



Archived at the Flinders Academic Commons:

<http://dspace.flinders.edu.au/dspace/>

This is the peer reviewed version of the following article:

Chen BN, Olsson C, Sharrad DF, Brookes SJ. Sensory innervation of the guinea pig colon and rectum compared using retrograde tracing and immunohistochemistry. *Neurogastroenterol Motil.* 2016 Sep;28(9):1306-16.

which has been published in final form at

doi: 10.1111/nmo.12825

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Self-Archiving.

**Sensory innervation of the guinea pig colon and rectum compared using retrograde
tracing and immunohistochemistry**

by

BN Chen¹, C Olsson², DF Sharrad¹ and SJH Brookes¹

¹Discipline of Human Physiology
FMST, School of Medicine
Flinders University
Bedford Park
South Australia 5042

²Department of Biological & Environmental Sciences
University of Göteborg
Box 463, 40530 Göteborg

Address for Correspondence

Simon Brookes, simon.brookes@flinders.edu.au,
Tel: +61 8 8204 4201; Fax: +61 8 8204 5768
Discipline of Human Physiology, FMST, School of Medicine
Flinders University, Sturt Road, Bedford Park, SA 5042

Running Title:

Extrinsic sensory nerves to rectum and colon

Abbreviations:

DiI; DiIC12(3) (1,1'-Didodecyl-3,3',3'-Tetramethylindocarbocyanine Perchlorate) or a 1:1 mixture of DiIC12(3) with CellTracker™ CM-DiI Dye. CGRP: calcitonin gene-related peptide; TRPV1: transient receptor potential vanilloid 1

Word count: 4250

ABSTRACT (250 words)

Background: Neurons in lumbar and sacral dorsal root ganglia comprise extrinsic sensory pathways to the distal colon and rectum but their relative contributions are unclear. In this study in the guinea pig, sensory innervation of the rectum and distal colon were directly compared using retrograde labelling combined with immunohistochemistry. **Methods:** The lipophilic tracer, DiI was injected in either the rectum or distal colon of anaesthetised guinea pigs, then dorsal root ganglia (T6 to S5) and nodose ganglia were harvested and labelled using antisera for Calcitonin Gene-Related Peptide (CGRP) and Transient Receptor Potential Vanilloid 1 (TRPV1). **Key Results:** More primary afferent cell bodies were labelled from the rectum than from the distal colon. Vagal sensory neurons, with cell bodies in the nodose ganglia comprised fewer than 0.5% of labelled sensory neurons. Spinal afferents to the distal colon were nearly all located in thoracolumbar dorsal root ganglia, in a skewed unimodal distribution (peak at L2); fewer than 1% were located in sacral ganglia. In contrast, spinal afferents retrogradely-labelled from the rectum had a bimodal distribution, with one peak at L3 and another at S2. Fewer than half of all retrogradely-labelled spinal afferent neurons were immunoreactive for CGRP or TRPV1 and these included the larger traced neurons, especially in thoracolumbar ganglia. **Conclusions & Inferences:** In the guinea pig, both the distal colon and the rectum receive a sensory innervation from thoracolumbar ganglia. Sacral afferents innervate the rectum but not the distal colon. CGRP-immunoreactivity was detectable in less than half of afferent neurons in both pathways.

Keywords: pelvic nerves, splanchnic nerves, thoracolumbar, lumbosacral, dorsal root ganglia

Key Messages:

We show that thoracolumbar spinal sensory neurons that project via splanchnic nerves, and lumbosacral sensory neurons that project via pelvic nerves, make very different contributions to the sensory innervation of the guinea pig distal colon and rectum. We used retrograde tracing combined with immunohistochemistry for CGRP and TRPV1 to characterise the two pathways in 20 guinea pigs. The rectum received a dense sensory innervation by both thoracolumbar and lumbosacral pathways. The distal colon received a less dense innervation overall, which only arises from thoracolumbar pathways. CGRP-containing neurons comprised fewer than half of all spinal sensory neurons in both pathways.

INTRODUCTION (532 words)

Extrinsic primary afferent (sensory) neurons from the gastrointestinal tract activate peripheral, spinal and brainstem reflex circuits, with sympathetic or parasympathetic autonomic as the efferent pathways to the gut. While the upper gut receives a substantial vagal sensory innervation, the colorectum is primarily innervated by sensory neurons with cell bodies in dorsal root ganglia (DRG). Spinal afferent neurons also give rise to noxious and non-noxious sensations from the gut, such as discomfort, pain and urge to defaecate. Several classes of extrinsic spinal afferent neurons innervating the gut can be distinguished functionally¹⁻⁴.

Spinal afferents have a rough viscerotopic distribution; proximal regions of gut are innervated by sensory neurons with cell bodies in more rostral dorsal root ganglia (DRG), whilst more distal gut regions are innervated from more caudal segments⁵. For the lower gut, axons of spinal afferent neurons project via two distinct pathways: splanchnic nerves, which supply most of the gut from the lower oesophagus to the rectum; and pelvic nerves, which largely supply the distal colorectum, anal canal and internal anal sphincter^{6, 7}. Splanchnic and pelvic pathways contain different proportions of the various functional classes of spinal afferent neurons. Splanchnic nerves contain many high-threshold mechanoreceptors which reach the colon via the lumbar colonic nerves, forming a major visceral pain pathway^{1, 2, 8}. For example, in humans, pain perception from the gut is reduced after section of splanchnic nerves⁹. High-threshold mechanoreceptors are less abundant in pelvic nerves, but there are many pelvic mechanoreceptors with relatively low thresholds, which project to the distal bowel via the rectal nerves^{1, 4, 10-12}. These low-threshold mechanoreceptors respond to mechanical stimuli within the physiological range and may contribute to non-noxious reflexes such as defaecation¹³.

In the guinea pig, anterograde tracing of lumbar colonic and rectal nerves has been used to characterise autonomic and sensory neurons to the distal colon and rectum^{14, 15}. Surprisingly, in

both distal colon and rectum, a substantial proportion of extrinsic axons lacked any of the common markers (calcitonin gene-related peptide, vesicular acetylcholine transporter, tyrosine hydroxylase, vasoactive intestinal polypeptide, nitric oxide synthase, somatostatin, and vesicular glutamate transporters 1 and 2) that have previously been used to define autonomic and extrinsic sensory neurons^{14, 15}. Retrograde tracing from the distal colon and rectum was used to analyse sympathetic and parasympathetic efferent pathways and the presence of key markers (tyrosine hydroxylase, nitric oxide synthase and choline acetyltransferase) in their nerve cell bodies¹⁶. To date, no study has compared the spinal sensory innervation of distal colon and rectum in the guinea pig using retrograde tracing. Such a study could clarify the contributions of splanchnic and pelvic pathways and identify whether these might contribute some of the immunohistochemically undefined extrinsic axons to the distal bowel. In this study, we applied the retrograde tracer, DiI to either the distal colon or rectum of the guinea pig *in vivo*. The nodose ganglia and DRG from T6 to S5 were then collected, up to 12 days later. Retrogradely-labelled and -unlabelled DRG were immunohistochemically-labelled with antibodies against common markers of spinal afferent neurons, the ion channel TRPV1 and the neuropeptide, calcitonin gene-related peptide (CGRP), enabling quantification of their expression in colorectal-projecting sensory neurons compared to the population of all spinal afferent neurons.

