Title: Electrophysiological Characterization of Human Rectal Afferents

Short Title: Electrophysiology of Human Rectal Afferents

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ABSTRACT

Introduction:
It is presumed that extrinsic afferent nerves link the rectum to the central nervous system. However, the anatomical / functional existence of such nerves has never previously been demonstrated in humans. Therefore, we aimed to identify and make electrophysiological recordings in vitro from extrinsic afferents, comparing human rectum to colon.

Methods:
Sections of normal rectum and colon were procured from anterior resection and right hemicolecctiony specimens, respectively. Sections were pinned and extrinsic nerves dissected. Extracellular visceral afferent nerve activity was recorded. Neuronal responses to chemical (capsaicin and ‘inflammatory soup’ [IS]) and mechanical (Von Frey probing) stimuli were recorded and quantified as peak firing rate [range] in one-second intervals.

Results:
28 separate nerve trunks from 8 rectums were studied. Of these, spontaneous multi-unit afferent activity was recorded in 24 nerves. Peak firing rates increased significantly following capsaicin (median 6 [range 3-25] spikes/sec vs. 2 [1-4], P<0.001) and IS (median 5 [range 2-18] spikes/sec vs. 2 [1-4], P<0.001). Mechanosensitive ‘hot-spots’ were identified in 16 nerves (median threshold 2.0g [range 1.4–6.0g]). In 8 of these, the threshold decreased after IS (1.0g [0.4–1.4g]). By comparison, spontaneous activity was recorded in only 3/30 nerves studied from 10 colons and only one ‘hot-spot’ (threshold 60g) was identified.
Conclusions:

This study confirms the anatomical / functional existence of extrinsic rectal afferent nerves and characterizes their chemo- and mechano-sensitivity for the first time in Man. They have different electrophysiological properties to colonic afferents and warrant further investigation in disease states.

NEW & NOTEWORTHY

This study confirms the existence of extrinsic nerves supplying the human rectum for the first time and demonstrates differences in the sensory innervation between the rectum and colon with rectal afferents being more mechanically and chemically sensitive than colonic afferents. As sensitization of gut afferent pathways appears important in the development of chronic pain in patients with functional bowel disorders, this ‘in vitro’ model will allow evaluation of potential therapeutic agents on human visceral afferents.

Keywords: Electrophysiology; Human; Rectal Afferents
INTRODUCTION

The importance of normal function of the human rectum is often only truly appreciated when the impact of symptoms resulting from dysfunction of this highly specialized, terminal segment of the gastrointestinal tract is considered. Abnormal sensation appears to be particularly relevant in the development of rectal dysfunction and is most obvious, in the clinical setting, in the context of inflammatory bowel disease where heightened sensation (rectal hypersensitivity)(18) occurs following mucosal and / or transmural inflammation. Inflammatory sensitization often manifests as an irrepressible urge to defecate, leading to severe faecal urgency, frequently associated with episodes of incontinence(18). Further, stimulation of nociceptive afferents may contribute to the development of pain(16). However, abnormal rectal sensation can affect function in the absence of organic disease, manifesting as disorder(s) of evacuation and / or storage of feces. Rectal hyposensitivity(22, 23) and hypersensitivity(11) are prevalent and are associated with chronic constipation and / or faecal incontinence. Abnormal rectal sensitivity has also been proposed as a biomarker of the irritable bowel syndrome(36, 38). Collectively, these functional bowel disorders account for up to 41% of diagnoses in specialty practices(13) and have been estimated to affect over one-third of the Western adult population in community studies(28). Additionally, a significant proportion of patients develop hindgut dysfunction following complete or partial excision of the rectum (proctectomy), termed ‘anterior resection syndrome’(42), which has been in part attributed to disturbances of (neo)rectal sensory function(9).

Despite the functional and clinical importance of the rectum, detailed understanding of normal rectal physiology, particularly its sensorimotor function remains incompletely understood and thus the ability to manage these clinical conditions remains suboptimal. The rectum provides graded sensory information to the brain, reflecting varying degrees of distension(18, 37, 45), pointing to the existence of a specialized extrinsic afferent pathway in humans. However, early histopathological
studies failed to identify specific receptors in the human rectum(17), leading to the belief that it lacked specialized sensory receptors and that ‘rectal sensation’ was mediated solely by nerve-endings and receptors in adjacent pelvic structures and pelvic floor musculature(39), particularly since patients still experience sensations of fullness and impending defaecation following excision of the entire rectum (complete proctectomy) and coloanal anastomosis(29, 44). However, it has come to be appreciated that these ‘neorectal’ sensations are not typical of normal rectal filling(44). Thus, awareness of filling is preserved after the anorectum is transposed on its neurovascular pedicle to the anterior abdominal wall(46) (despite being anatomically distinct and remote to the pelvis), strongly suggesting that the rectum (and / or its mesentery) itself is innervated by extrinsic afferent nerves.

