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IS SEROTONIN IN ENTERIC NERVES REQUIRED FOR DISTENSION-EVOKED PERISTALSIS AND PROPULSION OF CONTENT IN GUINEA-PIG DISTAL COLON?

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Abstract—Recent studies have shown genetic deletion of the gene that synthesizes 5-HT in enteric neurons (tryptophan hydroxylase-2, Tph-2) leads to a reduction in intestinal transit. However, deletion of the Tph-2 gene also leads to major developmental changes in enteric ganglia, which could also explain changes in intestinal transit. We sought to investigate this further by acutely depleting serotonin from enteric neurons over a 24-h period, without the confounding influences induced by genetic manipulation. Guinea-pigs were injected with reserpine 24 h prior to euthanasia. Video-imaging and spatio-temporal mapping was used to record peristalsis evoked by natural fecal pellets, or slow infusion of intraluminal fluid. Immunohistochemical staining for 5-HT was used to detect the presence of serotonin in the myenteric plexus. It was found that endogenous 5-HT was always detected in myenteric ganglia of control animals, but never in guinea-pigs treated with reserpine. Interestingly, peristalsis was still reliably evoked by either intraluminal fluid, or fecal pellets in reserpine-treated animals that also had their entire mucosa and submucosal plexus removed. In these 5-HT depleted animals, there was no change in the frequency of peristalsis or force generated during peristalsis. In control animals, or reserpine treated animals, high concentrations (up to 10 μM) of ondansetron and SDZ-205-557, or granisetron and SDZ-205-557 had no effect on peristalsis. In summary, acute depletion of serotonin from enteric nerves does not prevent distension-evoked peristalsis, nor propulsion of luminal content. Also, we found no evidence that 5-HT3 and 5-HT4 receptor activation is required for peristalsis, or propulsion of contents to occur. Taken together, we suggest that the intrinsic mechanisms that generate peristalsis and entrain propagation along the isolated guinea-pig distal colon are independent of 5-HT in enteric neurons or the mucosa, and do not require the activation of 5-HT3 or 5-HT4 receptors. © **2013 Published by Elsevier Ltd. on behalf of IBRO.**

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Abbreviations: CMMCs, colonic-migrating motor complexes; EC, enterochromafiin; GI, gastrointestinal; PBS, phosphate-buffered saline; Tph-2, tryptophan hydroxylase-2.

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INTRODUCTION

Antagonists of 5-HT3 receptors, such as ondansetron (Zofran) and alosetron (Lotrinex), have been commonly prescribed in clinics for relief of symptoms in patients with diarrhea-predominant irritable bowel syndrome (D-IBS). While it is clear that these antagonists can prolong gastrointestinal (GI) transit in both humans and laboratory animals, the mechanisms by which these drugs inhibit motility and reduce transit is still poorly understood. The long standing presumption is that 5-HT antagonists inhibit GI-motility and prolong GI-transit by preventing endogenous 5-HT from activating 5-HT receptors on enteric nerves or enterochromafiin (EC) cells in the mucosa (see Galligan et al., 2000; Gershon, 2000; Grundy, 2008 for review). Indeed, there is substantial circumstantial evidence to support a role for endogenous 5-HT and/or 5-HT receptors in the control of GI-motility, in healthy and diseased bowel (see Gershon, 2000). This is based largely on the fact that the largest quantity of serotonin in the body is synthesized in the intestinal mucosa (Erspamer, 1954) and high concentrations of 5-HT can be dynamically released from the mucosal layer of the colonic wall (Keating and Spencer, 2010; Spencer et al., 2011). In addition, 5-HT-mediated synaptic potentials have been reported to occur in a small subset of enteric neurons (Galligan et al., 2000; Nurgali et al., 2003a; Furness, 2006) and, finally, a wide variety of antagonists of 5-HT receptors can potently inhibit, or block peristalsis and reduce transit (Grider et al., 1996; Kadowaki et al., 1996).

Despite abundant circumstantial evidence that endogenous 5-HT is involved in the control of GI-motility, the idea that endogenous 5-HT plays a major role in peristalsis and the control of GI-transit was substantially revised in the last couple of years, in light of recent findings from two different laboratories. The first of these studies employed the first real time recordings of 5-HT release from the colonic mucosa during peristalsis (Spencer et al., 2011) and showed that when the mucosa was completely removed from the colon, it abolished all release of 5-HT, but did not