MATERIALS AND METHODS

Retrograde tracings: surgical procedures

Guinea pigs of either sex (150-400 g) were anaesthetised either with xylazine (8 mg kg⁻¹ i.p.) and ketamine (60 mg kg⁻¹ i.p.) or 5% halothane in O₂ delivered at 1.5 l.min⁻¹ (maintained with 2% halothane in O₂). An abdominal incision was made, and 1.5-3 µl DiI(C₁₂) (2 mg mL⁻¹, Molecular Probes, Invitrogen, Life Technologies Australia Pty Ltd VIC, Australia) was injected into the distal colon or rectum. In the latter half of the study, 3-4 µl of a 50:50 mixture of DiI(C₁₂) (2 mg mL⁻¹ and

CellTracker CM-DiI(C₁₈) (2.5 mg mL⁻¹, Molecular Probes,) was injected. The two tracers used labelled similar numbers of neurons but CellTracker CM-DiI(C₁₈) persisted better in the tissue during immunohistochemical labelling. For this reason, results from the two tracer solutions were pooled. DiI was dissolved in N, N-dimethylformamide (Sigma, St. Louis, MO) and applied from a glass micropipette (tip diameter approximately 10 µm) via pressure injection (100 kPa for 4 ms) into two to six closely spaced sites¹⁶. Rectal injections were made approximately 2-3 cm from the anal sphincter. Colonic injections were made in the upper part of the distal colon (distinguished by pelleted contents), at least 15 cm from the anal end, as measured in fixed tissue. Care was taken not to inject the dye into blood vessels, and visible leakage was removed immediately. The animals were given an intramuscular injection of analgesics and antibiotics (0.02ml each of Flunixin (Troy Labs, Glendenning, NSW, Australia) and enrofloxacin (Baytril, Bayer, Pymble, NSW, Australia) and allowed to recover for 6-12 days. During recovery, they were given 15g L⁻¹ of Oxytetracycline HCl (Tetravet, Bayer, Pymble, NSW, Australia) in the drinking water for 3 days. All experiments were carried out in accordance with ethical requirements by the Animal Welfare Committee of the Flinders University, South Australia (#330/99N).

Tissue fixation and processing

The animals were given a lethal injection (i.p.) of sodium pentobarbitone (0.5 ml kg⁻¹ of 325 mg mL⁻¹) followed by 10 units of heparin in saline into the heart before they were perfused with warm saline (0.15 M NaCl) to flush out the blood. Subsequently, the animals were perfused with cold 2% formaldehyde (in 0.1M phosphate buffer, pH 7.2) and the following tissues were collected for this study: nodose ganglia, dorsal root ganglia (DRG; from T6 to S5), and distal colon or rectum where the dye was injected. Tissue was post-fixed overnight in 2% formaldehyde at 4°C. After rinsing with 0.1 M phosphate buffered saline (PBS; 0.15 M NaCl, pH 7.2), whole dorsal root ganglia were cleared through 50%, 70%, and 100% glycerol (1–3 hours in each) and mounted on slides in bicarbonate-buffered glycerol (pH 8.5) for counting of DiI-labelled somata. In addition, DRG (L4

and S2/3) were taken from unoperated animals (n=5), fixed and processed as described above and used for quantifying populations of immunohistochemically labelled cells in the ganglia.

Immunohistochemistry

DRG were placed overnight in PBS containing 30% sucrose as cryoprotectant before being frozen in isopentane pre-chilled in liquid nitrogen. The ganglia were cut in 16 μ m sections on a cryostat and thaw-mounted onto polyethyleneimine-coated slides. Every sixth section was collected on the same slide to avoid double counting of cells and approximately 10 sections were counted for each ganglion. Slides were allowed to dry overnight at room temperature and were subsequently stored at 4°C, protected from light. Antisera against TRPV1 and calcitonin gene-related peptide (CGRP) were used to examine expression in DRG nerve cell bodies (Table 1). In addition, control ganglia (L4 and S2) from unoperated animals were fixed, sectioned, and labelled with antisera against CGRP and TRPV1, as described above. Sections were incubated with primary antisera at room temperature overnight. The preparations were then washed with PBS (three 10-minute washes) and incubated with appropriate secondary antibodies (Table 1) for 2-4 hours. After washing with PBS, the preparations were mounted as described above. Controls for double-labelling were performed by omitting one or more primary antibodies from the procedure and ensuring that all combinations of primary and secondary antisera were free of cross-reactivity.

Microscopy and image analysis

Preparations were viewed on an Olympus AX70 epifluorescence microscope and micrographs were taken via a Hamamatsu Orca digital camera (model C4742-95) on an Apple Macintosh computer with IPLab software (Scanalytics Inc.). The total number of DiI-labelled neurons in each ganglion was counted in wholemount preparations. Cells were scored as being labelled with DiI when they had a clear outline, a visible nucleus and could be readily distinguished from background fluorescence. Immunohistochemically-labelled cells were analyzed in sectioned tissue. All DiI-

labelled cells in non-consecutive sections were tested for immunoreactivity for CGRP or TRPV1. Micrographs taken on a Sony CCD-IRIS camera were then used to measure the size of DiI-labelled cell bodies in NIH Image (National Institute of Health, MD, USA). Data are presented as mean values \pm 95% confidence intervals, with n representing the number of animals used for each observation. Counts and size measurements of cells in control L4 and S2 ganglia were carried out by identifying all immunoreactive cell bodies with a nucleus in several non-consecutive sections and measuring their profiles in NIH Image.

Statistical analysis

Statistical analysis was performed in SPSS19 for PC (SPSS, Chicago, IL). One-way analysis of variance (ANOVA) with Tukey's HSD Test was used to compare the means of more than two samples and the means of two independent samples were compared by a Student's unpaired *t*-test. Differences were considered significant at $P < 0.05$. Errors are provided as 95% confidence intervals.

RESULTS

Retrograde tracing from the distal colon and rectum

Injection of DiI into the gut wall labelled structures in all layers, with intense granular red fluorescence¹⁶. Injection sites were confirmed by examination of the myenteric plexus of distal colon or rectum for the presence of DiI-labelled nerve cells. In enteric nerve cells that accumulated DiI, fluorescence was most intense in their nerve cell bodies, with dendrites and axons exhibiting finer diffuse or punctate labelling (Figure 1A). Similar to *in vitro* fills, the nucleus of DiI-labelled enteric nerve cells consistently lacked fluorescence¹⁷.

After application of DiI to either the distal colon or rectum, nerve cell bodies labelled with DiI were clearly visible in wholemount preparations of thoracic, lumbar and sacral DRG (fig 1B,C). In one

of 6 animals in which DiI was applied to the colon, 9 nerve cell bodies were labelled in the nodose ganglia, but in none of the other 11 animals studied (after either rectal or colonic DiI applications sites) were any neurons filled in the nodose ganglia. These 9 vagal sensory neurons were excluded from further analysis.

The number of DiI-labelled cell bodies in DRG was counted in wholemount preparations of ganglia. On average, after application of DiI to the distal colon, 169 ± 63 (95% CI) sensory neurons were labelled ($n=6$). These were located in a skewed unimodal distribution extending from T6 to S3, with the great majority of cells ($>90\%$) located between T7 and L3 (see Figure 2). Fewer than 1% of afferent neurons labelled by DiI applied to the distal colon were located in sacral DRG. The average numbers of cells on the right and left sides were not significantly different (right: 96 ± 29 , left: 75 ± 40 , $df=5$, $p=0.234$).

DiI application to the rectum also labelled spinal afferent neurons, but the pattern of labelling differed from colonic fills. First, the total number of afferent neurons filled from the rectum was much larger (1166 ± 249 cells per preparation, $n=9$). Second, these neurons were distributed with a clear bimodal distribution, with peaks at S2/S3 and at L2-L4 (see Figure 2). More neurons were labelled in sacral DRG (S1-S5: 757 ± 172 cells, $n=9$) than in thoracolumbar DRG (T6:L6: 409 ± 92 cells, $df=8$, $t=5.423$, $p=0.00063$). There were no significant differences between numbers of cells on right and left sides (550 ± 108 on right, 616 ± 233 on left; $df=8$, $t=1.406$, $p=0.197$, NS).