The anorectum is innervated by somatic and autonomic pathways(8) and animal studies have demonstrated that the hindgut is innervated via two afferent populations that arise from different levels of the spinal cord (thoracolumbar and lumbosacral)(14). However, studies have shown that the rectum receives different classes of extrinsic sensory neurons to the colon(6) and this includes specialized low-threshold mechanoreceptors(32), several classes of high-threshold nerve endings and mucosal receptors (1, 6). Specialized rectal afferent nerve endings (rectal intraganglionic laminar endings [rIGLEs]) are the sites of mechanotransduction of low-threshold mechanoreceptors in the guinea pig rectum(32). Comparable studies of human rectal preparations have not been carried out, although electrophysiological studies have recorded sensory nerve activity from the human colon and appendix in vitro(35). Additionally, two very recent studies have utilized ex vivo electrophysiological recording techniques to identify functionally distinct subpopulations of human visceral afferents, from different parts of the lower gastrointestinal tract (including the ileum). However, much of the focus was again placed on colonic tissue (33, 48). Therefore, this study aimed to identify and record visceral afferent activity from extrinsic nerves supplying the human rectum in vitro and to characterize their response to mechanical and chemical stimulation, making
comparison between rectal and colonic afferents.
MATERIALS AND METHODS

Subjects

This study was approved by the Sydney Local Health District Human Research Ethics Committee (LNR/12/CRGH/41). Fresh, non-inflamed rectal and colonic tissues were procured from specimens of consecutive patients undergoing elective anterior resection (proctectomy) or right hemicolectomy surgery for malignant or benign pathology between July 2013 and November 2013. Exclusion criteria included patients: (i) younger than 18 years; (ii) where there was possibility of a functional disorder (e.g. slow transit constipation, rectal prolapse); and (iii) previously diagnosed with inflammatory bowel disease.

Tissue Procurement

Specimens were resected according to standard surgical protocol. Immediately following specimen removal, full-thickness circumferential sections (1 to 2 cm width) of rectal or colonic tissue with attached mesentery (>3 cm) were isolated from the resection margins. Specifically, rectal tissue was acquired from the distal (aboral) portion of an anterior resection (proctectomy) specimen, and colonic tissue from either the proximal (oral) end of anterior resection (proctectomy) specimen or the distal (aboral) end of a right hemicolectomy specimen (Figure 1). All tissue samples obtained were macroscopically free of disease. If scattered diverticula were evident in left-sided specimens, these were avoided in specimen procurement. Tissues were then immediately immersed in Krebs solution (in mmol/L: NaCl 118; KCl 4.75; NaH₂PO₄ 1.0; NaHCO₃ 25; MgSO₄ 1.2; CaCl₂ 2.5; glucose 11; Sigma Aldrich, NSW, Australia) at room temperature to minimise ischemia time; the Krebs solution had been pre-oxygenated by bubbling with carbogen (95% O₂ / 5% CO₂; BOC, NSW, Australia). Tissues were then transported directly to the laboratory.
**Tissue preparation**

On arrival in the laboratory, tissues were transferred to a petri dish lined with Sylgard (Dow Corning, Dow Corning Corp., Midland, MI, USA) and oriented with respect to its mesentery. Epiploic fat was excised from each specimen, and the tissue opened by incising longitudinally along its anti-mesenteric border such that the mesentery lay along one edge of the open preparation. The preparation was pinned flat (mucosa side up) using stainless steel pins (200 μm diameter; Australian Entomological Supplies, NSW, Australia). Under a dissecting microscope (Olympus, Victoria, Australia), the mucosa was removed by sharp dissection from its submucosal attachments, and then the tissue was turned over and re-pinned serosa upward (Figure 2).

