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Neurochemical characterisation of nerve fibres in colonic nerves to myenteric ganglia of the guinea-pig distal colon

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by

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ABSTRACT

Extrinsic nerves to the gut influence the absorption of water and electrolytes and expulsion of waste contents, largely via regulation of enteric neural circuits; they also contribute to the control of blood flow. The distal colon is innervated by extrinsic sympathetic and parasympathetic efferent and spinal afferent neurons, via axons in colonic nerve trunks. In the present study, biotinamide tracing of colonic nerves was combined with immunohistochemical labeling for markers of sympathetic, parasympathetic and spinal afferent neurons to quantify their relative contribution to the extrinsic innervation. Calcitonin gene-related peptide, vesicular acetylcholine transporter and tyrosine hydroxylase, which selectively label spinal afferent, parasympathetic and sympathetic axons, respectively, were detected immunohistochemically in $1 \pm 0.5\%$ ($n = 7$), $15 \pm 4.7\%$ ($n = 6$) and $24 \pm 4\%$ ($n = 7$) of biotinamide-labeled extrinsic axons in myenteric ganglia. Immunoreactivity for vasoactive intestinal polypeptide, nitric oxide synthase, somatostatin, vesicular glutamate transporters 1 and 2 accounted for a combined maximum of 14% of biotinamide-labeled axons in myenteric ganglia. Thus, a maximum of 53% of biotinamide-labeled extrinsic axons in myenteric ganglia were labeled by antisera to one of these eight markers. Viscerofugal neurons were also labeled by biotinamide, and shown to have distinct morphologies and spatial distributions that correlated closely with their immunoreactivity for nitric oxide synthase and choline acetyltransferase. As reported for the rectum, nearly half of all extrinsic nerve fibers to the distal colon lack the key immunohistochemical markers commonly used for their identification. Their abundance may therefore have been significantly underestimated in previous immunohistochemical studies.

65 INTRODUCTION

The gastrointestinal (GI) tract has anatomically distinct regions that perform discrete functions, which include absorption of nutrients, water and electrolytes and expulsion of waste contents. These functions are under tight temporal and spatial control, mediated directly via the enteric nervous system (Furness and Costa, 1987). However, several populations of extrinsic neurons also innervate the GI tract; parasympathetic and sympathetic efferents, and spinal afferent neurons can modulate activity in enteric circuits that regulate local GI functions and thus coordinate their activity between regions of gut (Jänig, 2006). Motor behavior that occurs along the GI tract is highly dependent on such extrinsic innervation. For example, defecation, gastric accommodation and receptive relaxation all involve extrinsic autonomic pathways (Cannon and Lieb, 1911; De Groat and Krier, 75 1978; Wei et al., 1997). Damage to the extrinsic innervation of the GI tract is implicated in a number of disorders that affect GI function (Knowles et al., 2001; Pfeiffer, 2011; Vinik et al.,

2003). Quantitative characterization of the extrinsic innervation along the length of the GI tract may assist in understanding the mechanisms that control motility, in health and disease.

80 The distal bowel of guinea-pigs can be divided into two discrete regions, separated by a transition zone, on the basis of the sources of extrinsic innervation (see Figure 1). The rectum is innervated by parasympathetic neurons located in pelvic ganglia and some neurons in the sacral spinal cord, which reach the distal bowel primarily via the rectal nerves. In addition, rectal nerves contain sympathetic axons originating from: post-ganglionic cell bodies in lumbar sympathetic chain (via
85 hypogastric nerves); cell bodies in prevertebral ganglia; sacral sympathetic chain ganglia (via pelvic nerves); and from some sympathetic neurons located in pelvic ganglia (Luckensmeyer and Keast, 1995). Axons of spinal afferent neurons, from both lumbar and sacral segments, also run in the rectal nerves. The distal colon, on the other hand, receives sympathetic input primarily from post-ganglionic neurons in prevertebral ganglia, with a smaller contribution from sympathetic chain
90 ganglia. It also receives axons from some parasympathetic neurons from pelvic ganglia (Olsson et al., 2006). Extrinsic primary afferent neurons, with cell bodies in lumbar dorsal root ganglia, innervate both regions, with the rectum receiving an additional innervation from sacral segments (Robinson et al., 2004). Whilst the distal colon is specialised for absorption of water and electrolytes from luminal contents, the rectum controls expulsion of waste from the GI tract. Along
95 the length of the GI tract, many common features have been characterised in the enteric neuronal circuits and the motor behaviours that they generate (Brookes et al., 1999; Costa et al., 1996; Lomax and Furness, 2000; Schemann et al., 1995; Spencer et al., 2002). However, the sources and density of extrinsic innervation vary along the GI tract (Berthoud et al., 1991; Berthoud et al., 1990; Olsson et al., 2006; Trudrung et al., 1994). Nevertheless, in all regions, the enteric ganglia,
100 especially the myenteric plexus, are major targets of extrinsic nerve fibres in the gut wall (Holst et al., 1997; Olsson et al., 2004; Tassicker et al., 1999).

In this study, rapid anterograde tracing of lumbar colonic nerves to the distal colon of the guinea-pig was combined with immunohistochemical labeling to quantify the different types of extrinsic
105 innervation of myenteric ganglia. These results are compared to a previous study of extrinsic innervation of the rectum using identical techniques (Olsson et al., 2004) and to retrograde labeling studies of the same preparations (Olsson et al., 2006).

MATERIALS AND METHODS

110 *Tissue collection*

41 adult guinea-pigs of either sex (weight 220–360g) were killed in the morning (between 8am-

11.30am) by a blow to the back of the head and exsanguinated via the carotid arteries, in a manner approved by the Animal Welfare Committee of Flinders University, South Australia. Animals were opened along the ventral midline and the distal colon with the attached mesentery and pelvic ganglia were removed, intestinal contents were flushed and the preparation placed in Krebs solution (118mM NaCl, 4.75 mM KCl, 1.0mM NaH₂PO₄, 25 mM NaHCO₃, 1.2mM MgSO₄, 11.1 mM D-glucose, 2.5mM CaCl₂, bubbled with 95%O₂/5%CO₂). Tissue, at least 15cm from the anal margin, was pinned in a Sylgard-lined Petri dish (Dow Corning, Midland, MI) before the gut was opened longitudinally alongside the rectal artery. The mucosa and submucosa were carefully removed and the remaining wholemount preparation was pinned serosal-side up, with the major pelvic ganglia secured loosely on either side. Krebs solution was regularly changed during dissection.

Rapid anterograde tracing

Lumbar colonic nerve trunks were identified as those that entered the colonic wall, but which did not emanate from the major pelvic plexus. In many cases, these could be visually traced back to the inferior mesenteric ganglion; in others, fat obscured their source. These nerve trunks were dissected free of connective tissue over a length of at least 5mm and the extrinsic ganglia were removed. The preparation (20-30mm in length) was then pinned in a chamber lined with Sylgard. The nerve trunk ran under a glass coverslip and was pinned in a small Perspex isolation chamber sealed with high-vacuum silicon grease (Ajax, Auburn, NSW Australia). The nerve was washed in 3-4 changes of artificial intracellular medium solution (150 mM monopotassium, L-glutamic acid, 7 mM MgCl₂, 5 mM glucose, 1 mM ethylene glycolbis-[β-aminoethyl ether] N,N,N',N'-tetra-acetic acid [EGTA], 20 mM hydroxyeicosapentaenoic acid [HEPES] buffer, 5 mM disodium adenosine-5-triphosphate [ATP], 0.02% saponin, 1% dimethyl sulphoxide [DMSO], 100 IU/ml penicillin, 100 µg/ml streptomycin, 20 µg/ml gentamycin sulphate; (Tassicker et al., 1999), before the chamber was filled with paraffin oil. A small drop of 5% biotinamide (N-[2-aminoethyl] biotinamide, hydrobromide; Molecular Probes, Eugene, OR) dissolved in the same artificial intracellular solution was placed on the nerve. The Krebs solution in the main chamber was replaced with sterile culture medium and placed on a rocking tray in a humidified incubator at 37°C and in 5% CO₂ in air.

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Tissue fixation

After 16 hours, preparations were maximally stretched and fixed in modified Zamboni's fixative (2% formaldehyde, 0.2% saturated picric acid in 0.1M phosphate buffer, pH 7.0) for approximately 24 hours at 4°C. Tissue was then cleared with three washes of 100% dimethylsulphoxide (DMSO), and stored in PBS at 4°C. For immunohistochemical labeling of myenteric ganglia, the circular muscle layer was removed by sharp dissection.

Immunohistochemistry

Preparations of myenteric plexus and longitudinal muscle were incubated with antisera to combinations of markers (Table 1) at room temperature for two days. Preparations were rinsed three
150 times in PBS and incubated with secondary antisera (Table 2) for 4 hours at room temperature. Biotinamide was labeled with streptavidin conjugated to either Alexa Fluor488 or Cy3 (dilution 1:2,000; Molecular Probes). After a final rinse with PBS, preparations were equilibrated with 50%, 70%, and 100% carbonate-buffered glycerol, and mounted in 100% carbonate-buffered glycerol (pH 8.6). All antibodies were diluted in 0.1 M PBS (0.3 M NaCl) containing 0.1% sodium azide.
155 Controls for double-labeling were performed by omitting one or more primary antibodies from the procedure, and by ensuring that the associated secondary antisera no longer labeled structures in the tissue.

Antibody characterization

160 **Calcitonin gene-related peptide** The calcitonin gene-related peptide (CGRP) antibody (Peninsula Laboratories, Belmont CA; Cat. No. IHC6006; Lot. No. 040826-2; RRID: AB_2314156) was polyclonal; raised in rabbit against rat CGRP (sequence: HSCATATCVTHRLAGLLSRSGGVVKNFVPTNVGSEAF-NH₂). It recognized a single band of 14 kDa, corresponding to CGRP's molecular weight, on western blots of horse ileum homogenates
165 (Russo et al., 2010). Preabsorption of the diluted antibody with the immunogen abolishes staining in horse spinal cord, dorsal root ganglia, and intestine (Domeneghini et al., 2004).