Immunohistochemistry of lumbar DRG neurons innervating distal colon

Thoracolumbar and sacral DRG containing the peak numbers of DiI-labelled cells were sectioned, labelled with antisera against CGRP and TRPV1, and the proportion of DiI-labelled cells containing immunoreactivity for the different combinations of markers was quantified. Immunohistochemistry without DiI labelling was carried out in separate preparations of L4 DRG ($n=5$) to determine the co-

existence of CGRP and TRPV1 in a random sample of all nerve cell bodies.

CGRP- and TRPV1 immunoreactivities were present in both DiI labelled and unlabelled nerve cell bodies in lumbar DRG (Fig. 3). Of the nerve cell bodies in L2-L4 ganglia filled by DiI applied to the distal colon, most ($70\% \pm 12\%$) lacked both CGRP and TRPV1 immunoreactivities (Figure 5A). Of all DiI-filled neurons, $30 \pm 14\%$ (mean \pm 95% confidence interval, $n=3$) were immunoreactive for CGRP and 90% of these were also immunoreactive for TRPV1. No lumbar cells (0%) labelled by DiI applied to the distal colon contained TRPV1-immunoreactivity without CGRP (see Figs 5A,5F). We used L4 as a comparison ganglion for thoracolumbar spinal afferents, since substantial numbers of cells in this segment were filled from both colon and rectum. The overall proportion of CGRP+/TRPV1+ cells in the L4 ganglion as a whole was significantly lower than the proportion of CGRP+/TRPV1+ cells labelled with DiI from the distal colon (Fisher's exact test; $P=0.0017$, Fig 5A,5D).

Colon-projecting cells in L2 and L3 with CGRP and TRPV1 immunoreactivity (CGRP+/TRPV1+) were the largest DiI-filled cells in the ganglia, averaging $1014 \pm 311\mu\text{m}^2$ (see Figure 5F). The average size of a random selection of cells in L4 DRG, with the various combinations of neurochemical coding, is shown in Figure 5F, for comparison (NB: control cells without either CGRP or TRPV1 immunoreactivity could not be reliably measured). CGRP+/TRPV1+ cells labelled by DiI were larger, on average, than the total population of CGRP+/TRPV1+ cells in lumbar ganglia ($P=0.0356$, $df=6$, $t=2.699$). Typical examples of colon-projecting neurons with combinations of CGRP- and TRPV1-immunoreactivity are shown in Figure 3.

Immunohistochemistry of lumbar DRG neurons innervating rectum

Sensory neurons filled by DiI applied to the rectum were located in both thoracolumbar and sacral DRG; these sources were analysed separately. Of the DiI-filled neurons in L2-L3 ganglia, almost

50% \pm 16% (n=7) lacked both CGRP- and TRPV1-immunoreactivity (see Figure 5B). Nearly half of the cells were CGRP-immunoreactive (49 \pm 18%, n=7) (see Figure 5B). Of these, most (78% of thoracolumbar DiI-filled cells that contained CGRP) were also immunoreactive for TRPV1. There was a very small population of DiI-labelled cells in thoracolumbar ganglia (~1%) that were TRPV1-immunoreactive without CGRP-immunoreactivity. Rectum-projecting cells in L2-L4 ganglia were enriched in CGRP-immunoreactivity compared to the overall population of lumbar spinal afferents (Fisher's exact test $P < 0.0001$). Lumbar CGRP+/TRPV1+ afferent cells filled from the rectum were not significantly larger (733 \pm 67 μm^2) than either CGRP-/TRPV1- (626 \pm 25 μm^2) or CGRP+/TRPV1- cells (649 \pm 83 μm^2).

Immunohistochemistry of sacral DRG neurons innervating rectum

Over half of the DiI-labelled cells in sacral ganglia (filled from the rectum) lacked immunoreactivity for both CGRP and TRPV1 (61 \pm 10%, n=7). On average, 38 \pm 10% of cells were CGRP-immunoreactive, half of which contained TRPV1-immunoreactivity (ie: 19% of all DiI-filled cells - see Figure 5C). Compared to the total population of S2/S3 neurons, among which 23 \pm 3% contained CGRP, this represents an enrichment of CGRP-containing neurons in the rectum-projecting population (Fisher's exact test, $P < 0.0001$), but not an enrichment of CGRP+/TRPV1+ neurons (Fisher's exact test $P = 0.657$, NS). There was a very small population of retrogradely-labelled nerve cell bodies (~1.5%) that were immunoreactive for TRPV1 without CGRP. In terms of cell size, somata containing the various combinations of CGRP- and TRPV1-immunoreactivity did not differ from one another in average size and no immunohistochemically-defined DiI-filled population was larger than the S2/S3 population as a whole. The size of DiI-filled CGRP-/TRPV1+ neurons was not calculated due to the small sample size (Figure 5F). Typical rectal-projecting neurons with immunoreactivity for combinations of TRPV1 and CGRP are shown in Figure 4.

DISCUSSION (1586 words)

Sensory neurons in splanchnic and pelvic pathways to the distal bowel differ in a number of characteristics. For example, a large population of low threshold mechanoreceptors innervates the rectum⁴; these are much sparser in the colonic/splanchnic pathway¹⁴. In contrast, splanchnic afferents innervating the colon are largely medium-to-high threshold mechanonociceptors¹⁸ which are not as abundant in pelvic pathways¹⁹. In the mouse large intestine, systematic studies have demonstrated significant differences in both mechanosensitivity^{1, 19} and chemosensitivity²⁰ between spinal afferents in pelvic and splanchnic pathways.

In the present report we used retrograde tracing techniques, combined with multiple labelling immunohistochemistry, to compare the spinal afferents innervating the distal colon and the rectum of the guinea pig. As in previous studies^{14, 16}, we defined the rectum as the region of distal bowel that received rectal nerve trunks, which were connected to the pelvic ganglia (the most distal 6-8cm of the bowel). Proximal to this was a transition zone, 4-6cm long^{4, 14}. Between the transition zone and the colonic flexure the colon typically contained discrete faecal pellets and was connected to lumbar colonic nerves arising from the inferior mesenteric ganglia; we refer to this region as "distal colon".

The distributions of DiI-labelled afferent nerve cell bodies innervating the rectum and distal colon were very striking. First, the vagal afferent innervation of both distal colon and rectum was very sparse. Previous studies in the rat, using anterograde tracing, reported a small but significant vagal afferent innervation, extending as far as the distal colon²¹. However, in a quantitative study, selective anterograde labelling of vagal afferents labelled nerve endings in the distal colon of the rat with about 10% of the density in the duodenum²². Vagal efferent fibres are similarly sparse in the distal bowel²³. In both species, the distal colon is primarily involved in propulsion of faecal pellets

rather than in the storage and mixing of content. Vagal innervation is denser in proximal colon where transit of the content is slower and may provide a neural substrate for the vagal interactions with the microbiome.²⁴

In the present study, the guinea pig distal colon was innervated by sensory neurons with cell bodies in thoracolumbar DRG. Filled cell bodies were found rostrally up to T6 (the extent of the analysis) but fewer than 1% were located in sacral dorsal root ganglia. Previous studies showed very small numbers of sacral spinal afferents (range: 2-10) projecting rostrally in the hypogastric nerves of guinea pigs²⁵, although larger numbers were reported in the cat, arising from S2 and S3 DRG²⁶. This suggests that, at least in the guinea pig, chemical and mechanical stimuli in the distal colon are not likely to be detected by sacral afferents. Thus, the major sources of afferent innervation of the guinea pig distal colon are lumbar DRG neurons, peaking at L2/L3, whose axons project via the lumbar splanchnic nerves, inferior mesenteric ganglion and colonic nerves. A similar exclusive thoracolumbar distribution has been described for sensory neurons innervating the proximal and mid colon of the mouse²⁷.