**Mesenteric nerve dissection**

Using a dissecting microscope, fine paravascular extrinsic nerve trunks were dissected free from surrounding connective tissue (Figure 2). Between 6 to 10 nerve trunks were isolated for a length of 10 to 20 mm, then pinned using gold-plated tungsten pins (50 μm diameter; Goodfellow, PA, USA). Finally, the bowel wall was cut down to dimensions of 20 mm × 20 mm according to the distribution of nerves isolated. During the entire dissection, the Krebs solution contained within the petri dish was regularly exchanged for fresh oxygenated solution, approximately every 10 minutes.

**Electrophysiology**

The entire preparation was transferred to a Sylgard-lined organ bath containing a nerve-recording chamber (Figure 3), machined from clear acrylic. The organ bath was continually superfused at 5 mL per minute with oxygenated Krebs solution. The preparation was pinned with steel pins (200 μm) along three sides, with the free edge connected to an array of hooks, attached to a pulley-weight system applying a 20mN load. The dissected mesenteric nerves were led into the recording chamber and the ends fixed with 50 μm tungsten pins. A thin strand of connective tissue was also led into the chamber and attached to a reference electrode. The recording chamber was then sealed.
with a coverslip sealed with silicon grease (Ajax, Taren Point, Australia). Krebs solution was then removed from the recording chamber and replaced with paraffin oil. The entire organ bath was then placed on a water-perfused heating plate to achieve a temperature of 34°C within a Faraday cage.

The mesenteric nerves were recorded via a silver hook electrode attached to a low-noise AC-coupled differential amplifier (gain × 10,000), band pass filtered from 0.3 to 10 kHz (World Precision Instruments, Florida, USA), and sampled at 20 kHz via a CED 1401 interface (Cambridge Electronic Design, Cambridge, United Kingdom). Data were stored on an IBM-compatible personal computer for off-line analysis. The amplified signal was also used for on-line audio monitoring.

Assessment of tissue mechano- and chemo-sensitivity

The following features were recorded in a standardized protocol: (i) spontaneous afferent nerve activity (for 5 minutes); (ii) response(s) to mechanical stimulation; (iii) response(s) to chemical stimulation; and (iv) response(s) to mechanical stimulation post-chemical stimulation. Mechanical stimulation was assessed by systematically probing the tissue at 5mm intervals with von Frey hairs of varying force (0.05g to 60g). A ‘descending methods of limits’ was employed, beginning with 60.0 grams force and progressively decreasing in increments as defined by the Semmes-Weinstein monofilament set(30). Von Frey hairs were applied to the preparations in a grid extending over the full width and length of the preparations. Small (typically less than 200 μm in diameter), responsive sites, where firing was evoked, were identified as ‘hot-spots’, and their position marked on the tissue with fine carbon particles attached to the tip of the von Frey hair. The ‘threshold’ for each ‘hot-spot’ was determined.

Responses to chemical stimulation were assessed by superfusing the main chamber with: (i) a hyperkalemic Krebs solution ([K+] = 6 mM) with an ‘inflammatory soup’ (IS; bradykinin, serotonin, and histamine from frozen aqueous aliquots and prostaglandin E2 dissolved in
dimethylsulfoxide (19) all at 10 µM final concentration); and (ii) Krebs solution containing capsaicin (10 µM from ethanolic stock solution) applied at the end of the study protocol to avoid desensitization. The preparation was systematically re-probed with von Frey hairs following exposure to IS to assess sensitisation of receptors. Specifically, ‘hot-spots’ identified on initial probing (as marked by carbon particles) were re-assessed and changes in threshold and emergence of new ‘hot-spots’ were determined. This protocol was employed for each nerve trunk recorded. Between recordings, chamber was superfused with oxygenated Krebs for at least five minutes to flush away any residual chemical stimulants.

**Nerve activity analyses**

Action potentials were analyzed off-line using Spike2 software (version 7, CED, Cambridge, United Kingdom). Actions potentials were discriminated by waveform characteristics; the threshold was set to the smallest identifiable spike. Nerve activity was expressed as a rate histogram with one-second bin widths or as instantaneous frequency plots. Firing was analyzed according to (i) peak firing rate, defined as the maximum number of action potentials in a one second bin; and (ii) mean firing rate, defined as the average number of action potentials per second averaged over 10 seconds. Paired comparisons of these firing rates were compared to baseline rates using the Wilcoxon signed-rank test with significance set at P < 0.05.