Choline acetyltransferase The choline acetyltransferase (ChAT) antibody (Schemann; Cat. No. P3YEB; RRID: AB_2314176), generously provided by Dr. M. Schemann of Technische Universitat Muenchen, was polyclonal; raised in rabbit against a 22 amino acid peptide fragment of purified
170 porcine ChAT (peptide sequence: GLFSSYRLPGHTQDTLVAQKSS). It labels a single band on western blots of guinea pig inferior mesenteric ganglion and pelvic ganglia of 65 kDa, compared with the ChAT theoretical molecular weight of 72 kDa (Olsson et al., 2006).

Neuronal nitric oxide synthase The neuronal nitric oxide synthase (NOS) antibody (Emson; Cat. No. K205; RRID: AB_2314960), generously provided by Dr. P. Emson, was polyclonal; raised in
175 sheep against recombinant rat brain nNOS. It labels an intense band at 160 kDa (theoretical molecular weight of 160 kDa) and a fainter band at 40 kDa on western blots of guinea pig inferior mesenteric ganglion and pelvic ganglia (Olsson et al., 2006).

Somatostatin The somatostatin antibody (Brown; Cat. No. Soma 8; RRID: AB_2313828) was monoclonal; raised in mouse against somatostatin-14 conjugated to keyhole limpet hemocyanin
180 using carbodiimide (Buchan et al., 1985). Preabsorption of the diluted antibody with 100 µg/ml somatostatin-14 abolished labeling in the rat amygdala (Muller et al., 2007).

Tyrosine hydroxylase The tyrosine hydroxylase (TH) antibody, (DiaSorin, Stillwater, MN; Cat. No. 22941; Lot. No. 136032; RRID: AB_572268), was monoclonal; raised in mouse against TH extracted from rat PC12 cells. The antibody labels an intense band of 60 kDa (theoretical molecular weight of 56 kDa) and a faint band of 52–54 kDa in western blots of guinea pig inferior mesenteric ganglion and pelvic ganglia (Olsson et al., 2006).

Vesicular acetylcholine transporter The vesicular acetylcholine transporter (VACHT) antibody, (Phoenix Pharmaceuticals, Belmont, CA; Cat. No. HV007; Lot No. 417149; RRID: AB_2315530), was polyclonal; raised in goat against the C-terminal of human VACHT (peptide sequence: CTRSRSERDVLLDEPPGLYDAVRLRE). Preabsorption of the antibody with 50 µm of the immunogen abolished labeling in the dentate gyrus of monkeys (Shamy et al., 2007).

Vesicular glutamate transporter 1 The vesicular glutamate transporter 1 (VGluT1) antibody, (Synaptic Systems, Göttingen, Germany; Cat. No. 135002; RRID: AB_2315546) was polyclonal; raised in rabbit against recombinant GST-fusion protein containing amino acid residues 456-561 of rat VGluT1. Preabsorption with the immunogen according to the manufacturer's datasheet completely abolished all labeling within the myenteric neuropil, myenteric neuronal cell bodies, muscularis mucosae, and muscularis propria in the mouse esophagus (Kraus et al., 2007).

Vesicular glutamate transporter 2 The vesicular glutamate transporter 2 (VGluT2) antibody, (Synaptic Systems, Göttingen, Germany; Cat. No. 135102; RRID: AB_2315570) was polyclonal; raised in rabbit against recombinant GST-fusion protein containing amino acid residues 510-582 of rat VGluT2. Preabsorption with 1.5-fold excess of antigen abolished staining of IGLEs in the rat and mouse esophagus (Raab and Neuhuber, 2004).

Vasoactive intestinal peptide The vasoactive intestinal peptide (VIP) antibody (Chemicon; Cat. No. AB1581, Lot No. 0611044826; RRID: AB_2288587) was polyclonal; raised in sheep against synthetic peptide VIP (1-28) coupled to keyhole limpet hemocyanin with glutaraldehyde. Previous studies have shown that the VIP antibody used in this study gives an identical labeling profile to other VIP antibodies on guinea-pig intestinal tissue (Sharrad et al., 2013b).

Microscopy, image analysis and processing

Specimens were examined on an Olympus IX71 microscope (Japan) equipped with epifluorescence and highly discriminating filters (Chroma Technology Co., Battledore, VT). Images were captured using a Roper scientific (Coolsnap) camera at 1392 x 1080 pixels, using AnalySIS Imager 5.0 (Olympus-SIS, Münster, Germany) and saved as TIFF files. Some images were obtained from an Olympus AX70 epifluorescence microscope, captured with a Hamamatsu digital camera (model C4742-95, Japan) using IPLab software (Scanalytics Inc., Fairfax, VA, USA; RRID: rid_000102). Matched micrographs of immunohistochemically-labeled nerve structures were captured using a

40x objective water-immersion lens and displayed in ImageJ (NIH, Bethesda, MD; RRID: nif-0000-30467) as a stack. We used a previously validated method to identify coexistence of biotinamide with immunohistochemical markers in varicosities (Sharrad et al., 2013a; Sharrad et al., 2013b; Sharrad et al., 2013c). In brief, a biotinamide-labeled varicosity was selected by moving the cursor to a random site in a ganglion and selecting the closest varicosity. Using the stacked images, we tested whether the varicosity was immunoreactive for any of the other markers. Varicosities were considered immunoreactive when they were clearly discernible above background labeling: a criterion that corresponded to at least three standard deviations above the mean value of background fluorescence. Biotinamide-labeled varicosities were excluded when:

- (i) they could not be easily distinguished above background labeling;
- (ii) were out-of-focus;
- (iii) were overlying nerve cell bodies or other immunoreactive structures.

Otherwise, sampling was unbiased and not influenced by either the size of the varicosity nor the intensity of biotinamide labeling. Either five or ten varicosities (depending on density of labelling) were selected at widely separated sites in each image to minimize double counting of axons and either 20 or 10 stacks were examined, giving total counts of 100 varicosities, in each of the guinea-pigs. Group data are expressed as percentage means (mean number of varicosities from a sample of 100) \pm standard error of the mean (SEM), with n referring to the number of animals. All biotinamide-labeled cell bodies were sampled and tested for immunoreactivity for ChAT and NOS. Viscerofugal nerve cell bodies from eight animals were also characterized in this way. Figures were generated from grayscale images adjusted for contrast and brightness in Adobe Photoshop CS5 and cropped and resized to improve display of varicosities of interest. Statistical analysis was performed by one-way ANOVA with Bonferroni post-hoc analyses or X^2 tests using IBM SPSS Statistics 20 for Microsoft Windows (release 20.0.0, IBM Corp., USA). Differences were considered significant if $P < 0.05$.

245 RESULTS

Biotinamide filling in layers of the colon

Biotinamide-labeled axons were intensely labeled after organ culture overnight. Labeled axons were abundant in the myenteric plexus near the point of entry of the colonic nerve, with each ganglion containing numerous branching varicose axons (Figure 2A). Further from the point of entry, the density of varicose axons in ganglia decreased markedly. Unlike the rectum, dense arrays of biotinamide-filled extrinsic axons in circular or longitudinal muscle layer were very rare (Olsson et al., 2004) but occasional fibres of passage through the circular muscle layer were observed (Fig 2A arrow). Viscerofugal nerve cell bodies were also labelled in some myenteric ganglia in most preparation (Fig 2A, open arrowhead). Biotinamide-filled axons were much sparser in the submucosa, with a few branching axons in submucous ganglia (Fig 2B arrowheads), but many fibres running parallel to submucosal blood vessels and sometimes single axons branching on their surface (Fig 2B open arrow). There was also a very sparse innervation of the mucosa, with a few rare axons meandering around the bases of the colonic glands (Figure 2C). D. Outside the gut wall, bundles of axons-of-passage, together with perivascular and paravascular fibres associated with the blood vessels, were labeled, although these were often obscured by mesenteric fat. Paravascular bundles running near mesenteric vessels could be traced visually to be continuous with bundles of axons associated with intramural blood vessels in the submucosa. Consistently, the major target of biotinamide filled axons in the distal colon (judged by density of varicose branching nerve fibres) were the myenteric ganglia. For this reason, axons in this layer were investigated immunohistochemically and compared to a similar previous study of extrinsic nerves to the guinea pig rectum (Olsson et al., 2004).

Markers of sympathetic, parasympathetic and spinal afferent axons

The proportions of biotinamide-labeled axonal varicosities containing well-characterised immunohistochemical markers of extrinsic neuronal populations were quantified from stacked micrographs of epifluorescent images (see methods and Fig 3). Calcitonin gene-related peptide (CGRP) labels the axons of many extrinsic spinal afferent neurons (Gibbins et al., 1985); tyrosine hydroxylase (TH) is present in noradrenergic sympathetic neurons (Macrae et al., 1986), and vesicular acetylcholine transporter (VAcHT) is localised in the axons of cholinergic parasympathetic neurons (Olsson et al., 2006; Olsson et al., 2004). On average, $1 \pm 0.5\%$ ($n = 7$) of biotinamide-labeled varicosities in myenteric ganglia contained CGRP-immunoreactivity, whereas $15 \pm 4.7\%$ ($n = 6$) contained VAcHT-immunoreactivity and $24 \pm 4\%$ ($n = 7$) contained TH-immunoreactivity. Assuming that there was no coexistence, these values showed that up to $39 \pm 9.2\%$ of biotinamide-labeled varicose axons contained one of these three major

280 immunohistochemical markers of extrinsic nerve populations. These counts assume that immunoreactivity for these markers did not change during the period of 16 hours during which biotinamide labeling took place. Qualitatively, the density of TH- and CGRP-immunoreactive fibres in the myenteric plexus appeared similar in tissue maintained in organ culture for 16 hours compared to freshly fixed tissue (see Figure 4), although a slight increase in background
285 fluorescence was noted after culture. A blinded observer was unable to distinguish reliably fresh-fixed preparations from biotinamide-filled preparations on the basis of the appearance of varicose axons immunohistochemically labeled for VAcHT, TH or CGRP (Figure 4).