In contrast to the distal colon, the guinea pig rectum was innervated by both lumbar and sacral spinal afferent neurons, with a distinctive bimodal distribution. This pattern of innervation is similar to that described previously for pelvic organs including mouse colorectum^{28, 29} rat colorectum³⁰⁻³², pig bladder³³, porcine testis³⁴, porcine vas deferens³⁵ and mouse uterus³⁶. Thoracolumbar spinal afferents probably reach the rectum by projecting caudally via hypogastric nerves^{37, 38} to pelvic ganglia, then into the bowel via rectal nerves. Sacral afferents project via pelvic nerves, pelvic ganglia and rectal nerves to the distal bowel.

Immunohistochemical labelling.

In the present study, CGRP-immunoreactivity was detected in fewer than half of the spinal afferents

projecting to the gut wall, which was less than rats and mice. Approximately 30% of thoracolumbar afferents projecting to the guinea pig colon contained CGRP-immunoreactivity; nearly 50% of thoracolumbar afferents filled from the rectum were CGRP-immunoreactive while 40% of sacral afferents projecting to the rectum contained CGRP. In the mouse more colorectal-projecting afferents are CGRP immunoreactive; 78 - 79% of thoracolumbar afferents CGRP positive^{39, 40}. For lumbosacral afferents in mice, 56.5% were CGRP immunoreactive⁴⁰. In rats, 82 - 87% of thoracolumbar spinal afferents to the distal bowel were CGRP immunoreactive⁴⁰ although an earlier study reported a lower count (46+24%³²). For lumbosacral afferents, CGRP immunoreactivity was reported in 60-91% of colorectal-projecting afferents^{32, 40, 41}. In comparison, our results suggest that both thoracolumbar and lumbosacral pathways contain lower proportions of CGRP immunoreactive neurons than in either rats and mice. Where present, TRPV1 nearly always coexisted with CGRP- in colorectal-projecting neurons in the guinea pig.

The significance of species differences in CGRP expression is not clear. CGRP is a potent vasodilator⁴² and mediates sensory vasodilation⁴³. Activation of extrinsic sensory endings in guinea pig colon cause an axon-reflex vasodilation of upstream mesenteric arteries⁴⁴, likely mediated by CGRP. Electrical activation of extrinsic spinal afferents evokes slow excitatory post-synaptic potentials in guinea pig enteric neurons that are mediated, in part, by CGRP⁴⁵. Thus, even though guinea pigs have lower CGRP expression in colorectal spinal afferents than other rodents, this neuropeptide contributes to the physiological function of peripheral sensory endings. In guinea pig colorectal afferents, CGRP immunoreactivity has been detected in axons of medium-high threshold mechanonociceptors with endings on intramural and extramural blood vessels⁸ but not in specialised low threshold mechanoreceptors^{4, 14} nor in muscular mucosal mechanoreceptors or intramuscular afferents (unpublished observations). This suggests that CGRP expression is probably confined to particular functional classes of afferents.

The total proportion of DiI filled cells that contained CGRP immunoreactivity was greater than the overall population of afferent cell bodies in either L4 or S2 (Figure 5). This reflects the filling of specific populations of sensory neurons by DiI applied to the gut wall. In rats and mice, many non-peptidergic spinal afferents express Isolectin B4 (IB4) binding^{46, 47}. However, preliminary cell counts revealed that in L4 in the guinea pig, the great majority of IB4-binding is in CGRP and TRPV1-immunoreactive spinal afferents; fewer than 3% of afferents were IB4+ without CGRP or TRPV1. This suggests that IB4 would not be a useful marker for a large population of non-CGRP containing colorectal afferents. Furthermore, in anterograde tracing studies, IB4-binding was not detectable in extrinsic axons innervating the guinea pig colon¹⁵.

The guinea-pig spinal sensory neurons that lack both CGRP and TRPV1 immunoreactivity in their cell bodies probably also lack these markers in their axons. Anterograde tracing of peripheral axons to the guinea pig rectum and colon showed that close to half of the filled axons in the myenteric plexus lacked any of the commonly used markers for sympathetic, parasympathetic or extrinsic sensory axons (ie: tyrosine hydroxylase, vesicular acetylcholine transporter, vasoactive intestinal polypeptide, nitric oxide synthase, somatostatin, vesicular glutamate transporters 1 and 2 or CGRP)^{14, 15}. Since spinal afferents constitute about one quarter of all extrinsic neurons projecting to the distal bowel of the guinea pig¹⁶, it is likely that spinal afferents that lack CGRP may contribute to this pool of "unlabelled" extrinsic axons.

In the present study, colorectal-projecting spinal afferent neurons were comparable in size to those afferents projecting to other viscera (ureters) in the guinea pig⁴⁸. They were rarely the smallest neurons in the ganglia and were generally the same size or slightly larger than other (non-DiI-containing) CGRP+/TRPV1+ neurons (Figure 5). This is similar to the situation in murine jejunal and colonic spinal afferents, which are predominantly of medium size rather than being the smallest in the ganglia²⁹. Cell body size correlates positively with conduction velocity in spinal afferents⁴⁹,

suggesting that colorectal afferents in the guinea pig are all likely to be in the C and A δ range, but are probably not the slowest conducting sensory neurons.

Possible roles of thoracolumbar/splanchnic and sacral/pelvic afferents

Splanchnic and pelvic afferent neurons project in parallel to sympathetic and parasympathetic efferent pathways to pelvic organs, respectively. Many pelvic afferents have low mechanical thresholds^{1,4}, which are strongly activated by distensions and contractions, within the physiological range⁵⁰. These are likely to activate parasympathetic efferent pathways to the distal bowel, which are important in the physiology of defaecation. In contrast, high amplitude distensions reliably activate pain pathways from all regions of the gut⁵¹. This may be largely mediated by splanchnic afferents, which have medium-to-high thresholds, and are selectively activated by noxious stimuli. The transduction sites of these afferents appear to be located on intramural and extramural blood vessels, particularly arteries⁸. The present study has shown that there is a significant innervation of the rectum by splanchnic afferents. This appears to suggest that pain from noxious stimulation of both colon and rectum is primarily mediated by splanchnic afferents. However, at least in the mouse, selective lesions of pelvic pathways abolish visceromotor responses activated by noxious colorectal distension, whereas lesions of hypogastric and/or colonic nerves have little effect²⁷. One explanation for this might be that noxious distension of the rectum may be encoded, at least in part, by sacral afferents with wide dynamic ranges, that can encode into the noxious range, although a small number with high thresholds may be present¹⁹.

Conclusions

This study has shown that both the distal colon and rectum are both innervated by a population of thoracolumbar spinal afferents, concentrated in the mid lumbar segments. The rectum receives an additional sensory innervation from spinal afferent neurons with cell bodies located in sacral spinal ganglia, which do not innervate the distal colon. It is likely that the sacral afferents represent a

specialised population, including low threshold mechanoreceptors, which may contribute to the physiological control of defaecation and non-noxious sensations.

ACKNOWLEDGMENTS, FUNDING & DISCLOSURES

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health; Grant number: 56986, and NHMRC (Australia) grant #1048195. We would like to thank Dr Sarahlouise Jones for assistance with surgery and tracer injections.

BNC and CA carried out experimental studies and analysed data, with assistance from DS and SJHB. SJHB assisted with study conception and design. All authors contributed to the writing and presentation of the paper.

Competing interests: the authors have no competing interests.