Firing was also compared between rectum and colon; to minimise bias, discrimination of action potentials and calculation of firing rates was performed by an observer (KSN) blinded to the tissue type or treatment. Discrimination parameters were then cross-checked by an independent reviewer (DM), who was similarly blinded to the tissue type and treatment.

**Electrical stimulation**

In preparations where there was neither spontaneous firing nor mechanically-induced firing, focal
electrical stimulation was used to confirm nerve integrity from evoked compound action potentials with appropriate latencies; this check was performed for six colonic nerves (randomly selected) where nerve activity was not initially recorded. A round-tipped concentric electrode (external diameter 0.55mm, internal diameter 0.125mm; FHC, Bowdoin, ME, USA, 0.5 ms pulse duration, 0.3Hz, up to 10mA) was micromanipulated in light contact with the tissue. A stimulus intensity of 10mA was used to excite afferent endings. The electrode was moved systematically (2 – 3 mm steps) across the preparation until action potentials were recorded. Then, electrode position was adjusted to pinpoint the site of maximum activation. Conduction velocity was calculated from the straight-line distance between the stimulating cathode and the recording site, divided by the latency of the compound action potential. A conduction velocity of less than 2 meters per second was considered to reflect activation of afferent C-fibers.
RESULTS

Description of Tissues Procured

Colonic and rectal tissues were available from consecutive resections, as no patients declined participation in the study. All tissues appeared macroscopically normal on arrival in the laboratory, evidenced by their color and the spontaneous contractility. Overall, eight rectums and ten colons were procured for the study, from which 28 rectal and 30 colonic nerves were studied, respectively. The patients from whom rectal and colonic tissues were procured were comparable for age (rectum: median 67 years [range 38 – 82 years]; colon: 73 years [57 – 82 years], P = 0.244) and gender (rectum: 6 males; colon: 7 males, P = 0.618) (Table 1).

Of rectal samples \((n=8)\), seven patients underwent surgery for rectal or sigmoid adenocarcinoma and one for diverticular disease. All colonic samples \((n=10)\) were from patients undergoing surgery for adenocarcinoma. The distance of rectal samples from the anal verge was variable, as dictated by the surgical extent of the resection. Five samples were obtained from the upper rectum \((11 – 15 \text{ cm from the anal verge})\), one from the mid-rectum \((6 – 10 \text{ cm})\), and two from the low-rectum \((1 – 5 \text{ cm})\). Of the ten colonic samples procured, five were from the proximal transverse colon at the aboral end of right hemicolectomy specimens, and five were from the distal descending colon at the oral end of anterior resection (proctectomy) specimens. Two (of eight) patients from whom rectum was procured received neoadjuvant radiotherapy (Table 1). Whilst no patients had any formal documented neurological conditions, two rectal and two colonic samples were taken from patients with type II diabetes mellitus without associated neurological complications.

Spontaneous Nerve Activity

A typical trace of spontaneous activity is shown in Figure 4A, with characteristic irregular spontaneous firing, which always persisted throughout the initial 5 minutes of recording, although occasional periods of quiescence without neuronal activity for up to 30 seconds were occasionally
observed. Spontaneous firing was recorded in 24 of the 28 rectal nerves studied, with nerve activity recorded in nerve trunks from all patients ($n=8$). The median mean firing rate (calculated over 10s) was 0.4 Hz (range 0.0 – 0.5 Hz), and the median peak firing rate was 2 Hz (range 1 – 4 Hz). By contrast, spontaneous firing was recorded from only three of the 30 colonic nerves studied in 3 of 10 patients. Spontaneous colonic nerve activity was noted to be less intense than rectal nerves, with a median mean firing rate of 0.14Hz (calculated over 10s; range 0.14 – 1.12 Hz) and a median peak firing rate of 1 Hz (range 1 – 4 Hz).

**Nerve Responses to Mechanical Stimulation**

Typical responses to mechanical probing of a mechanosensitive ‘hot-spot’ are shown in Figures 4B and 4C, with reproducible firing on repeated probing, even after several minutes. ‘Hot-spots’ were typically punctate and recorded activity often appeared to be multi-unit rather than single unit, reflecting firing by multiple neurons. Of the 28 rectal nerves studied, 18 ‘hot-spots’ were identified in 16 nerves. The median threshold probing force required to elicit nerve activity was 2.0 grams (range 1.4 – 6.0g), and firing rates varied with force (i.e. nerve discharges decreased as probing force decreased towards threshold), as shown in Figure 4C. Generally, only one ‘hot-spot’ was identified per nerve; however, in two nerves, two separate ‘hot-spots’ were identified. By contrast, only one ‘hot-spot’ was identified from the 30 colonic nerves trunks recorded, which had a threshold of 60 g to elicit firing.