Other markers of extrinsic neuronal populations

290 Other markers have previously been reported in nerve cell bodies of sympathetic, parasympathetic or spinal afferent neurons; the presence of these markers in anterogradely labeled extrinsic axons in myenteric ganglia of the guinea-pig distal colon was also investigated (Fig 5). Somatostatin is contained in some nerve cell bodies in pelvic ganglia (Morris and Gibbins, 1987), celiac ganglia (Keast et al., 1993), superior mesenteric ganglia (Keast et al., 1993) and inferior mesenteric ganglia
295 (Parr and Sharkey, 1996). Vasoactive intestinal polypeptide (VIP) is expressed in pelvic ganglia (Morris and Gibbins, 1987) and inferior mesenteric ganglia (Parr and Sharkey, 1996). Nitric oxide synthase (NOS) is expressed in pelvic ganglia (Olsson et al., 2006) and inferior mesenteric ganglia (Parr and Sharkey, 1996). Vesicular glutamate transporter 1 (VGluT1) is expressed in the cell bodies of some dorsal root ganglion neurons, as is vesicular glutamate transporter 2 (VGluT2)
300 (Morris et al., 2005).

In biotinamide-filled extrinsic axons in myenteric ganglia of guinea-pig distal colon, somatostatin was present in $2 \pm 1.1\%$ ($n = 4$), VIP in $5 \pm 2.5\%$ ($n = 5$) and VGluT1 in $5 \pm 1.4\%$ ($n = 5$) of biotinamide-labeled axons. Rarely was VGluT2 detected in biotinamide-labeled axons ($1 \pm 0.3\%$, $n = 4$). NOS was not detectable in biotinamide-labeled axons at all ($0 \pm 0\%$, $n = 8$). When these
305 proportions are summed together with the proportion of biotinamide-labeled axons that contained CGRP-, TH- and VAcHT-immunoreactivity (assuming no coexistence) a maximum of $53 \pm 14.5\%$ of biotinamide-labeled axons contained one of the eight substances studied. We also examined binding of isolectin B4 from *Bandeiraea simplicifolia*; a marker for a population of sensory neurons, although many are probably cutaneous afferents (Gerke and Plenderleith, 2002). IB4
310 labeled a dense plexus of nerve fibres and enteric nerve cell perikarya in enteric ganglia. However the labeling was both dense and somewhat diffuse quality, and did not allow co-existence with biotinamide to be quantified. IB4 labeling was not visible in axons on mesenteric or intramural blood vessels, indicating that the lectin binding site is not expressed at measurable levels in axons of either sympathetic efferent or peptidergic spinal afferent neurons in the guinea pig colon.

Biotinamide-labeled viscerofugal enteric nerve cell bodies

Application of biotinamide to colonic nerve trunks labeled an average of 20 ± 11 viscerofugal nerve cell bodies per preparation (7 preparations, $n = 5$). Pooling this data, 57 cells had axons that could be traced through the myenteric plexus; all had a single axon (Hibberd et al., 2012; Sharkey et al., 1998; Tassicker et al., 1999) most with a smooth appearance (43 of 57, $n=5$); the remaining 14 had a varicose appearance (average: $74 \pm 7\%$ with smooth axons, $n=5$, $\text{mean} \pm \text{SD}$). Of 138 filled cell bodies ($n = 5$), 81 had small cell bodies (area: $521 \pm 241 \mu\text{m}^2$) with few or no dendrites, resembling “simple cells” (Furness et al., 1988). The remaining 57 had medium-sized cell bodies (area: $767 \pm 236 \mu\text{m}^2$) with the lamellar dendrites of Dogiel type I “lamellar” morphology (Furness et al., 1988). Averaged per preparations, $59 \pm 10\%$ ($n=5$) had “simple” soma-dendritic morphology; the remainder were Dogiel type I cells; none had multi-axonal Dogiel type 2 morphology (Ermilov et al., 2003).

Preparations were immunohistochemically-labeled with antisera against choline acetyltransferase (ChAT) and NOS (Fig. 6). Of 137 biotinamide-labeled viscerofugal cell bodies (pooled from $n = 5$), 53 contained immunoreactivity for both ChAT and NOS; 59 contained immunoreactivity for ChAT without NOS (ChAT+/NOS-); 15 were immunoreactive for NOS without ChAT (ChAT-/NOS+), and the remaining 10 lacked both ChAT- and NOS-immunoreactivity (ChAT-/NOS-). When averaged per preparation $40 \pm 8\%$ ($n=5$) were immunoreactive for both markers, $40 \pm 11\%$ were ChAT+/NOS-, $11 \pm 7\%$ were ChAT-/NOS+ and the remaining $9 \pm 9\%$ were ChAT-/NOS-. Pooled data showed that viscerofugal nerve cell body morphology correlated with immunohistochemical coding (chi squared, $df = 3$, $P < 0.001$). ChAT+/NOS- cell bodies had “simple cell” morphology (standardized residual: ± 2.4), and ChAT+/NOS+ cell bodies were associated with Dogiel type I lamellar morphology (standardized residual: $+ 3.5$). ChAT-/NOS+ and ChAT-/NOS- viscerofugal neurons were not significantly associated with either type of morphology. Fig. 7).

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The locations of viscerofugal nerve cell bodies were mapped (7 preparations, $n = 5$, Fig. 8) revealing that ChAT+/NOS- simple cells had a different distribution from ChAT+/NOS+ Dogiel type I cells; the former lying further from the mesenteric border than ChAT+/NOS- neurons (3.5 ± 2.2 mm vs 1.9 ± 1.8 mm, respectively, $n = 5$, $F = 5.4$, $df = 3$, $P < 0.001$). In addition, ChAT+/NOS+ Dogiel type I nerve cell bodies were located, on average, 1.5 ± 3.5 mm oral to the nerve trunks that their axons projected in, whereas ChAT+/NOS- viscerofugal nerve cell bodies averaged 1.3 ± 2.2 mm aboral (pooled data from $n = 5$, $F = 8.0$, $df = 3$, $P < 0.001$). In combination, these data suggest the presence of at least 2 distinct morphological subtypes of viscerofugal neurons.

350 **DISCUSSION**

Extrinsic spinal afferent neurons that project to the gut can give rise to conscious sensations or they can activate peripheral and central reflexes; parasympathetic and sympathetic axons forming the efferent arm of these reflex loops. Most of the effects of parasympathetic and sympathetic input are mediated via enteric neuronal circuits, rather than through direct control of effector cells, although control of blood vessels and sphincteric muscles are notable exceptions. In particular, myenteric ganglia are the major target of extrinsic autonomic fibres involved in gut motility. In this study, we investigated axons in colonic nerves that innervate myenteric ganglia using immunohistochemical markers widely used to distinguish sensory and autonomic neurons. Viscerofugal neurons (an identified class of primary afferent neuron(Hibberd et al., 2012)) that have axons in colonic nerves, were also filled and characterised. Results of the present study were compared with a similar study using the same biotinamide anterograde tracing approach to characterise populations of extrinsic neurons innervating the guinea-pig rectum (Table 3`)(Olsson et al., 2004).

Sympathetic innervation of the distal bowel

365 The relative sizes of populations of axons labeled by various markers in the two regions are compared in Table 3. As expected, sympathetic axons make up a larger proportion of the extrinsic innervation of the distal colon ($24\pm 4\%$, $n=8$) than the rectum ($4\pm 1\%$; $n=8$) (Olsson et al., 2004). This may reflect a contribution by sympathetic neurons that project via colonic nerves to the gut where they descend to innervate the rectum and colon, probably via the myenteric plexus(Yamanouchi et al., 2002). Many sympathetic fibres to the distal colon originate in the inferior mesenteric ganglion (Costa and Furness, 1973). The results of a previous study in the guinea pig, comparing retrograde labelling from the rectum and colon (Olsson et al., 2006) are informative (pathways summarized in Fig 1). This showed that the rectum is supplied by larger absolute numbers of autonomic (sympathetic + parasympathetic) neurons, approximately 6-times as many to any given area than the distal colon ($3,232 \pm 787$ vs 482 ± 94), (Olsson et al., 2006). Approximately 70% of autonomic neurons projecting to the distal colon came from the major prevertebral (coeliac, superior and inferior mesenteric) sympathetic ganglia, compared to 16% for the rectum. About 16% of extrinsic nerve fibres to the distal colon arose from neurons in the sympathetic chain ganglia compared to 37% for the rectum. Most sympathetic nerve cell bodies (in both prevertebral and sympathetic chain ganglia (81-85%) were immunoreactive for tyrosine hydroxylase and presumed noradrenergic (81-85%(Olsson et al., 2006)). In summary; the colon receives a denser innervation by sympathetic neurons, mostly from prevertebral ganglia - and most of these neurons are noradrenergic, based on the presence of tyrosine hydroxylase-immunoreactivity in their cell bodies.

Parasympathetic innervation of the distal bowel

After biotinamide-labeling of colonic nerves, 15% of labeled axons were immunoreactive for vesicular acetylcholine transporter (VACHT) which is very similar to their proportion of axons with this marker in the guinea pig rectum (see Table 3) (Olsson et al., 2004). As with the rectum, most of
390 the parasympathetic fibres to the colon probably originate in cell bodies in the pelvic ganglia, which project via the hypogastric nerves, then colonic nerves, to the gut wall. Consistent with this, previous studies have identified a population of pelvic plexus neurons that project rostrally in the hypogastric nerves of the cat and guinea-pig (Baron and Janig, 1988; McLachlan, 1985).

395 Sensory innervation of the distal bowel

The peptide-containing sensory nerve supply to the rectum (CGRP-immunoreactive) comprised a smaller proportion of the extrinsic innervation to the colon compared to the rectum (Table 3) (Olsson et al., 2004). Non-peptide-containing axons that contained VGluT1 or VGluT2 were also more abundant in the rectum than colon (Table 3) and are likely to include non-peptidergic sensory
400 neurons, since VGluT1 and VGluT2 are expressed in rectal intraganglionic laminar endings: the transduction site of non-peptidergic extrinsic mechanoreceptors (Lynn et al., 2003). VGluT1 and VGluT2 are detectable in many dorsal root ganglion neurons in guinea-pigs, where they rarely coexist with CGRP (Morris et al., 2005).