REFERENCES

1. Brierley SM, Jones RC, 3rd, Gebhart GF, Blackshaw LA. Splanchnic and pelvic mechanosensory afferents signal different qualities of colonic stimuli in mice. *Gastroenterology* 2004;**127**:166-78.
2. Brookes SJH, Spencer NJ, Costa M, Zagorodnyuk VP. Extrinsic primary afferent signalling in the gut. *Nature Reviews Gastroenterology & Hepatology* 2013;**10**:286-96.
3. Lynn PA, Blackshaw LA. In vitro recordings of afferent fibres with receptive fields in the serosa, muscle and mucosa of rat colon. *J Physiol* 1999;**518**:271-82.
4. Lynn PA, Olsson C, Zagorodnyuk V, Costa M, Brookes SJH. Rectal intraganglionic laminar endings are transduction sites of extrinsic mechanoreceptors in the guinea pig rectum. *Gastroenterology* 2003;**125**:786-94.
5. Beyak MJ, Bulmer DCE, Jiang W, Keating C, Rong W, Grundy D. Extrinsic sensory afferent nerves innervating the gastrointestinal tract. In: Johnson LR, ed. Physiology of the gastrointestinal tract. Volume 1. 5th Edition ed. Amsterdam: Academic Press, 2006:685-725.
6. Berthoud HR, Blackshaw LA, Brookes SJH, Grundy D. Neuroanatomy of extrinsic afferents supplying the gastrointestinal tract. *Neurogastroenterology & Motility* 2004;**16 Suppl 1**:28-33.
7. Cervero F. Sensory innervation of the viscera: peripheral basis of visceral pain. *Physiol Rev* 1994;**74**:95-138.
8. Song X, Chen BN, Zagorodnyuk VP, Lynn PA, Blackshaw LA, Grundy D, Brunsden AM, Costa M, et al. Identification of medium/high-threshold extrinsic mechanosensitive afferent nerves to the gastrointestinal tract. *Gastroenterology* 2009;**137**:274-84.
9. Bentley FH, Smithwick RH. Visceral pain produced by balloon distension of the jejunum. *Lancet* 1940;**240**:389-391.
10. Feng B, La JH, Schwartz ES, Tanaka T, McMurray TP, Gebhart GF. Long-term sensitization of mechanosensitive and -insensitive afferents in mice with persistent colorectal hypersensitivity. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2012;**302**:G676-83.
11. Feng B, La JH, Tanaka T, Schwartz ES, McMurray TP, Gebhart GF. Altered colorectal afferent function associated with TNBS-induced visceral hypersensitivity in mice. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2012;**303**:G817-24.
12. Zagorodnyuk VP, Kyloh M, Nicholas S, Peiris H, Brookes SJ, Chen BN, Spencer NJ. Loss of visceral pain following colorectal distension in an endothelin-3 deficient mouse model of Hirschsprung's disease. *Journal of Physiology* 2011;**589**:1691-706.
13. Yamanouchi M, Shimatani H, Kadowaki M, Yoneda S, Nakagawa T, Fujii H, Takaki M. Integrative control of rectoanal reflex in guinea pigs through lumbar colonic nerves. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2002;**283**:G148-56.
14. Olsson C, Costa M, Brookes SJH. Neurochemical characterization of extrinsic innervation of the guinea pig rectum. *Journal of Comparative Neurology* 2004;**470**:357-71.
15. Chen BN, Sharrad DF, Hibberd TJ, Zagorodnyuk VP, Costa M, Brookes SJH. Neurochemical characterization of extrinsic nerves in myenteric ganglia of the guinea pig distal colon. *Journal of Comparative Neurology* 2015;**523**:742-56.
16. Olsson C, Chen BN, Jones S, Chataway TK, Costa M, Brookes SJH. Comparison of extrinsic efferent innervation of guinea pig distal colon and rectum. *Journal of Comparative Neurology* 2006;**496**:787-801.
17. Brookes SJH. Retrograde tracing of enteric neuronal pathways. *Neurogastroenterology & Motility* 2001;**13**:1-18.
18. Blumberg H, Haupt P, Janig W, Kohler W. Encoding of visceral noxious stimuli in the discharge patterns of visceral afferent fibres from the colon. *Pflugers Arch* 1983;**398**:33-40.

19. Feng B, Brumovsky PR, Gebhart GF. Differential roles of stretch-sensitive pelvic nerve afferents innervating mouse distal colon and rectum. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2010;**298**:G402-9.
20. Brierley SM, Carter R, Jones W, 3rd, Xu L, Robinson DR, Hicks GA, Gebhart GF, Blackshaw LA. Differential chemosensory function and receptor expression of splanchnic and pelvic colonic afferents in mice. *Journal of Physiology* 2005;**567**:267-81.
21. Berthoud HR, Patterson LM, Neumann F, Neuhuber WL. Distribution and Structure Of Vagal Afferent Intraganglionic Laminar Endings (IGLEs) In the Rat Gastrointestinal Tract. *Anatomy & Embryology* 1997;**195**:183-191.
22. Wang FB, Powley TL. Topographic inventories of vagal afferents in gastrointestinal muscle. *Journal of Comparative Neurology* 2000;**421**:302-24.
23. Berthoud HR, Jedrzejewska A, Powley TL. Simultaneous labeling of vagal innervation of the gut and afferent projections from the visceral forebrain with dil injected into the dorsal vagal complex in the rat. *J-Comp-Neurol* 1990;**301**:65-79 issn: 0021-9967.
24. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nature Reviews Neuroscience* 2012;**13**:701-12.
25. McLachlan EM. The components of the hypogastric nerve in male and female guinea pigs. *Journal of the Autonomic Nervous System* 1985;**13**:327-42.
26. Baron R, Janig W. Neurons projecting rostrally in the hypogastric nerve of the cat. *J Auton Nerv Syst* 1988;**24**:81-6.
27. Kyloh M, Nicholas S, Zagorodnyuk VP, Brookes SJ, Spencer NJ. Identification of the visceral pain pathway activated by noxious colorectal distension in mice. *Frontiers in Neuroscience* 2011;**5**:16.
28. Robinson DR, McNaughton PA, Evans ML, Hicks GA. Characterization of the primary spinal afferent innervation of the mouse colon using retrograde labelling. *Neurogastroenterology & Motility* 2004;**16**:113-24.
29. Tan LL, Bornstein JC, Anderson CR. Distinct chemical classes of medium-sized transient receptor potential channel vanilloid 1-immunoreactive dorsal root ganglion neurons innervate the adult mouse jejunum and colon. *Neuroscience* 2008;**156**:334-43.
30. Hicks GA, Coldwell JR, Schindler M, Ward PA, Jenkins D, Lynn PA, Humphrey PP, Blackshaw LA. Excitation of rat colonic afferent fibres by 5-HT(3) receptors. *Journal of Physiology* 2002;**544**:861-9.
31. Vizzard MA, Brisson M, de Groat WC. Transneuronal labeling of neurons in the adult rat central nervous system following inoculation of pseudorabies virus into the colon. *Cell & Tissue Research* 2000;**299**:9-26.
32. Keast JR, De Groat WC. Segmental distribution and peptide content of primary afferent neurons innervating the urogenital organs and colon of male rats. *Journal of Comparative Neurology* 1992;**319**:615-23.
33. Russo D, Clavenzani P, Sorteni C, Bo Minelli L, Botti M, Gazza F, Panu R, Ragionieri L, et al. Neurochemical features of boar lumbosacral dorsal root ganglion neurons and characterization of sensory neurons innervating the urinary bladder trigone. *Journal of Comparative Neurology* 2013;**521**:342-66.
34. Sienkiewicz W. Sources of the porcine testis innervation. *Andrologia* 2010;**42**:395-403.
35. Kaleczyc J, Scheuermann DW, Pidsudko Z, Majewski M, Lakomy M, Timmermans JP. Distribution, immunohistochemical characteristics and nerve pathways of primary sensory neurons supplying the porcine vas deferens. *Cell & Tissue Research* 2002;**310**:9-17.
36. Herweijer G, Kyloh M, Beckett EA, Dodds KN, Spencer NJ. Characterization of primary afferent spinal innervation of mouse uterus. *Frontiers in Neuroscience* 2014;**8**:202.
37. Dalsgaard CJ, Elfvin LG. Structural studies on the connectivity of the inferior mesenteric ganglion of the guinea pig. *Journal of the Autonomic Nervous System* 1982;**5**:265-78.