**Nerve Activity Following Chemical Stimulation**

In many nerves studied, chemical stimulation (either by application of an inflammatory soup or capsaicin) resulted in an increase in firing. Responses of rectal nerves to chemical stimulation are presented quantitatively in Figures 5 and 6. Of the rectal nerves with spontaneous firing (24 nerve trunks), application of IS resulted in a significant increase in mean neuronal firing from 0.37 Hz (median) (range: 0.02 – 0.54 Hz) to 0.53 Hz (range 0.05 – 1.02 Hz); P<0.001. In addition, peak
firing rates increased from 2 Hz (range 1 – 4 Hz) to 5 Hz (range 2 – 18 Hz); P<0.001 (Figure 5). In rectal nerves where spontaneous activity was not present, application of IS did not induce any discernible nerve activity.

Application of capsaicin consistently evoked a significant increase in nerve activity in rectal nerves where spontaneous activity was initially recorded (n = 24). Specifically, mean firing rate increased from 0.35 Hz (median) (range 0.02 – 0.68) to 0.62 Hz (range 0.12 – 2.23) (P<0.001) and peak firing rates increased from 2 Hz (range 1 – 4 Hz) pre-capsaicin to 6 Hz (range 3 – 25 Hz) post-capsaicin (P<0.001) (Error! Reference source not found.). Application of capsaicin did not induce firing in rectal nerves where spontaneous activity was not present.

Colonic nerve activity following chemical stimulation

In the three colonic nerves where spontaneous activity was recorded, application of IS increased mean neuronal firing from 0.14 Hz (median) (range 0.14 – 1.12) to 0.19 Hz (range 0.13 – 1.49 Hz). Peak firing increased from 1 Hz (range 1 – 4 Hz) to 3 Hz (range 2 – 8 Hz). The significance of this observation is difficult to interpret given the small number of observations. In one case, application of IS evoked firing (peak firing rate of 2 Hz) in a colonic nerve that lacked spontaneous firing. Capsaicin also increased mean firing rate from 0.15 Hz to 0.23 Hz (n=3). Peak discharge rate increased from 2 to 9 Hz in one of the three colonic nerves where spontaneous activity was initially recorded; in the other two colonic nerves, there was no increase in peak neuronal firing rate. As observed with IS, capsaicin evoked nerve activity in one colonic nerve trunk that had previously had no spontaneous firing (a different trunk to that activated by IS).

Mechanosensitivity to von Frey hairs after IS

Overall, there was a significant decrease in the mechanosensitivity thresholds during von Frey hair probing following chemical stimulation with IS (median pre-IS 2.0g [range 1.4 – 6.0g], median
post-IS 1.4g [range 0.4 – 6.0g]; P = 0.011). More specifically, the threshold was decreased in eight of the 18 ‘hot-spots’ identified in rectal tissue following chemical stimulation with IS (median pre-IS threshold: 1.7g [range 1.4 – 2.0]; median post-IS: 1.0g [range 0.4 – 1.4]). Additionally, three additional ‘hot-spots’ were identified on probing following IS application that were not identified prior to chemical stimulation (median threshold post-IS 1.4g). In the one colonic nerve where a ‘hot-spot’ was identified, application of IS did not alter the threshold force of probing required to elicit nerve activity.