405 Functional significance of differences in innervation between rectum and colon

The differences in the relative contributions of sympathetic and parasympathetic extrinsic neuronal populations to the guinea-pig distal colon and rectum are likely to reflect the specialized functions of each region. In guinea-pig small and large intestines, sympathetic efferent neurons contact enteric neurons and release noradrenaline onto adrenoreceptors, reducing post-synaptic cell
410 excitability and causing presynaptic inhibition (Crema et al., 1970; Hirst and McKirdy, 1974; Spencer et al., 1999; Stebbing et al., 2001). The result is a decrease in reflex activity in enteric circuits and a net reduction in propulsive motility. The predominance of sympathetic innervation in the distal colon is consistent with the slow mixing and propulsion of contents needed to ensure optimal reabsorption of water and electrolytes, as well as permitting storage of feces. In contrast,
415 the rectum is specialized for the intermittently activated, controlled expulsion of waste material. This is facilitated by propulsive waves of contraction, which can be evoked by strong activation of the sacral parasympathetic nervous system (Crema et al., 1970; De Groat and Krier, 1978). The axons of sacral parasympathetic efferents enter the gut wall via the rectal nerves and release acetylcholine onto nicotinic receptors present on myenteric neurons (Tamura, 1997), increasing

420 their excitability and enhancing activity in myenteric circuits that underlie propulsive motility (De
Groat and Krier, 1978). The present study has shown that there is a comparable cholinergic, and
hence parasympathetic, input to the distal colon as well as rectum. This pathway is likely to extend
the influence of sacral parasympathetic pathways up the large intestine, and coordinate propulsive
motility to propel contents down to the rectum. This pathway may account for the pan-colonic
425 excitatory effects of sacral nerve stimulation (Dinning et al., 2007).

Lack of immunohistochemical markers in extrinsic axons to colon

Nearly half of the extrinsic axons innervating myenteric ganglia of the guinea-pig distal colon lack
any of the widely used immunohistochemical markers of sensory and autonomic neurons.
430 Assuming no coexistence between CGRP, TH, VACHT, VGluT1, VGluT2, somatostatin and VIP,
only about half of all axons contained one of these markers ($53 \pm 14\%$). The most obvious
explanation would be that immunohistochemical labeling of axons was inadequate, but several lines
of evidence suggest that this is unlikely to be the full explanation. The antisera against CGRP, TH,
VACHT and ChAT have all been rigorously characterized on guinea-pig intestinal tissue previously
435 (Olsson et al., 2004; Sharrad et al., 2013a; Sharrad et al., 2013b; Sharrad et al., 2013c) and all give
high contrast labeling of axons and cell bodies using epifluorescence microscopy. This methodology
has been validated previously against confocal microscopy(Sharrad et al., 2013b). All varicosities
that were classified as immunoreactive for a marker had a fluorescence intensity at least 3 standard
deviations above mean background. The density of CGRP- and TH-immunoreactivity in the
440 myenteric plexus did not visibly differ between fresh fixed tissue and tissue cultured for 16 hours
by a blinded observer. Thus it is unlikely that immunohistochemical markers disappeared during
the period of axonal filling. In theory labeling with biotinamide may have interfered with
immunohistochemical labeling of axons in the present study, but the presence of intense
immunoreactivity in many axon profiles labeled with biotinamide argues against this. Furthermore,
445 retrograde tracing from the guinea-pig distal colon demonstrated that most of the labeled extrinsic
autonomic cell bodies in prevertebral, paravertebral or pelvic ganglia, contained either tyrosine
hydroxylase or choline acetyltransferase, as predicted for sympathetic and parasympathetic neurons
(Olsson et al., 2006).

450 Put together, these results identify a mismatch between immunohistochemical markers in cell
bodies and in the axons of many autonomic neurons that project to the distal gut. Comparable
observations were reported for extrinsic innervation of the guinea pig rectum, in which nearly 60%
of extrinsic axons lacked detectable CGRP, TH, VACHT, VGluT1, or VGluT2 (Olsson et al., 2004).
Similar findings also apply to some enteric neurons, but not all. Thus, axons of enteric interneurons

455 and sensory neurons often lack markers that are detectable in their cell bodies, but enteric motor neurons do not show such a marked disparity (Sharrad et al., 2013a).

We speculate that these results could be functionally significant. There may be considerable variation in the levels of proteins used to synthesise, concentrate and release neurotransmitters from the axons of some autonomic neurons. This could be (i) a lifelong feature of some neurons, (ii) a cyclical pattern of expression or (iii) may reflect “spare capacity” for transmission within classes of autonomic neurons that can be activated at times of need (e.g. during inflammation, or ischaemia). The idea of “spare capacity” has been raised previously. Some sympathetic axons have action potential-related calcium transients that rarely release neurotransmitter (Brain et al., 2002).
465 Inflammation can awaken synaptic connections in the spinal cord from subsets of spinal afferent axons (Torsney, 2011). Inflammation also confers mechanosensitivity on previously mechanically insensitive spinal afferent endings (Feng and Gebhart, 2011) and can upregulate neurotransmitters and related enzymes in enteric neurons (Belai et al., 1997; Belai and Burnstock, 1999; Neunlist et al., 2003).

470

Viscerofugal nerve cell bodies in the distal colon

In the present study, viscerofugal cell bodies which all function as primary afferent mechanoreceptors (Hibberd et al., 2012) were also labeled by biotinamide applied to colonic nerve trunks. Several populations could be distinguished: 42% expressed both ChAT and NOS; 37% had
475 ChAT without NOS; 16% had NOS without ChAT and 5% of cell bodies expressed neither ChAT nor NOS. It is striking that these 2 markers were localized in the great majority of these cell bodies, unlike immunohistochemical markers in biotinamide-filled axons. In previous studies, all guinea-pig viscerofugal neurons projecting to the coeliac ganglion (Mann et al., 1995) and to the inferior mesenteric ganglion (Sharkey et al., 1998) were reportedly immunoreactive for choline
480 acetyltransferase. From 60-70% of colonic viscerofugal neurons projecting to prevertebral ganglia were NOS-immunoreactive (Anderson et al., 1995). Our results are comparable to these previous reports, but may distinguish a small population that lacks ChAT-immunoreactivity, and another small population that lacks both ChAT- and NOS-immunoreactivity. It is unclear why these two populations were not identified previously; NOS-immunoreactive and ChAT-immunoreactivity in
485 cell bodies were readily distinguished from background in the present study. It is possible that these small populations project to targets other than the coeliac and inferior mesenteric ganglia; feasibly the superior mesenteric ganglion (Messenger and Furness, 1993), the pelvic ganglia (Luckensmeyer and Keast, 1995), or the spinal cord (Neuhuber et al., 1993; Suckow and Caudle, 2008). In the present study we demonstrated that ChAT+/NOS+ viscerofugal neurons are differentiated from

490 ChAT+/NOS- viscerofugal neurons by their neurochemical content, cell body morphology, and in
their spatial distributions in both circumferential and longitudinal axes. In addition, some evidence
of functional differences between these populations of viscerofugal neurons has been reported
(Hibberd et al., 2014). Whether these populations of viscerofugal neurons serve different
physiological roles remain to be shown.

495

In summary, we report that nearly half of all extrinsic axons to the guinea-pig distal colon targeting
myenteric ganglia lack markers that identify extrinsic neuronal populations in healthy adult animals.
These unlabeled axons are likely to be of both efferent and afferent origin. Whether the levels of
markers in extrinsic axons to the colon can be upregulated under pathophysiological conditions,
500 such as inflammation, or physiological circumstances, such as ageing, remains to be determined.

Role of Authors

Study concept and design: SJHB; Acquisition of data: BNC, DFS and TJH; Data analysis and
interpretation: DFS, TJH, MC, VZ and SJHB; Drafting and correcting the manuscript: DFS, TJH,
505 BNC, MC, VZ and SJHB.

Conflict of interest

The authors declare no conflict of interest.

510 FIGURE LEGENDS

Figure 1. Schematic diagram showing major extrinsic neuronal pathways to rectum and colon. Sympathetic neurons are shown in black; parasympathetic neurons as white and extrinsic afferent neurons as grey cell bodies with axons shown as dashed black lines. The biotinamide application site was restricted to colonic nerves that run between the inferior mesenteric ganglion and the distal colon (bottom left of diagram). Note that colonic nerves contain axons of extrinsic (spinal) afferent neurons, paravertebral and prevertebral sympathetic neurons and parasympathetic axons originating from cell bodies in pelvic ganglia. Schema is based on previous published data(Olsson et al., 2006).

Figure 2. Biotinamide filling in the distal colon was usually most dense in the myenteric plexus. **A.** micrograph of the myenteric plexus, close to the entry point of a biotinamide-filled colonic nerve trunk. Note extensive branching axons within the ganglion, a single viscerofugal nerve cell body (open arrowhead) and many axons running vertically in internodal strands. Relatively few axons ran in either the circular or longitudinal muscle layers (arrows). **B.** filling in the submucous plexus was generally sparser, with modest branching in submucous ganglia (arrowheads) but many usually some perivascular and paravascular fibres ran close to arteries and major arterioles (open arrow). **C.** Occasional fibres were seen branching around the base of the colonic glands - in this case a single axon shows extensive branching just below the epithelium. **D.** In the mesenteries outside the gut wall, extensive filling of bundles of axons of passage was observed in most preparations, with some axons branching extensively on the surface of blood vessels (asterisk).

Figure 3: effects of overnight organ culture on immunohistochemical labeling. Tissues were either fixed shortly after removal from the animal (Fresh fix, A,C,E) or after biotinamide labeling in organ culture overnight ("filled", B,D,F). Both preparations were then immunohistochemically labeled for vesicular acetylcholine transporter (VACHT; A,B), tyrosine hydroxylase (TH: C,D) or calcitonin gene related peptide (CGRP: E,F) and photographed, using identical exposures and protocols. There was a small but consistent increase in background fluorescence after organ culture, but no loss of labeling or reduction in density of varicosities was detectable to a blinded observer. This suggests that the low proportion of biotinamide labeled extrinsic nerve fibres containing immunoreactivity for one of these markers was not due to loss of immunohistochemical markers during the filling process.