38. Baron R, Janig W, McLachlan EM. The afferent and sympathetic components of the lumbar spinal outflow to the colon and pelvic organs in the cat. I. The hypogastric nerve. *Journal of Comparative Neurology* 1985;**238**:135-46.
39. Beyak MJ, Ramji N, Krol KM, Kawaja MD, Vanner SJ. Two TTX-resistant Na⁺ currents in mouse colonic dorsal root ganglia neurons and their role in colitis-induced hyperexcitability. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2004;**287**:G845-55.
40. Christianson JA, Traub RJ, Davis BM. Differences in spinal distribution and neurochemical phenotype of colonic afferents in mouse and rat. *Journal of Comparative Neurology* 2006;**494**:246-59.
41. Domoto T, Yang H, Bishop AE, Polak JM, Oki M. Distribution and origin of extrinsic nerve fibers containing calcitonin gene-related peptide, substance P and galanin in the rat upper rectum. *Neuroscience Research* 1992;**15**:64-73.
42. Brain SD, Williams TJ, Tippins JR, Morris HR, MacIntyre I. Calcitonin gene-related peptide is a potent vasodilator. *Nature* 1985;**313**:54-6.
43. Kawasaki H, Takasaki K, Saito A, Goto K. Calcitonin gene-related peptide acts as a novel vasodilator neurotransmitter in mesenteric resistance vessels of the rat. *Nature* 1988;**335**:164-7.
44. Meehan AG, Kreulen DL. A capsaicin-sensitive inhibitory reflex from the colon to mesenteric arteries in the guinea-pig. *J Physiol Lond* 1992;**448**:153-9.
45. Wang GD, Wang XY, Liu S, Qu M, Xia Y, Needleman BJ, Mikami DJ, Wood JD. Innervation of enteric mast cells by primary spinal afferents in guinea pig and human small intestine. *American Journal of Physiology - Gastrointestinal & Liver Physiology* 2014;**307**:G719-31.
46. Price TJ, Flores CM. Critical evaluation of the colocalization between calcitonin gene-related peptide, substance P, transient receptor potential vanilloid subfamily type 1 immunoreactivities, and isolectin B4 binding in primary afferent neurons of the rat and mouse. *Journal of Pain* 2007;**8**:263-72.
47. Silverman JD, Kruger L. Lectin and neuropeptide labeling of separate populations of dorsal root ganglion neurons and associated "nociceptor" thin axons in rat testis and cornea whole-mount preparations. *Somatosensory Research* 1988;**5**:259-67.
48. Semenenko FM, Cervero F. Afferent fibres from the guinea-pig ureter: size and peptide content of the dorsal root ganglion cells of origin. *Neuroscience* 1992;**47**:197-201.
49. Harper AA, Lawson SN. Conduction velocity is related to morphological cell type in rat dorsal root ganglion neurones. *Journal of Physiology* 1985;**359**:31-46.
50. Lynn PA, Zagorodnyuk VP, Hennig GW, Costa M, Brookes SJH. Mechanical activation of rectal intraganglionic laminar endings of the guinea pig distal gut. *J Physiol* 2005;**564.2**:589-601.
51. Ness TJ, Gebhart GF. Visceral pain: a review of experimental studies. *Pain* 1990;**41**:167-234.

TABLES

Table 1. Primary and secondary antisera used in the study

Abbreviations: CGRP, calcitonin gene-related peptide; TRPV1, transient receptor potential vanilloid 1 IgG, immunoglobulin G; AMCA, aminomethylcoumarin; Cy5, indodicarbocyanine;

Primary Antibody	Immunising antigen	Raised	Dilution	Source/cat. #/lot #
CGRP	Rat CGRP, peptide sequence: HSCATATCVTHRLAGLLSRS GGVVKNNFVPTNVGSEAF-NH2	Rabbit	1:1600	Peninsula/IHC6006 /030687-3/ RRID: AB_2314156
TRPV1	VR1 (R-18) is an affinity purified goat polyclonal antibody raised against a peptide mapping at the carboxy terminus of Vanilloid Receptor 1 of rat origin	Goat	1:100	Santa Cruz/sc-8671 /H1803
Secondary Antibody	Company and cat. #	Raised	Dilution	Conjugated fluorophore
Donkey anti-goat IgG	Jackson, 705 155 003	Donkey	1:200	AMCA
Donkey anti-goat IgG	Jackson, 705 175 147	Donkey	1:100	Cy5
Donkey anti-rabbit IgG	Jackson, 711 155 152	Donkey	1:200	AMCA
Donkey anti-rabbit IgG	Jackson, 711 175 152	Donkey	1:200	Cy5

FIGURE LEGENDS

Figure 1. Images of DiI-filled neurons. A shows neurons in a myenteric ganglion less than 10mm oral to a DiI injection site in the gut wall. B: DiI-filled spinal sensory neurons in a wholemount dorsal root ganglion from segment L2 after application to the rectum. C: Large numbers of neurons in S3 filled by DiI applied in the rectum. The thickness and opacity of dorsal root ganglia caused images of cells to have poorly defined outlines in 1B and 1C; nevertheless, high signal: noise ratios allowed counting of filled nerve cell bodies.

Figure 2. Distribution of spinal sensory neurons filled by DiI applied to the distal colon (black bars, n=6) and rectum (white bars, n=9). The Y-axis shows the average number of cells in each pair of spinal ganglion at each level. Much larger numbers of cells were filled from the rectum than the distal colon, despite approximately similar numbers and volumes of DiI injection. In addition, nerve cell bodies filled from the colon were confined to thoracolumbar ganglia, with a peak at L2/L3, whereas DiI applied to the rectum filled neurons with a bimodal distribution peaking at L3 and S3.

Figure 3. Typical examples of retrogradely-traced neurons filled by DiI applied to the distal colon, labelled for CGRP- and TRPV1-immunoreactivity. In the upper pictures, a DiI filled neuron in L2 is immunoreactive for both CGRP and TRPV1. In the lower triplet, a DiI-filled nerve cell body is immunoreactive for CGRP but not TRPV1.

Figure 4. Cells labelled with DiI applied to the rectum, immunohistochemically-labelled for CGRP- and TRPV1-immunoreactivity. The upper panels show two cells labelled in L3 (one with intense DiI (arrow), the other faintly labelled with punctate fluorescence (arrowhead)). Arrowed cell is immunoreactive for both CGRP and TRPV1, whereas the faint cell (arrowhead) contains

neither marker. In the lower triplet, two cells with DiI fluorescence (arrowheads) are visible - both lack immunoreactivity for CGRP and TRPV1.