Electrical Volley

A random sample (n=6) of colonic nerves which lacked both spontaneous and chemically-stimulated nerve activity was investigated using electrical stimulation. Suprathreshold electrical stimulation applied to the surface of the preparation evoked compound action potentials at small punctate sites. Typically, as the electrode was moved away from the center of a stimulation site, the electrical threshold increased abruptly. Conduction velocities calculated from compound action potentials were relatively consistent (mean: 1.1 m/s; range 0.8 – 1.5 m/s), suggesting the presence of axons conducting in the C-fiber range.
This study is the first to develop an *in vitro* model to successfully record afferent activity from nerves supplying the human rectum, and thus, functionally demonstrate human rectal innervation via extrinsic nerves. There were demonstrable differences in the sensory innervation between the rectum and colon with rectal afferents being more mechanically and chemically sensitive than colonic afferents. Much of our current understanding of the electrophysiology of rectal afferent nerves was based on findings from animal studies with tracing and immunocytochemical studies revealing the distribution of afferent fibers and their terminals within the gut wall(15, 21). Indeed, elegant *in vitro* studies of mouse colon and rectum have provided a sophisticated description & electrophysiological classification of visceral afferent fibers, with those responding to: (i) contraction and physiological distension (<20 mmHg) being classified as ‘muscular’; (ii) fine mucosal stimulation (e.g. stroking) being referred to as ‘mucosal’; (iii) noxious levels of distension (>40 mmHg) being referred to as ‘serosal’ and ‘mesenteric’; and (iv) both tactile and distension stimuli being referred to as ‘muscular-mucosal’(1, 6). Further, endings of specialized low-threshold endings of extrinsic afferent neurons in the rectum in the guinea pig have been identified(31, 32, 34). These rectal intraganglionic laminar endings (rIGLEs) are located exclusively within the myenteric ganglia and consist of flattened leaflet-like endings, arising from branching axons, and are the mechanotransduction sites of specialized sacral mechanoreceptors(32).

While focus on human tissue studies has increased in the last decade in neurogastroenterology, few studies have successfully recorded from human extrinsic nerves conveying sensory information along the brain gut axis(43). In the 5 years since afferent nerve activity was first recorded from the colon and appendix in Man (26, 35), the same two laboratories have extended their preliminary work to identify functionally distinct subpopulations of human visceral afferents supplying different parts of the lower gastrointestinal tract (including the ileum). However, much of the focus was still placed on colonic tissue in these studies (33, 48) and thus no studies have specifically sought to
record and characterize activity from human rectal afferents in non-inflamed states. Consequently, that was the aim of the present study with the additional goal of a direct comparison between afferents innervating the human rectum and colon using identical protocols. Spontaneous activity was readily recorded from rectal nerves, but less so from colonic nerves and mechanosensitive ‘hot-spots’ were also more abundant in rectal nerve recordings and had lower median thresholds than for colonic nerves, which were rarely responsive to this form of mechanical stimulation. Notably, most recorded activity was multi-unit, reflecting firing by multiple sensory neurons, as with many of the recordings made in previous human afferent studies(26, 35). This is in contrast to recordings made in animal studies, where single-units can often be discriminated. This difference probably reflects the anatomical differences encountered in human bowel (e.g. increased mural thickness with a possible more complex three-dimensional arrangement of nerve endings, increased mesenteric adipose and connective tissue [as evident in Figure 2] etc.) and the greater difficulty of dissection of extrinsic nerve trunks. It remains to be seen if single-unit recordings can be routinely made with refinements in tissue dissection protocols; this warrants evaluation in future studies.

The sparse distribution of colonic ‘hot-spots’ identified in the present study is comparable to the two previous reports, which both reported few mechanosensitive sites (2 / 9 and 4 / 27) in colonic preparations (26, 35). Furthermore, differences in nerve activity between rectum and colon corroborates well with previous animal studies(6, 32). In the guinea-pig colorectum, fewer colonic nerve units were found be low threshold mechanoreceptors than rectal nerve units, consistent with the findings of the present study, and the few colonic nerve units which could be activated by von Frey hair stimulation had substantially higher force thresholds(32). Furthermore, anterograde labeling of colonic nerve and rectal nerve trunks revealed substantially fewer rIGLEs filled per colonic nerve compared to rectal nerves in the guinea pig distal bowel (34). All aspects of our experimental protocol, such as tissue preparation, nerve dissection and degree of tissue stretch applied, were standardized between rectal and colonic tissue. Additionally, differences between
rectal and colonic afferents were unlikely to be due to damage by the dissection, since compound
action potentials could be evoked by focal electrical stimuli in 6/6 colonic nerve trunks,
demonstrating that the neural connections with the preparation were intact. The resulting
conduction velocities recorded were in the range expected of C-afferent fibers.