Figure 4. Paired fluorescence images of guinea-pig distal colon myenteric ganglia containing biotinamide-labeled extrinsic axons labeled with antisera against CGRP, TH, VACHT, or VGluT1. Arrows indicate biotinamide-labeled varicosities that were immunoreactive for one of these markers, and arrowheads indicate varicosities lacking immunoreactivity for a marker. Only a minority of the biotinamide-labeled axons in these preparations was labeled by antisera against any of these four markers. Scale bars = 20 μ m. Abbreviations: CGRP, calcitonin gene-related peptide; TH, tyrosine hydroxylase; VACHT, vesicular acetylcholine transporter; VGluT1, vesicular glutamate transporter 1.

Figure 5 Paired fluorescence images of guinea-pig distal colon myenteric ganglia containing

biotinamide-labeled extrinsic axons labeled with antisera against NOS, SOM, VGluT2 or VIP. Arrows indicate biotinamide-labeled varicosities that were immunoreactive for one of these markers, and arrowheads indicate varicosities lacking immunoreactivity for a marker. Only a minority of the biotinamide-labeled axons in these preparations was labeled by antisera against any of these four markers. Scale bars = 20 μm . Abbreviations: NOS, nitric oxide synthase; SOM, somatostatin; VGluT2, vesicular glutamate transporter 2; VIP, vasoactive intestinal polypeptide.

Figure 6. Matched fluorescence images of guinea-pig distal colon myenteric ganglia containing biotinamide-labeled viscerofugal nerve cell bodies immunohistochemically double-labeled with antisera against ChAT and NOS. There were four neurochemical classes of viscerofugal neurons distinguished on the basis of the combinations of ChAT- and NOS-immunoreactivity expressed in their cell bodies. There was a class that contained ChAT-immunoreactivity alone (**A, B & C**), NOS-immunoreactivity alone (**D, E & F**), ChAT- and NOS-immunoreactivity together (**G, H & I**), and a small class that lacked both ChAT- and NOS-immunoreactivity (**J, K & L**). Scale bars = 20 μm . Abbreviations: ChAT, choline acetyltransferase; NOS, nitric oxide synthase.

Figure 7. The sizes of viscerofugal nerve cell bodies for each of the four immunohistochemical groups are shown in **A**. The horizontal bars indicate the average nerve cell body size within each group. Viscerofugal nerve cell bodies containing both ChAT- and NOS- immunoreactivity were significantly larger than viscerofugal nerve cell bodies than the next largest group which contained ChAT-immunoreactivity alone ($*P < 0.001$, $F = 27.8$, $df = 3$). The distributions of soma-dendritic area of these two groups are shown below in a frequency histogram (**B**). Abbreviations: ChAT, choline acetyltransferase; NOS, nitric oxide synthase.

Figure 8. A composite map with frequency histograms, showing the distribution of biotinamide-labeled viscerofugal nerve cell bodies in 7 preparations ($n = 5$). The viscerofugal nerve cell bodies containing both ChAT and NOS-immunoreactivity (solid black circles and solid black bars) were distributed mainly oral to the entry point of the colonic nerve trunk from which they were filled; viscerofugal nerve cell bodies containing ChAT alone (open circles and bars) were mainly aboral to the entry point of the filled colonic nerve trunk. In the circumferential axis, viscerofugal nerve cell bodies containing both ChAT and NOS immunoreactivity were more evenly distributed around the gut than viscerofugal nerve cell bodies containing ChAT alone, which were concentrated near the mesenteric border. The dashed lines indicate the point of entry of the biotinamide-labelled colonic nerve into the gut, in both axes. Abbreviations: ChAT, choline acetyltransferase; NOS, nitric oxide synthase.

REFERENCES

595

- Anderson CR, Furness JB, Woodman HL, Edwards SL, Crack PJ, Smith AI. (1995). Characterisation Of Neurons With Nitric Oxide Synthase Immunoreactivity That Project to Prevertebral Ganglia. *Journal of the Autonomic Nervous System*, 52, 107-116.
- Baron R, Janig W. (1988). Neurons projecting rostrally in the hypogastric nerve of the cat. *Journal of the Autonomic Nervous System*, 24, 81-86.
- 600
- Belai A, Boulos PB, Robson T, Burnstock G. (1997). Neurochemical coding in the small intestine of patients with Crohn's disease. *Gut*, 40, 767-774.
- Belai A, Burnstock G. (1999). Distribution and colocalization of nitric oxide synthase and calretinin in myenteric neurons of developing, aging, and Crohn's disease human small intestine. *Digestive Diseases & Sciences*, 44, 1579-1587.
- 605
- Berthoud HR, Carlson NR, Powley TL. (1991). Topography of efferent vagal innervation of the rat gastrointestinal tract. *Am J Physiol*.
- Berthoud HR, Jedrzejewska A, Powley TL. (1990). 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*, 301, 65-79 issn: 0021-9967.
- 610
- Brain KL, Jackson VM, Trout SJ, Cunnane TC. (2002). Intermittent ATP release from nerve terminals elicits focal smooth muscle Ca²⁺ transients in mouse vas deferens. *J Physiol*, 541, 849-862.
- Brookes SJH, Chen BN, Costa M, Humphreys CMS. (1999). Initiation of peristalsis by circumferential stretch of flat sheets of guinea-pig ileum. *Journal of Physiology London*, 516, 525-538.
- 615
- Buchan AM, Sikora LK, Levy JG, McIntosh CH, Dyck I, Brown JC. (1985). An immunocytochemical investigation with monoclonal antibodies to somatostatin. *Histochemistry*, 83, 175-180.
- 620
- Cannon WB, Lieb CW. (1911). The receptive relaxation of the stomach. *Am. J. Physiol.*, 27.
- Costa M, Brookes SJH, Steele PA, Gibbins I, Burcher E, Kandiah CJ. (1996). Neurochemical classification of myenteric neurons in the guinea-pig ileum. *Neuroscience*, 75, 949-967.
- Costa M, Furness JB. (1973). The origins of the adrenergic fibres which innervate the internal anal sphincter, the rectum, and other tissues of the pelvic region in the guinea-pig. *Z Anat Entwicklungsgesch*, 140, 129-142.
- 625
- Crema A, Frigo GM, Lecchini S. (1970). A pharmacological analysis of the peristaltic reflex in the isolated colon of the guinea-pig or cat. *British Journal of Pharmacology*, 39, 334-345.
- De Groat WC, Krier J. (1978). The sacral parasympathetic reflex pathway regulating colonic motility and defaecation in the cat. *J Physiol Lond*, 276, 481-500.
- 630
- Dinning PG, Fuentealba SE, Kennedy ML, Lubowski DZ, Cook IJ. (2007). Sacral nerve stimulation induces pan-colonic propagating pressure waves and increases defecation frequency in patients with slow-transit constipation. *Colorectal Disease*, 9, 123-132.

- 635 Domeneghini C, Radaelli G, Arrighi S, Bosi G, Dolera M. (2004). Cholinergic, nitrenergic and peptidergic (Substance P- and CGRP-utilizing) innervation of the horse intestine. A histochemical and immunohistochemical study. *Histology & Histopathology*, 19, 357-370.
- 640 Ermilov LG, Miller SM, Schmalz PF, Hanani M, Lennon VA, Szurszewski JH. (2003). Morphological characteristics and immunohistochemical detection of nicotinic acetylcholine receptors on intestinofugal afferent neurones in guinea-pig colon. *Neurogastroenterology & Motility*, 15, 289-298.
- Feng B, Gebhart GF. (2011). Characterization of silent afferents in the pelvic and splanchnic innervations of the mouse colorectum. *American Journal of Physiology - Gastrointestinal & Liver Physiology*, 300, G170-180.
- 645 Furness JB, Bornstein JC, Trussell DC. (1988). Shapes of nerve cells in the myenteric plexus of the guinea-pig small intestine revealed by the intracellular injection of dye. *Cell & Tissue Research*, 254, 561-571.
- Furness JB, Costa M. 1987. *The enteric nervous system*. Churchill-Livingstone, Edinburgh, pp. 290.
- 650 Gerke MB, Plenderleith MB. (2002). Analysis of the distribution of binding sites for the plant lectin *Bandeiraea simplicifolia* I-isolectin B4 on primary sensory neurones in seven mammalian species. *Anatomical Record*, 268, 105-114.
- Gibbins IL, Furness JB, Costa M, MacIntyre I, Hillyard CJ, Girgis S. (1985). Co-localization of calcitonin gene-related peptide-like immunoreactivity with substance P in cutaneous, vascular and visceral sensory neurons of guinea pigs. *Neurosci Lett*, 57, 125-130.
- 655 Hibberd TJ, Spencer NJ, Zagorodnyuk VP, Chen BN, Brookes SJH. (2014). Targeted electrophysiological analysis of viscerofugal neurons in the myenteric plexus of guinea-pig colon. *Neuroscience*, 275C, 272-284.
- Hibberd TJ, Zagorodnyuk VP, Spencer NJ, Brookes SJH. (2012). Identification and mechanosensitivity of viscerofugal neurons. *Neuroscience*, 225, 118-129.
- 660 Hirst GD, McKirdy HC. (1974). Presynaptic inhibition at mammalian peripheral synapse? *Nature*, 250, 430-431.
- Holst MC, Kelly JB, Powley TL. (1997). Vagal preganglionic projections to the enteric nervous system characterized with *Phaseolus vulgaris*-leucoagglutinin. *Journal of Comparative Neurology*, 381, 81-100.
- 665 Jänig W. 2006. *The integrative action of the autonomic nervous system; Neurobiology of homeostasis*. Cambridge University Press, pp. 600.
- Keast JR, McLachlan EM, Meckler RL. (1993). Relation between electrophysiological class and neuropeptide content of guinea pig sympathetic prevertebral neurons. *Journal of Neurophysiology*, 69, 384-394.
- 670 Knowles CH, Scott SM, Lunniss PJ. (2001). Slow transit constipation: a disorder of pelvic autonomic nerves? *Digestive Diseases & Sciences*, 46, 389-401.
- Kraus T, Neuhuber WL, Raab M. (2007). Distribution of vesicular glutamate transporter 1 (VGLUT1) in the mouse esophagus. *Cell & Tissue Research*, 329, 205-219.

