ganglia of guinea-pig colon and that the amount of 5-HT can be dynamically altered as a result of stimulation of the gut wall. This result infers a changing role of 5-HT in the myenteric plexus during periods of gut activity, such as after a meal. Whether this is the case and what the exact nature of such roles is will hopefully be the focus of future investigations.

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Figure Legends

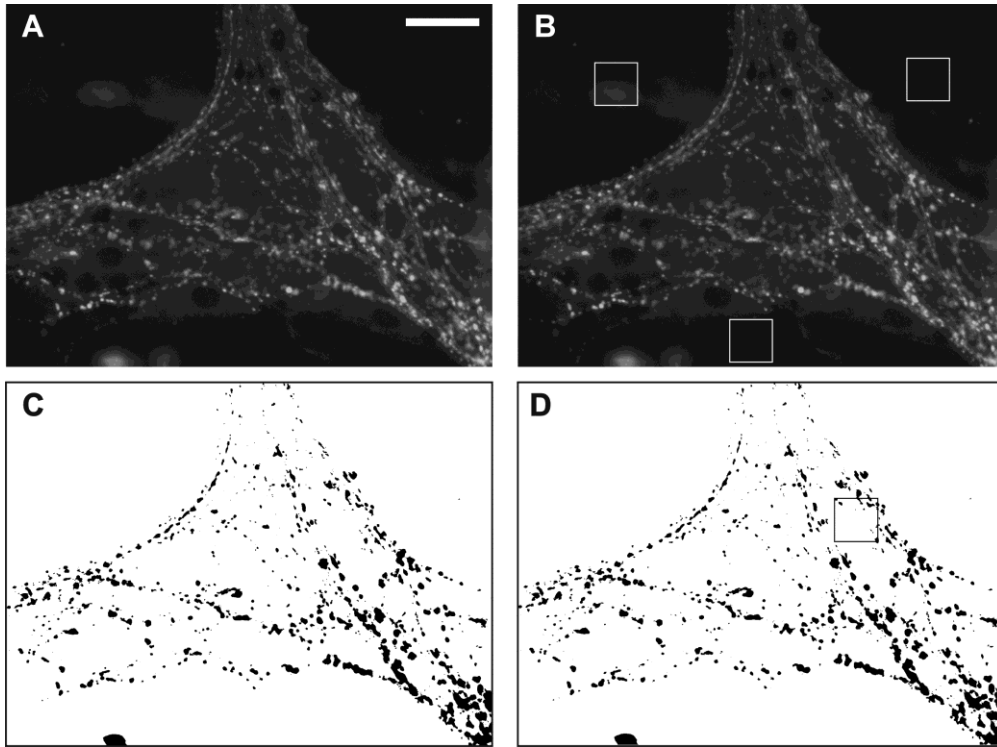


Figure 1: Method for quantifying 5-HT in the guinea pig colon myenteric ganglia from immunocytochemical images. (A) Original image captured using fluorescence microscopy. (B) Three regions of interest (boxes) of identical size measure the background staining of each image. (C) A working threshold is then set for this image combining the starting threshold for a single image with the visual threshold from all images. (D) The percentage of a single region of interest (box) within the ganglion above this threshold is then calculated to provide a measure of 5-HT staining in this sample. See methods for full details. Scale bar=30 μ m.