This study also used two algogenic stimuli to examine neuronal responses to chemical stimuli: (i)
an ‘inflammatory soup’ containing a combination of chemical mediators released from inflamed
tissue; and (ii) a naturally occurring ligand of the transient receptor potential vanilloid 1 (TRPV1),
capsaicin. Both caused significant increases in multi-unit firing frequency with increased activation
of spontaneously active units as well as recruitment of previously ‘silent’ units. The sensitivity to
capsaicin confirms that many human visceral afferents (especially in rectal nerves) express TRPV1
receptors, in keeping with the finding from of a previous immunohistochemical study(12). The
role(s) of TRP ion channels in visceral sensation(2) and nociception(4) have been well investigated
and described in small animals. For example, treatment with ruthenium red, a TRP channel blocker,
reduced distension responses in one study of guinea-pig colon afferents(49), whilst TRPV1 (TRPV1
/-), TRPV4 (TRPV4 -/-) and TRPA1 knockout mice show markedly attenuated behavioral and
afferent responses to colon distension(5, 7, 27, 40, 41). Additionally, TRPV4 has been described to
make a specific and major contribution to high threshold mechanosensory afferent function(7), and
as such is the only nociceptor-specific TRP channel as yet identified(2). It is interesting to note that
while the present study revealed a robust response to both IS and capsaicin, the increases in firing
rates were smaller than a previous study of human colon following capsaicin and ‘inflammatory
soup’ (35). It is possible that discarding the mucosa prior to recording may have removed some
classes of endings that were sensitive to these agents; the mucosa was left intact in the previous
studies (26, 35).

Sensitisation of visceral afferents was demonstrated in the current study by a decrease in thresholds
to von Frey hair probing following exposure to IS. Furthermore, three new ‘hot-spots’ were identified post-IS, suggesting that previously silent afferents had been activated. Sensitisation of visceral afferents by inflammatory mediators has previously been documented in animal studies (19, 47), with one study demonstrating a decrease in electrical stimulus thresholds following chemical stimulation (19). Furthermore, mechanically-insensitive afferents (so called ‘silent afferents’) may also be activated by chemical stimulation in small animals (3).

The observed differences in mechanosensitivity between rectal and colonic axons may reflect physiological differences in function between the two regions. Specifically, rectal distension typically elicits different graded rectal sensations, the nature of which qualitatively changes as the distension volume (or pressure) increases from an initial awareness, followed by a desire to defecate and lastly an intense, irrepressible urge to defecate (18, 37, 45). In contrast, colonic sensation which is less graded and manifests as visceral pain during high-pressure distension (24).

This study was limited by the technical and logistical difficulties encountered with human tissue samples. Patients were heterogeneous in age, gender, and co-morbidities and were undergoing surgery for organic disease (predominantly cancer) with the theoretic possibility that the tissue was not entirely “normal”, although all tissue specimens were procured from the resection margin as far away from the disease process as practical. Further, specimens were probably subject to a degree of ischemia prior to recording; this was minimized but could not be completely avoided. It was also unrealistic to study rectal and colonic tissues from the same patient (so comparisons were unpaired). Finally, the protocol involved removal of the bowel mucosa, so some classes of mucosally-projecting afferents were unlikely to be recorded. In addition, the variable signal-to-noise ratio made single unit recordings difficult. This meant that it has not been possible to develop of an electrophysiological classification scheme for human visceral afferent fibers. Previously, animal studies have revealed that splanchnic and pelvic pathways contain distinct populations of
mechanosensitive afferents; the proportions, receptive field distributions, and response properties differed greatly between the regions (6). This was not a realistic aim for the present study but it is likely that future studies will further characterize and electrophysiologically classify rectal afferent fibers as achieved in animals.

For the first time, afferent innervation of the human rectum via extrinsic nerves has been functionally demonstrated using \textit{in vitro} electrophysiological techniques. This study has also demonstrated differences in the sensory innervation of the rectum compared to the colon, consistent with the physiological differences between these parts of the gastrointestinal tract. Preparations from laboratory animals have been used to develop disease models, offering insight into the mechanisms of action for potential pharmacological treatments (10). Nevertheless, a number of novel drug candidates have failed to prove effective in clinical practice despite robust mechanistic data from animals (20, 25, 26, 43). The feasibility of an \textit{in vitro} model of human rectal sensory nerves may allow examination of the effects of novel therapeutic agents on visceral afferents, avoiding the issue of interspecies differences and providing a valuable supplement to animal models.
Table 1. Clinicopathological features of patients studied, divided into those from whom rectal or colonic tissues were acquired.