- 675 Lomax AE, Furness JB. (2000). Neurochemical classification of enteric neurons in the guinea-pig distal colon. *Cell & Tissue Research*, 302, 59-72.
- Luckensmeyer GB, Keast JR. (1995). Distribution and morphological characterization of viscerofugal projections from the large intestine to the inferior mesenteric and pelvic ganglia of the male rat. *Neuroscience*, 66, 663-671.
- 680 Lynn PA, Olsson C, Zagorodnyuk V, Costa M, Brookes SJ. (2003). Rectal intraganglionic laminar endings are transduction sites of extrinsic mechanoreceptors in the guinea pig rectum. *Gastroenterology*, 125, 786-794.
- Macrae IM, Furness JB, Costa M. (1986). Distribution of subgroups of noradrenaline neurons in the coeliac ganglion of the guinea-pig. *Cell Tissue Res*, 244, 173-180.
- 685 Mann PT, Furness JB, Pompolo S, Mader M. (1995). Chemical Coding Of Neurons That Project From Different Regions Of Intestine to the Coeliac Ganglion Of the Guinea Pig. *Journal of the Autonomic Nervous System*, 56, 15-25.
- McLachlan EM. (1985). The components of the hypogastric nerve in male and female guinea pigs. *Journal of the Autonomic Nervous System*, 13, 327-342.
- 690 Messenger JP, Furness JB. (1993). Distribution of enteric nerve cells projecting to the superior and inferior mesenteric ganglia of the guinea-pig. *Cell Tissue Res*, 271, 333-339.
- Morris JL, Gibbins IL. (1987). Neuronal colocalization of peptides, catecholamines, and catecholamine-synthesizing enzymes in guinea pig paracervical ganglia. *Journal of Neuroscience*, 7, 3117-3130.
- 695 Morris JL, Konig P, Shimizu T, Jobling P, Gibbins IL. (2005). Most peptide-containing sensory neurons lack proteins for exocytotic release and vesicular transport of glutamate. *Journal of Comparative Neurology*, 483, 1-16.
- Muller JF, Mascagni F, McDonald AJ. (2007). Postsynaptic targets of somatostatin-containing interneurons in the rat basolateral amygdala. *Journal of Comparative Neurology*, 500, 513-529.
- 700 Neuhuber WL, Appelt M, Polak JM, Baier KW, Abelli L, Ferri GL. (1993). Rectospinal neurons: cell bodies, pathways, immunocytochemistry and ultrastructure. *Neuroscience*, 56, 367-378.
- 705 Neunlist M, Aubert P, Toquet C, Oreshkova T, Barouk J, Lehur PA, Schemann M, Galmiche JP. (2003). Changes in chemical coding of myenteric neurones in ulcerative colitis. *Gut*, 52, 84-90.
- Olsson C, Chen BN, Jones S, Chataway TK, Costa M, Brookes SJ. (2006). Comparison of extrinsic efferent innervation of guinea pig distal colon and rectum. *Journal of Comparative Neurology*, 496, 787-801.
- 710 Olsson C, Costa M, Brookes SJ. (2004). Neurochemical characterization of extrinsic innervation of the guinea pig rectum. *Journal of Comparative Neurology*, 470, 357-371.
- Parr EJ, Sharkey KA. (1996). Immunohistochemically-defined subtypes of neurons in the inferior mesenteric ganglion of the guinea-pig. *Journal of the Autonomic Nervous System*, 59, 140-150.

- 715 Pfeiffer RF. (2011). Gastrointestinal dysfunction in Parkinson's disease. *Parkinsonism & Related Disorders*, 17, 10-15.
- Raab M, Neuhuber WL. (2004). Intraganglionic laminar endings and their relationships with neuronal and glial structures of myenteric ganglia in the esophagus of rat and mouse. *Histochemistry & Cell Biology*, 122, 445-459.
- 720 Robinson DR, McNaughton PA, Evans ML, Hicks GA. (2004). Characterization of the primary spinal afferent innervation of the mouse colon using retrograde labelling. *Neurogastroenterology & Motility*, 16, 113-124.
- Russo D, Bombardi C, Grandis A, Furness JB, Spadari A, Bernardini C, Chiocchetti R. (2010). Sympathetic innervation of the ileocecal junction in horses. *Journal of Comparative Neurology*, 518, 4046-4066.
- 725 Schemann M, Schaaf C, Mader M. (1995). Neurochemical Coding Of Enteric Neurons In the Guinea Pig Stomach. *Journal of Comparative Neurology*, 353, 161-178.
- Shamy JL, Buckmaster CA, Amaral DG, Calhoun ME, Rapp PR. (2007). Reactive plasticity in the dentate gyrus following bilateral entorhinal cortex lesions in cynomolgus monkeys. *Journal of Comparative Neurology*, 502, 192-201.
- 730 Sharkey KA, Lomax AE, Bertrand PP, Furness JB. (1998). Electrophysiology, shape, and chemistry of neurons that project from guinea pig colon to inferior mesenteric ganglia. *Gastroenterology*, 115, 909-918.
- Sharrad DF, Chen BN, Brookes SJH. (2013a). Neurochemical coding compared between varicose axons and cell bodies of myenteric neurons in the guinea-pig ileum. *Neurosci Lett*, 534, 171-176.
- 735 Sharrad DF, De Vries E, Brookes SJH. (2013b). Selective expression of α -synuclein-immunoreactivity in vesicular acetylcholine transporter-immunoreactive axons in the guinea-pig rectum and human colon. *J Comp Neurol*, 521, 657-676.
- Sharrad DF, Gai WP, Brookes SJ. (2013c). Selective coexpression of synaptic proteins, alpha-synuclein, cysteine string protein-alpha, synaptophysin, synaptotagmin-1, and synaptobrevin-2 in vesicular acetylcholine transporter-immunoreactive axons in the guinea pig ileum. *Journal of Comparative Neurology*, 521, 2523-2537.
- 740 Spencer N, McCarron SL, Smith TK. (1999). Sympathetic inhibition of ascending and descending interneurons during the peristaltic reflex in the isolated guinea-pig distal colon. *Journal of Physiology*, 519 Pt 2, 539-550.
- 745 Spencer NJ, Hennig GW, Smith TK. (2002). A rhythmic motor pattern activated by circumferential stretch in guinea-pig distal colon. *Journal of Physiology*, 545, 629-648.
- Stebbing M, Johnson P, Vremec M, Bornstein J. (2001). Role of alpha(2)-adrenoceptors in the sympathetic inhibition of motility reflexes of guinea-pig ileum. *Journal of Physiology*, 534, 465-478.
- 750 Suckow SK, Caudle RM. (2008). Identification and immunohistochemical characterization of colospinal afferent neurons in the rat. *Neuroscience*, 153, 803-813.

- 755 Tamura K. (1997). Synaptic Inputs to Morphologically Identified Myenteric Neurons In Guinea Pig Rectum From Pelvic Nerves. *American Journal of Physiology Gastrointestinal & Liver Physiology*, 36, G49-55.
- Tassicker BC, Hennig GW, Costa M, Brookes SJH. (1999). Rapid anterograde and retrograde tracing from mesenteric nerve trunks to the guinea-pig small intestine in vitro. *Cell & Tissue Research*, 295, 437-452.
- 760 Torsney C. (2011). Inflammatory pain unmasks heterosynaptic facilitation in lamina I neurokinin 1 receptor-expressing neurons in rat spinal cord. *Journal of Neuroscience*, 31, 5158-5168.
- Trudrung P, Furness JB, Pompolo S, Messenger JP. (1994). Locations and Chemistries Of Sympathetic Nerve Cells That Project to the Gastrointestinal Tract and Spleen. *Archives of Histology & Cytology*, 57, 139-150.
- 765 Vinik AI, Maser RE, Mitchell BD, Freeman R. (2003). Diabetic autonomic neuropathy. *Diabetes Care*, 26, 1553-1579.
- Wei JY, Wang YH, Go VLW, Tache Y. (1997). Esophageal Distension Induced Gastric Relaxation Is Mediated In Part By Vagal Peripheral Reflex Mechanism In Rats. *Journal of the Autonomic Nervous System*, 63, 12-18.
- 770 Yamanouchi M, Shimatani H, Kadowaki M, Yoneda S, Nakagawa T, Fujii H, Takaki M. (2002). Integrative control of rectoanal reflex in guinea pigs through lumbar colonic nerves. *American Journal of Physiology - Gastrointestinal & Liver Physiology*, 283, G148-156.