Figure 5. Immunoreactivity for CGRP and TRPV1 of spinal afferents filled from colon and rectum (A-C) compared to all cells in lumbar and sacral ganglia (D-E). **A:** Only 30% of thoracolumbar spinal afferents (in L2 and L3) filled from the colon contained immunoreactivity for CGRP, of which the great majority were also immunoreactive for TRPV1 (n=3). **B:** nearly 50% of thoracolumbar sensory neurons filled from the rectum were immunoreactive for CGRP and, again, the majority of these also contained TRPV1-immunoreactivity (n=7). **C:** Most sacral afferents filled from the rectum lacked CGRP (38% were CGRP-immunoreactive, of which approximately half were also TRPV1 immunoreactive; n=7). **D** shows overall proportions of immunohistochemical types of neurons from L4 DRG ganglia in guinea pigs (n=5), against which **A** and **B** should be compared. Note that CGRP+/TRPV1+ neurons are more abundant in the DiI-filled populations than in the ganglion as a whole. **E.** Proportions of CGRP- and TRPV1-immunoreactive neurons in control S2 DRG as a whole (n=4) against which **5C** can be compared. Overall, the results indicate that CGRP-containing peptidergic neurons make up a smaller proportion of spinal afferents than in rats and mice (see text). **F.** Soma size of neurochemically-defined colorectal afferents measured from vertical projections of cell bodies. The four combinations of CGRP- and TRPV1-immunoreactivity are shown for gut-projecting neurons (to rectum or colon) located in either lumbar or sacral dorsal root ganglia. Where available, cell sizes of a selection of all cells in lumbar or sacral ganglia are also shown for comparison (hatched bars). Note that it was not possible to measure the area of non-DiI-labelled cells that lacked both CGRP and TRPV1, which would be expected to include many of the largest cells in the ganglia. It has previously been reported that CGRP+/TRPV1+ neurons are small cells in dorsal root ganglia. In the present study this did not appear to be the case. In particular, DiI-labelled lumbar CGRP+/TRPV1+ neurons, filled from either rectum or colon, were slightly larger, on average, than

other DiI-filled cells. Lumbar cells that projected to the distal colon also tended to be larger, on average, than cells in other pathways. The sizes of DiI-filled, CGRP-/TRPV+ neurons are not shown due to inadequate sample sizes.



fig 1

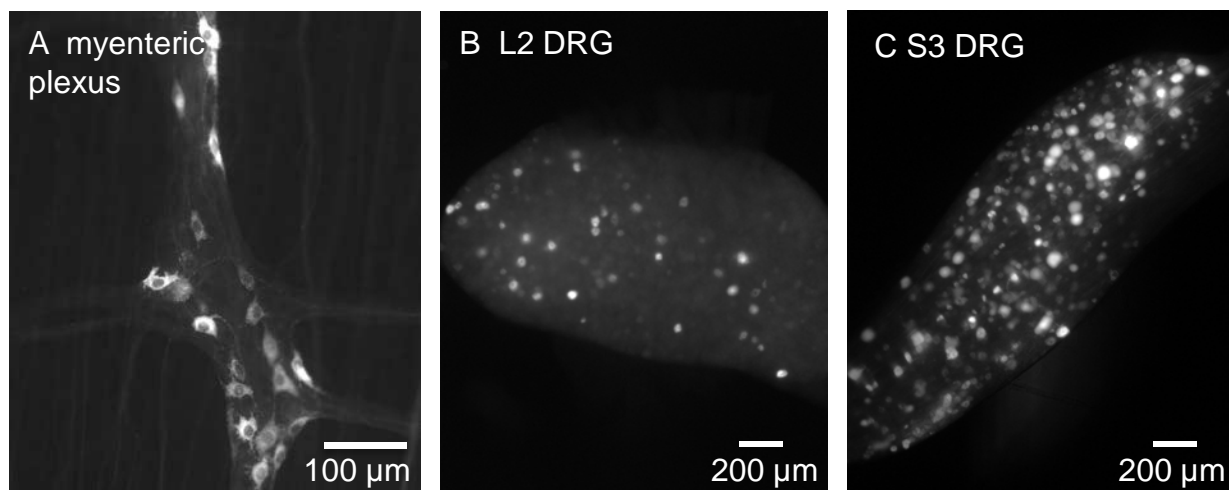
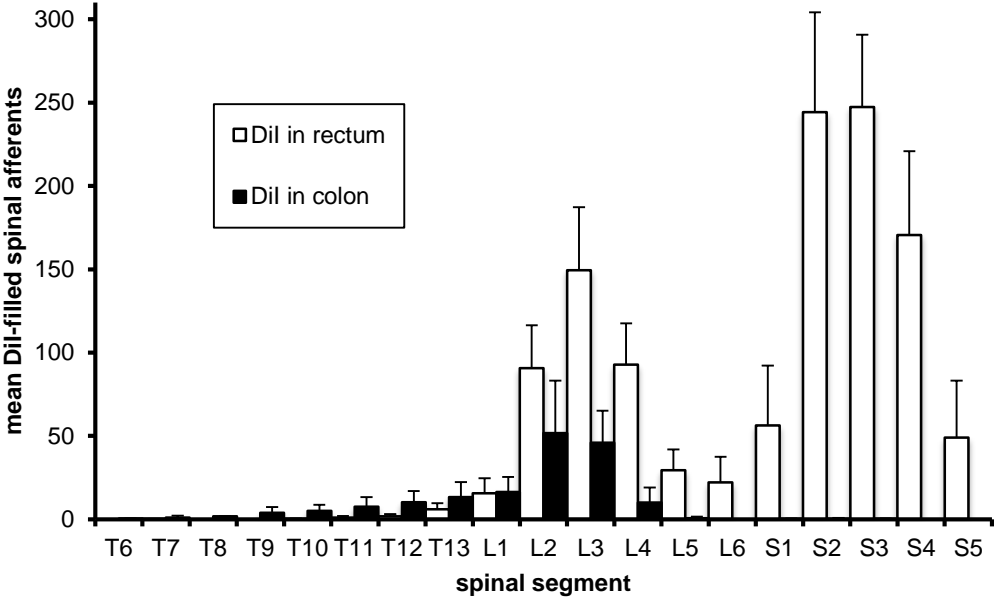