prevent the initiation of peristalsis (Spencer et al., 2011), nor the generation of colonic migrating motor complexes (CMMCs) (Keating and Spencer, 2010). This showed that the release of 5-HT from the mucosa was a consequence of peristalsis and CMMCs; and was clearly not the underlying cause of their generation (Keating and Spencer, 2010; Spencer et al., 2011). In support of this, another independent study demonstrated that selective inhibition of 5-HT synthesis from EC cells within the mucosa abolished 5-HT levels *in vivo*, but did not have any effect on transit time, gastric emptying or colonic motility (Yadav et al., 2010; Li et al., 2011). These recent findings are significant because the vast majority of 5-HT in the body (>95%) is synthesized within the intestinal mucosa, with only minor quantities synthesized in the enteric nervous system. In fact, fewer than 1% of enteric neurons synthesize 5-HT (Costa et al., 1996) and studies have shown that 5-HT-mediated fast synaptic potentials are rare in guinea-pig enteric neurons (Bornstein et al., 2004; Furness, 2006) and have never been detected in the mouse colon (Furukawa et al., 1986; Nurgali et al., 2004), rat small intestine (Brookes et al., 1988b) or human colon enteric neurons (Brookes et al., 1988a), where all fast excitatory postsynaptic potentials (FEPSPs) have been shown to be abolishd by nicotinic antagonists. In light of the difficulty in identifying fast synaptic transmission in enteric neurons that is mediated by endogenous 5-HT, it is puzzling why antagonists of 5-HT3 receptors can potently inhibit or abolish GI motility patterns in these species (Balfour et al., 2000; Bush et al., 2001).

Since recent studies have shown that removal of the mucosa does not prevent peristalsis (Spencer et al., 2011), or CMMCs *in vitro* (Keating and Spencer, 2010) and has no inhibitory effect on GI-transit *in vivo* (Yadav et al., 2010), this raises the fundamental question as to whether neuronal 5-HT plays an important role in the control of GI-motility of mammals? Could it be that mucosal 5-HT in the body has little effect (Keating and Spencer, 2010), or no effect on colonic motility (Yadav et al., 2010), but that neuronal 5-HT plays a major role? A recent study showed that genetic deletion of the enzyme that synthesizes 5-HT in enteric neurons Tph-2 leads to an increase in intestinal and colonic transit, suggesting that neuronally synthesized 5-HT may be important for regulating GI-propulsion (Li et al., 2011). However, in these knockout mice, there were major developmental and neurochemical changes that occurred in their enteric nervous system (Li et al., 2011), which could also have led to the changes in transit. Therefore, at present, it is still unclear whether the changes in GI-transit reported are due to the depletion of enteric neuronal 5-HT, or the development deficits that occurred in the enteric ganglia, as a consequence of deletion of the Tph-2 gene. This prompted us to determine whether the acute depletion of enteric neuronal 5-HT would lead to changes in colonic motility, but without the confounding influences caused by genetic deletions of specific genes.

EXPERIMENTAL PROCEDURES

Preparation of tissues

Adult male guinea pigs (350–450 g), were killed by a blow to the occipital region and exsanguinated, in a manner approved by the Animal Welfare Committee of the Flinders University. The distal colon (5–10 cm from the anus) was removed and placed in warm Krebs solution which was constantly bubbled with carbogen gas (95% O_2/5% CO_2). After a period of time (usually <20 min), fecal pellets naturally present were expelled from the colon. A segment of distal colon (6 cm in length) was mounted in an organ bath and left to equilibrate for 30 min. After this time, video imaging or mechanical recordings were made from the circular muscle using the protocol described below.

Technique to deplete endogenous 5-HT from the enteric nervous system

The enteric nervous system was depleted of endogenous 5-HT using the technique first demonstrated by Costa et al. (1982). This involves a single intraperitoneal injection of reserpine (at a concentration of 0.5 mg/kg) between 18 and 24 h prior to euthanasia. 5-HT immunoreactivity is not detected in the enteric nervous system after this procedure (Costa et al., 1982). However, because reserpine does not deplete 5-HT from the mucosa, we further employed our recently published method (Spencer et al., 2011) to remove the mucosa, submucosa and submucosal plexus by sharp dissection from the colon. This allows us to test whether acute depletion of 5-HT from enteric nerves and the absence of the mucosa and submucosal plexus impair colonic motility, without potential complications such as compensation induced in genetically modified animals.

Terminology used to define different types of preparations

Throughout the results we refer to control preparations as those which were not treated with reserpine and had their mucosa and submucosal plexus present. We refer to ‘‘reserpine treated’’ preparations as those which have been treated with reserpine and which also have their mucosa and submucosal plexus removed. Therefore, reserpine-treated preparations are devoid of all known sources of endogenously synthesized 5-HT.

Protocol used to measure fecal pellet propagation

To induce peristalsis *in vitro*, using fecal pellets we followed our recent protocol (Spencer et al., 2011). In brief, natural fecal pellets that had been expelled from guinea-pigs weighing between 350 and 450 g were obtained and coated in epoxy resin to form a hard coat. A single pellet was gently inserted into the oral end of colon every 5 min and the characteristics of peristalsis noted. The propagation velocity of inserted pellets was determined from spatio-temporal maps. In these cases, the propagation of pellets along the colon was characterized as either continuous (i.e. did not pause propagation at any point), or showed a staggered propagation – where pellets remained stationary for periods $\geqslant$10 s, >60 s, >5 min, or where inserted pellets did not propagate at all after insertion. Only preparations which showed continuous propagation of pellets were included in the analysis of propagation velocity.