Fig1

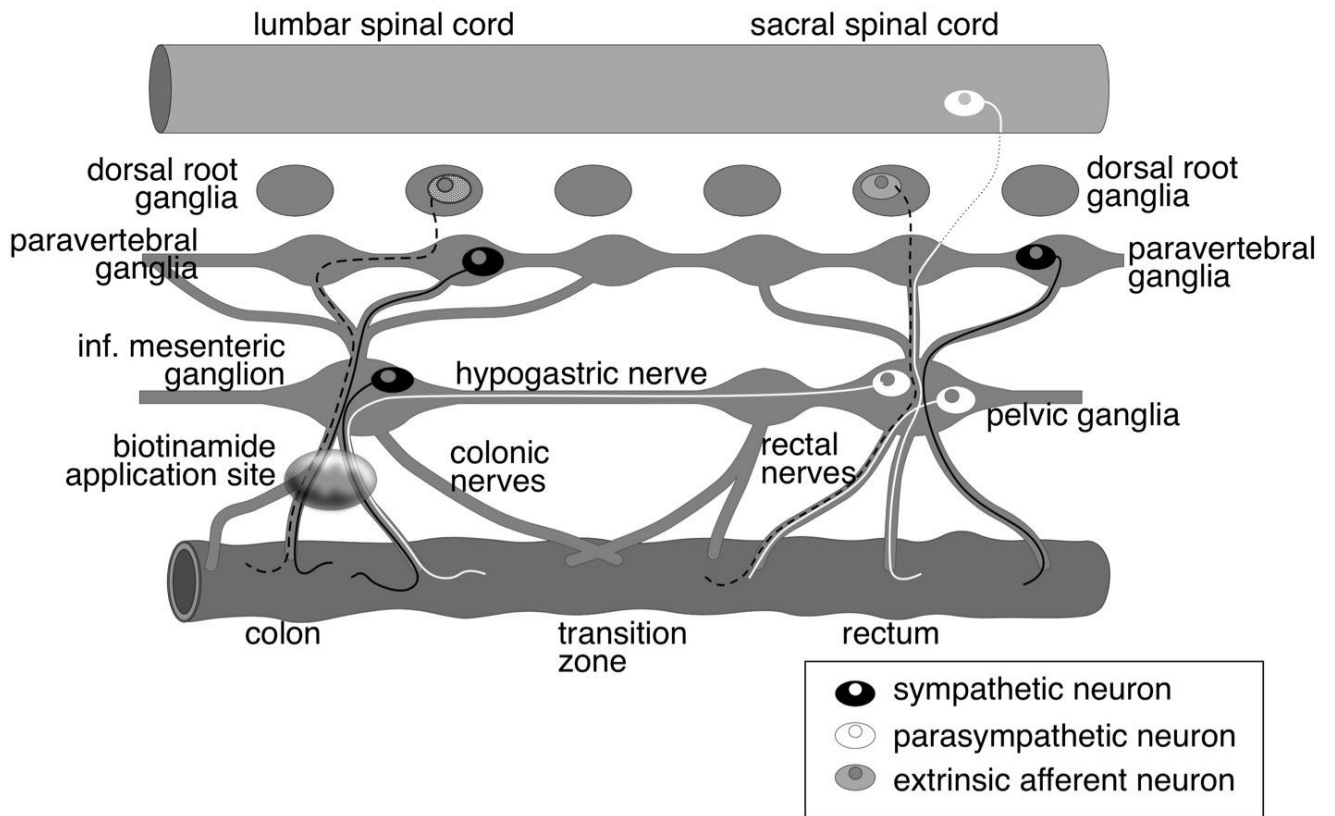


Fig2

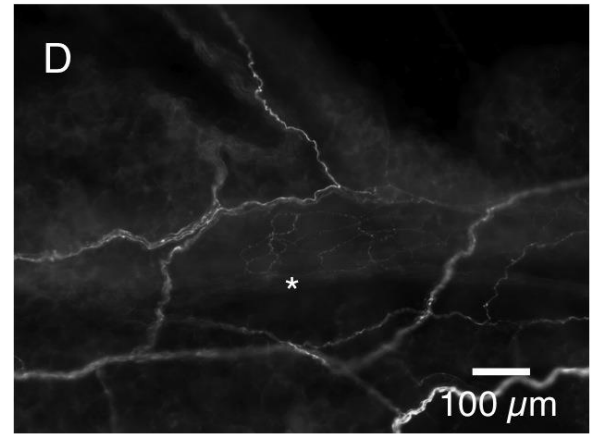
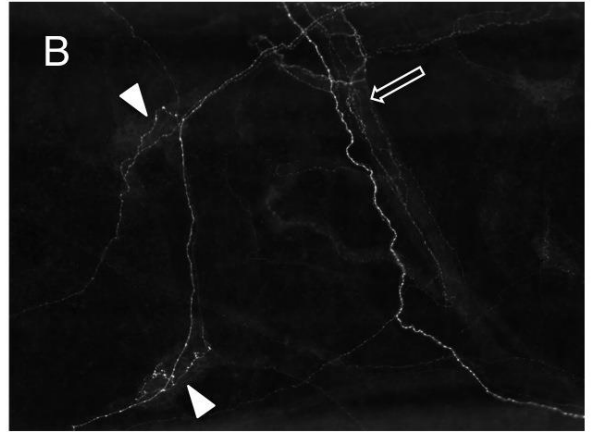
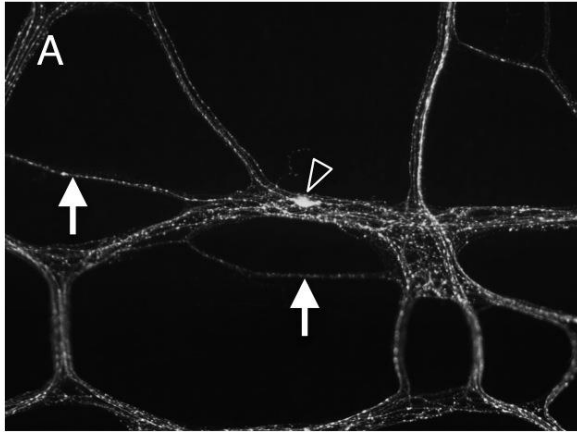


Fig3

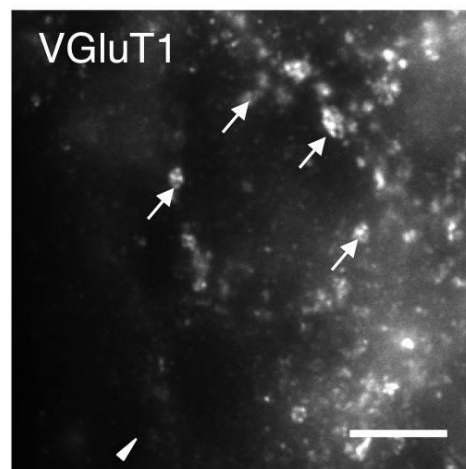
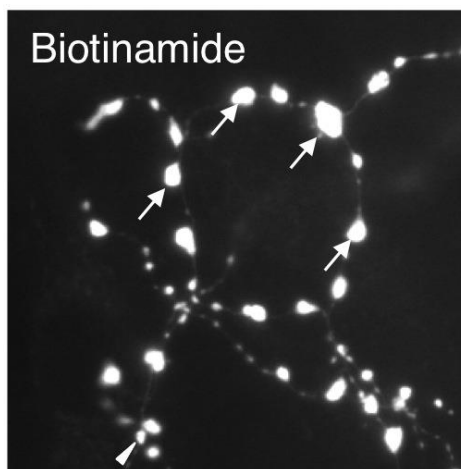
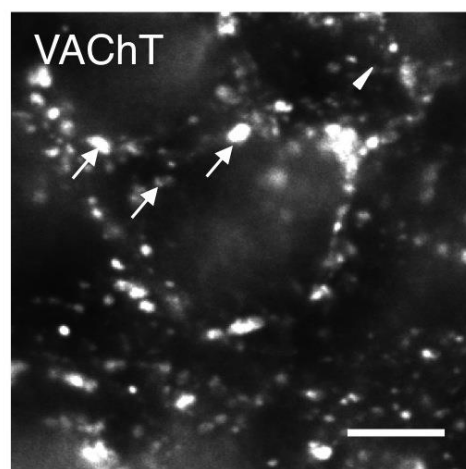
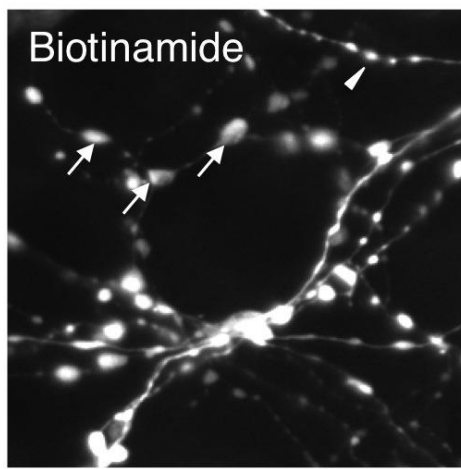
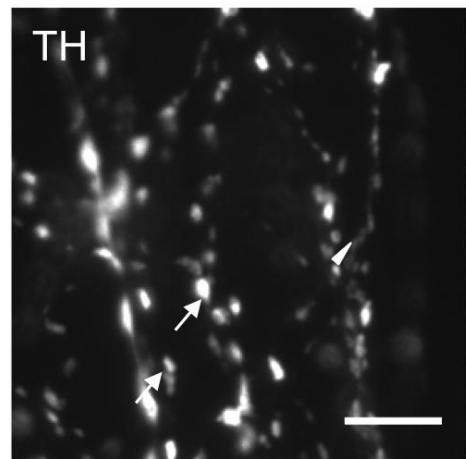
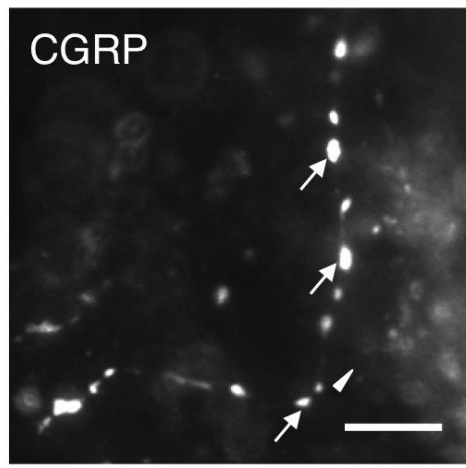
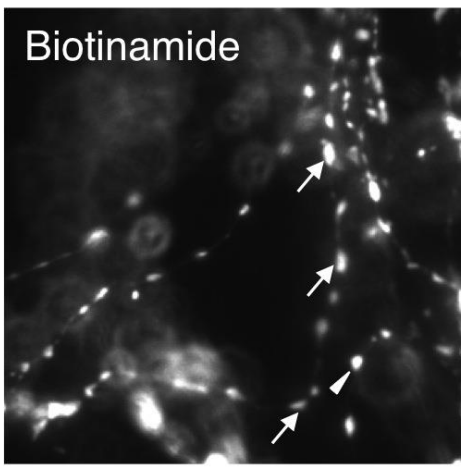