**Figure Legends**

Figure 1. Diagram showing tissues procured for the study. 
(A). Rectal tissue, acquired from the distal (aboral) end of an anterior resection specimen; 
(B). Colonic tissue, acquired from (i) the proximal (oral) end of an anterior resection specimen, or (ii) the distal (aboral) end of a right hemicolecotomy specimen.

Figure 2. (A). Tissue pinned serosa side up (1X magnification). Red arrows indicate mesenteric nerves running in neurovascular bundles. 
(B). View under a dissecting microscope (2X magnification). Red arrows indicate mesenteric nerves; 
(C). Nerve trunks dissected free from surrounding connective tissue.

Figure 3. Specialized tissue chamber containing a separate paraffin-filled nerve recording well that could be isolated from the main chamber.

Figure 4. (A). A typical trace of spontaneous nerve activity (horizontal axis: time [sec]; vertical axis: voltage [millivolts]), recorded from a rectal nerve (patient: 56 year old male. The specimen was of rectal tissue [8 cm from anal verge] acquired from anterior resection performed for rectosigmoid adenocarcinoma). Red arrows indicate
spontaneous nerve action potentials. The dotted lines show one of these action potentials at a faster time base, with a typical biphasic waveform. (B). A typical nerve response to repeated mechanical probing with a 4.0g von Frey hair applied to a single marked ‘hot-spot’ on the preparation. Application is indicated by the red interval bars (C). ‘Hot-spot’ responses to a decreasing series of mechanical stimuli applied by von Frey hairs of three different stiffnesses. Firing rates decreased as probing force was reduced, confirmed on corresponding plot of instantaneous frequency (Hz) which could be used to identify the threshold according to the ‘decreasing method of limits’.

Figure 5. Line graph showing changes in peak (A) and mean (B) firing rates in rectal nerve activity pre-/post- application of inflammatory soup (‘IS’). The individual lines (black) represent changes in firing rates for individual rectal nerves. The solid red line in each graph indicates median values of firing rates pre- and post chemical stimulation, with median values indicated in red text. The changes in firing rates pre- and post- chemical stimulation were all statistically significant (P<0.001).

Figure 6. Line graph showing changes in peak (A) and mean (B) firing rates in rectal nerve activity pre-/post capsaicin. The individual lines (black) represent changes in firing rates for individual rectal nerves. The solid red line in each graph indicates median values of firing rates pre- and post chemical stimulation, with median values stated in red. The changes in firing rates pre- and post- chemical stimulation were all statistically significant (P<0.001).
REFERENCES


CONFLICT OF INTEREST / STUDY SUPPORT

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- Dr David Mahns contributed to study design, data collection, analysis and interpretation, and manuscript preparation and review.
- Prof. Marc Gladman was responsible for study conception / design, data interpretation and review of the final manuscript.

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Potential competing interests: None
Main chamber (oxygenated Krebs solution)

Nerve recording well (paraffin)
Table 1. Clinicopathological features of patients studied, divided into those from whom rectal or colonic tissues were acquired.

<table>
<thead>
<tr>
<th></th>
<th>Rectum (n = 8)</th>
<th>Colon (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (median [range])</td>
<td>67 years (38 – 82)</td>
<td>73 years (57 – 82)</td>
</tr>
<tr>
<td>Male (number, %)</td>
<td>6 (75%)</td>
<td>7 (70%)</td>
</tr>
<tr>
<td>Pathology (number, %)</td>
<td>Cancer 7 (88%)</td>
<td>Cancer 10 (100%)</td>
</tr>
<tr>
<td></td>
<td>Diverticular disease 1 (12%)</td>
<td></td>
</tr>
<tr>
<td>Distance from anal verge (number, %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>11-15 cm (upper rectum) 5 (63%)</td>
<td></td>
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<tr>
<td></td>
<td>6-10 cm (mid rectum) 1 (12%)</td>
<td></td>
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<tr>
<td></td>
<td>1-5 cm (low rectum) 2 (25%)</td>
<td></td>
</tr>
<tr>
<td>Neoadjuvant radiotherapy (number, %)</td>
<td>2 (25%)</td>
<td></td>
</tr>
<tr>
<td>Tissue type</td>
<td>Transverse colon 5 (50%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Descending colon 5 (50%)</td>
<td></td>
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</table>