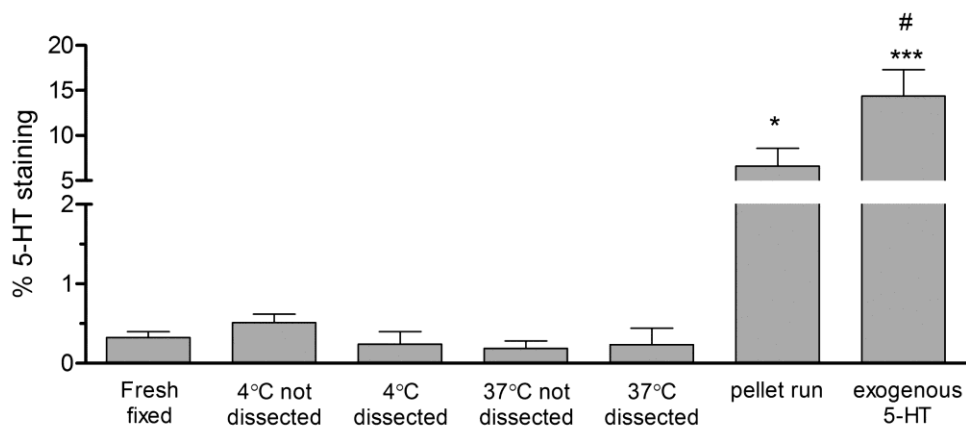
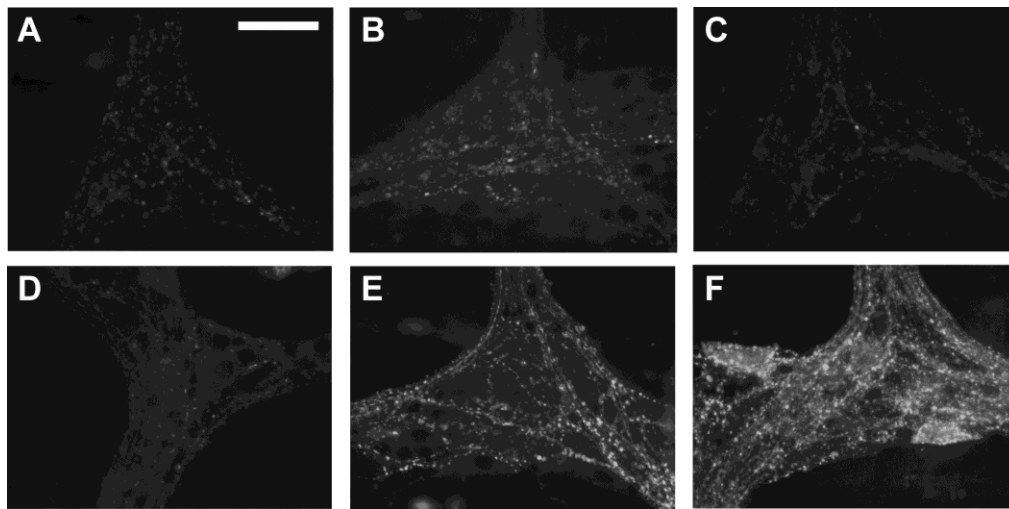


Figure 2: Quantifying 5-HT levels in colonic myenteric ganglia under various conditions. Fluorescence microscopy identified punctate 5-HT localisation in the myenteric plexus in (A) tissue freshly fixed, (B) undissected tissue at 4°C, (C) dissected tissue at 4°C, (D) undissected tissue at 37°C, (E) tissue with continuous pellet runs and (F) tissue with exogenous 5-HT in the bath. Scale bar=30µm. (G) Using this approach we see that myenteric ganglia exposed to either continuous pellet runs or exogenous 5-HT contain more 5-HT than the other treatment groups. N=4-5 animals and n=4-5 samples, *P<0.05 compared to all groups except the exogenous 5-HT group, **P<0.01 compared to the pellet run group, #P<0.001 compared to all other groups.