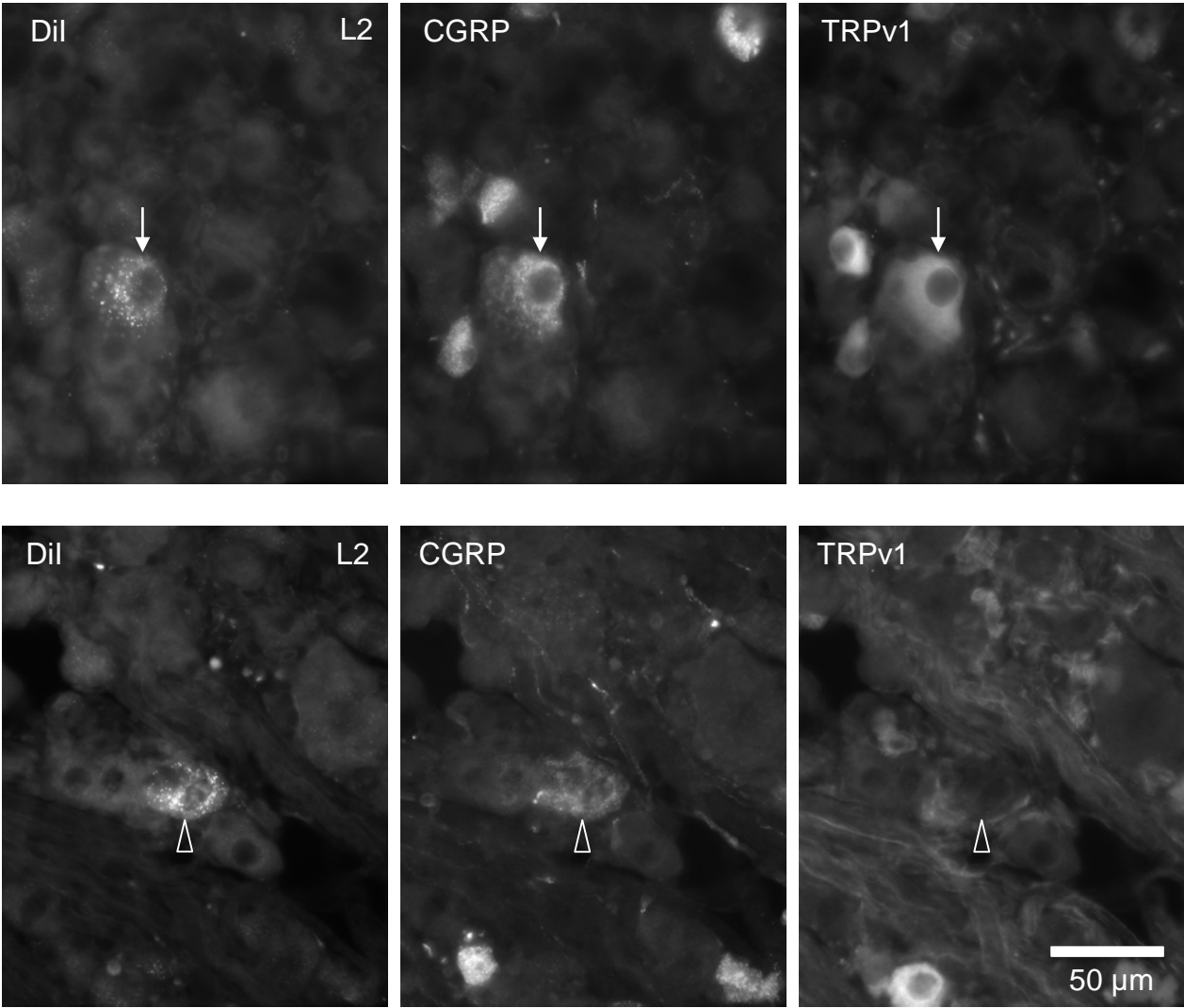
fig 2

A



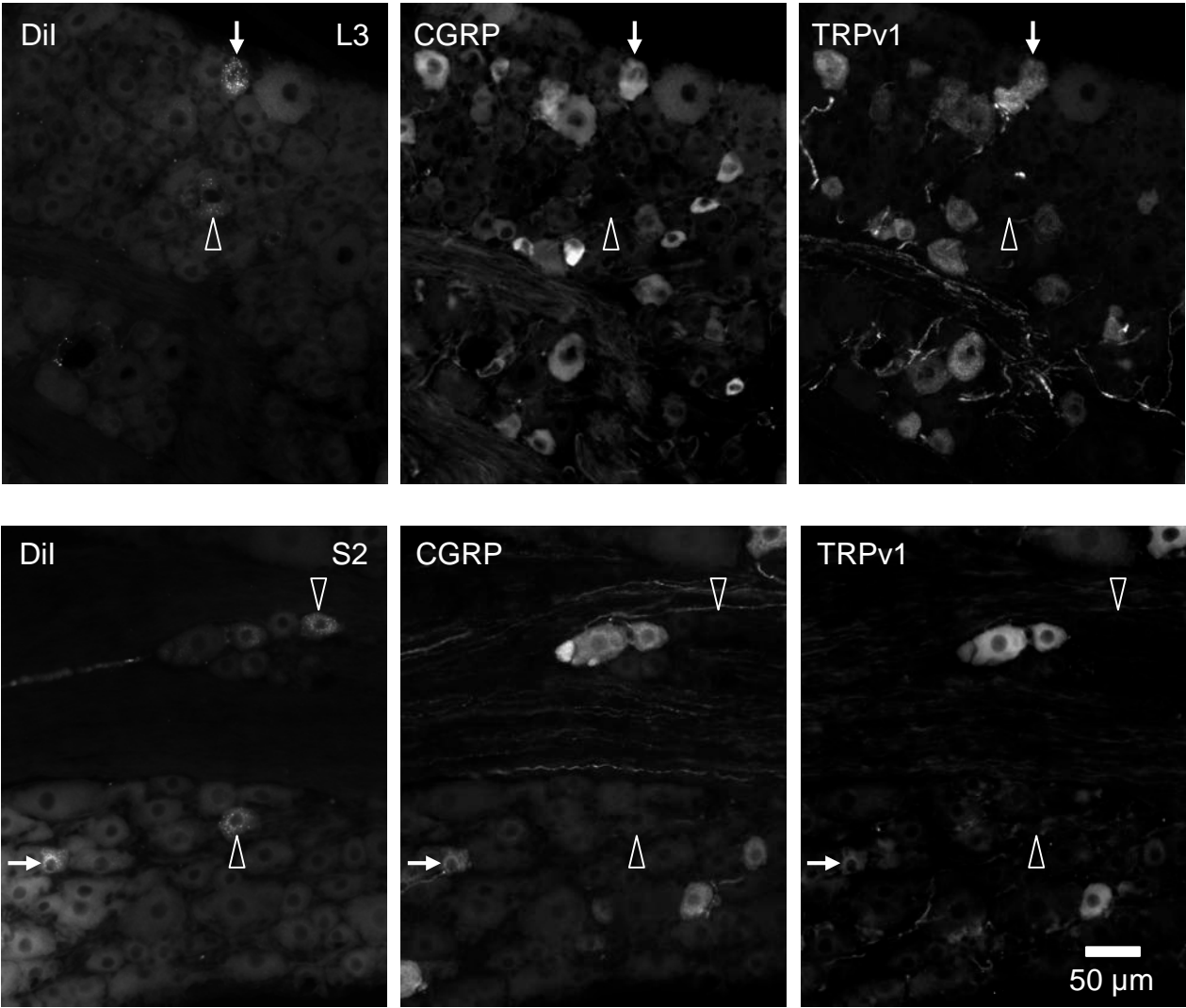
Dil colon

fig 3

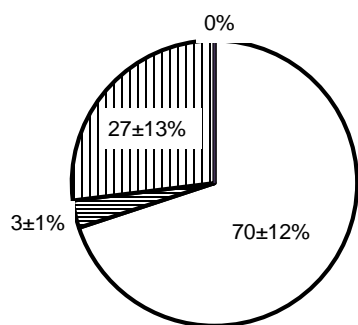


Dil rectum

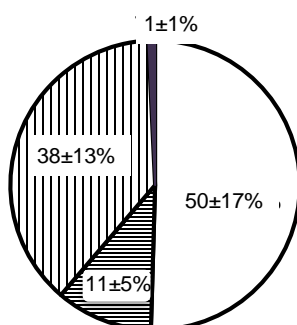
fig 4



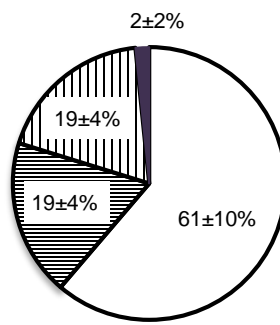
A Dil cells L2/L3
from colon



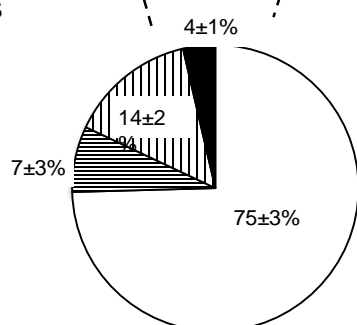
B Dil cells L2/L3
from rectum



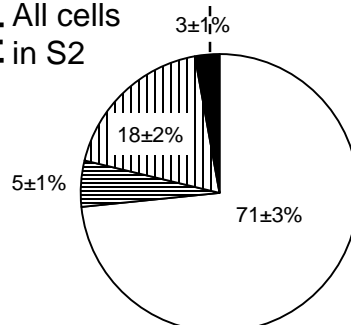
C Dil cells S2/S3
from rectum



D All cells
in L4



E All cells
in S2



F