Supporting Information

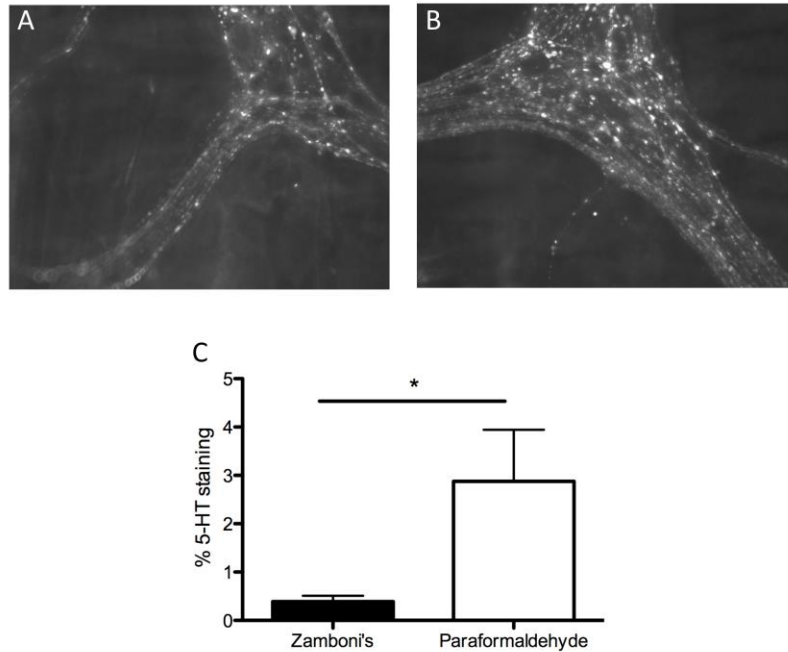


Figure S1: Comparison of 5-HT staining in identical colonic preparations using 2 different fixation/permeabilisation protocols. (A) image of myenteric plexus prepared using the fixation protocol identical to the method used in this manuscript while (B) is an image from tissue fixed in 4% paraformaldehyde (details below). (C) Quantification of 5-HT labelling in each group. Tissue in each group is from the same animals (n=5), *p<0.05.

Method for paraformaldehyde fixation of tissue

Full thickness guinea pig colon was opened along mesenteric border and pinned flat in a petri dish. Tissue was fixed for 2 hours at room temperature in 4% paraformaldehyde, cleared in DMSO (3 x 10 mins) and PBS (3 x 10 mins). The

mucosa, sub-mucosa and circular muscle was then removed and the remaining tissue permeabilised in PBS + 1% Triton X-100. 1.5 cm square preparations were cut and labelled with an antibody against 5-HT (Immunostar, USA) at 1:1500 dilution overnight. Tissue was then washed in PBS (3 x 10 mins) and the secondary antibody (DaG CY3, Jackson Immunoresearch) was applied at 1:400 dilution for 2 hours. Tissue was finally washed in PBS and mounted in bicarbonate buffered glycerol (ph 8.6).

Detailed method for the analysis of 5'HT stained images using ImageJ

ImageJ version 1.46r or later can be used for the analysis of these images. For the purpose of analysis all images should be taken at the same magnification and constant exposure time. Images may be in either an eight bit or sixteen bit format, but should be constant for the entire experiment.

Loading Images: File > Open > Select desired image from source folder. For an eight bit image the area should be 1392 x 1040 Arbitrary Units.

Measurement of Starting Threshold: With the image open: Image > Adjust > Threshold. The upper threshold (the second scroll bar) should always be at 255. Move the lower threshold scroll bar (the first scroll bar) from left to right (progressively increasing the lower threshold value) until all regions of 5'HT staining with the tissue have been captured on the image and no false positive staining (background or artefacts) has been captured. This value is defined as the starting threshold of this image.

Measurement of Background Intensity: Select the rectangular drawing tool and draw a square in a region within the tissue with no 5-HT staining. The area of the square will vary depending on the type of tissue, magnification used for image capture and the staining patterns of various antibodies. The area should be carefully set prior to beginning the analysis and should take into consideration variations across treatment groups. For the analysis described in this paper a square with dimension 0.5 x 0.5 (Arbitrary Units) was used for the measurement of background intensity. Mean background intensity within the defined square can be measured by clicking Analyse > Measure. In the results table the "Mean" column describes the mean fluorescence intensity within the square. Background intensity should be measured from three regions within the image and averaged for each image.

Measurement of percentage 5-HT staining: Adjust the threshold of the image by moving the upper threshold (second scroll bar) to 255 and the lower threshold (first scroll bar) to the working threshold value (starting threshold + mean background intensity). Once the threshold has been set draw a square to define the region of interest to be measured and click Analyse > Measure. In the results table the value in the "% Area" column is the percentage of 5-HT staining within the defined region. For the purpose of this analysis the region of interest was a square 0.5 x 0.5 and was within the myenteric plexus.