Fig4

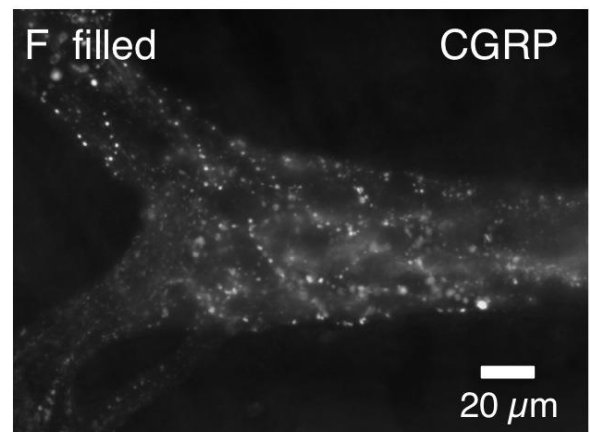
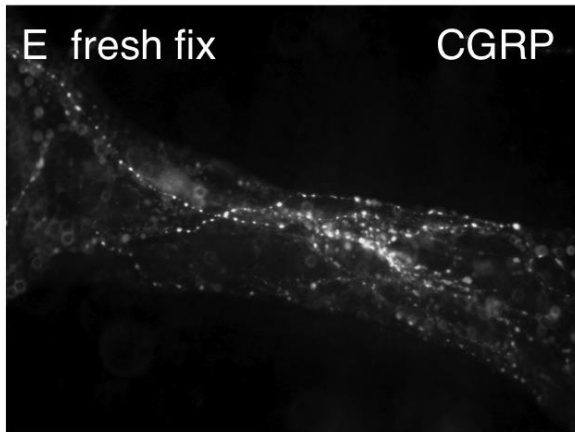
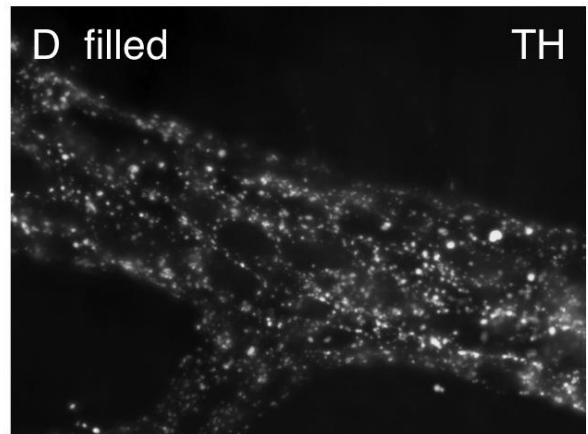
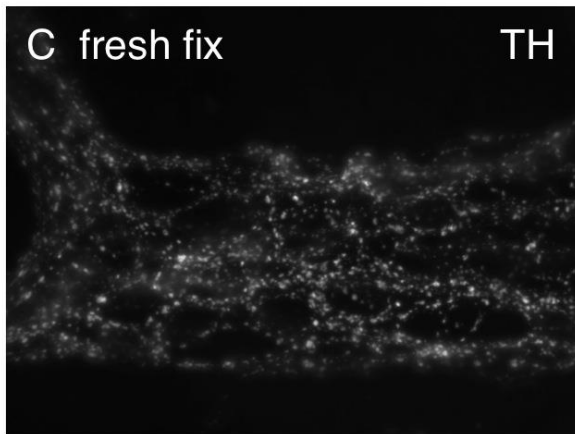
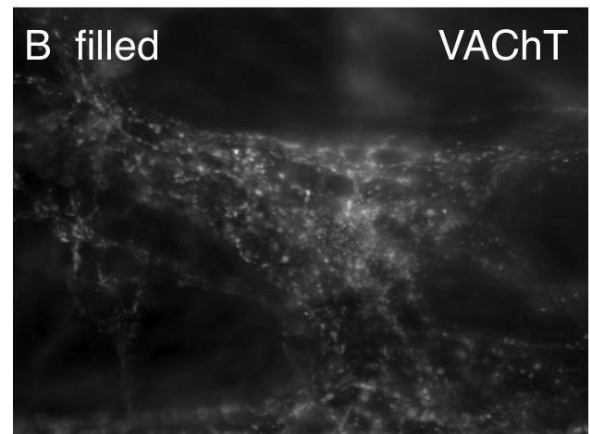
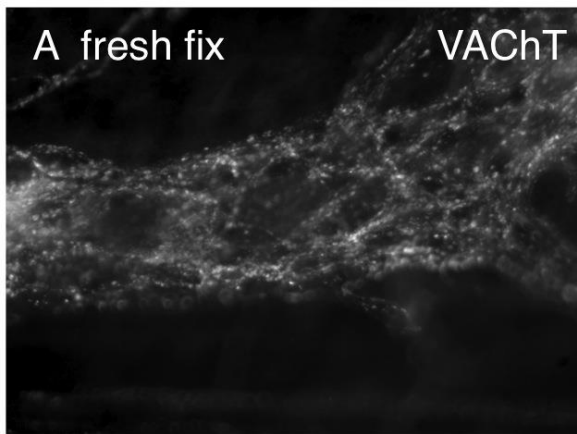
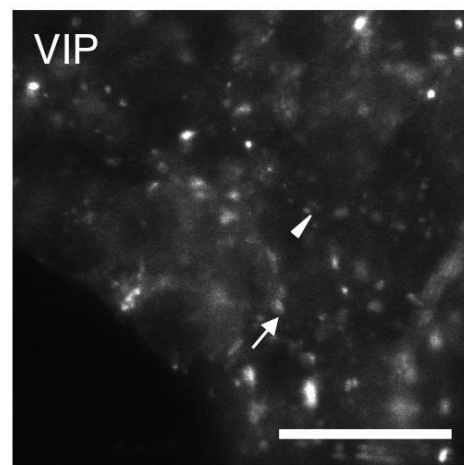
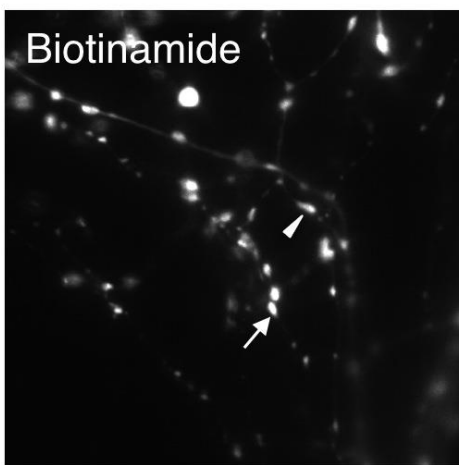
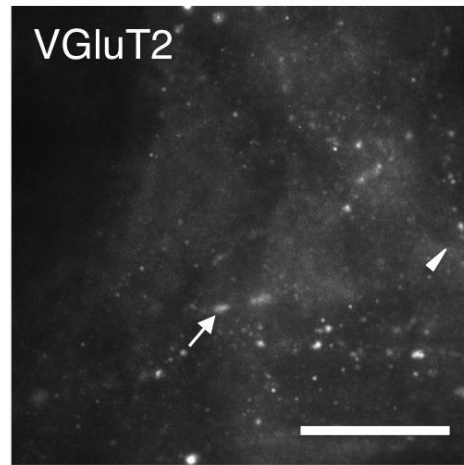
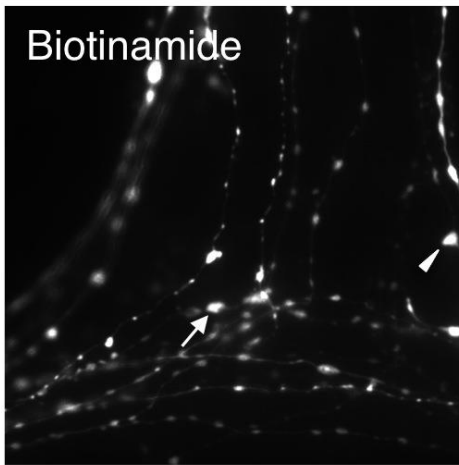
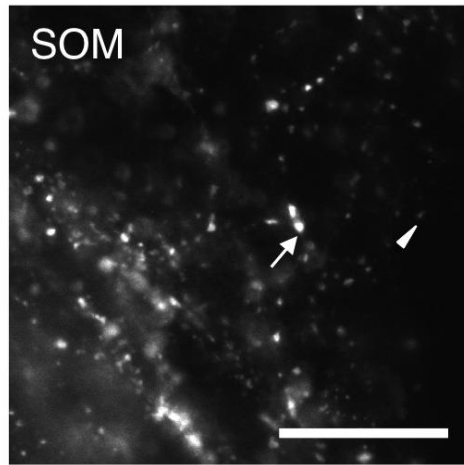
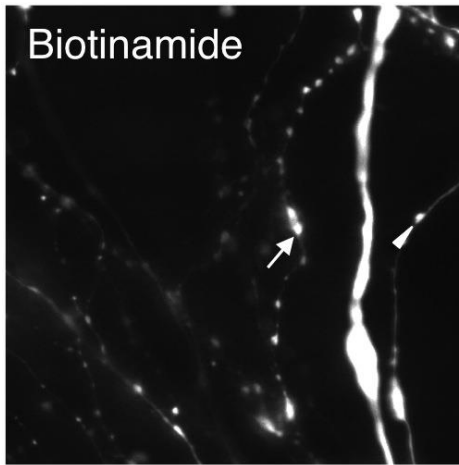
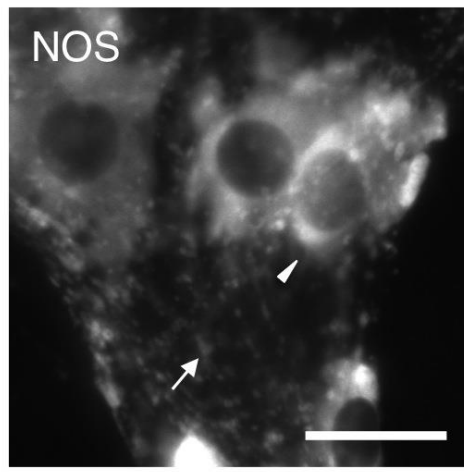
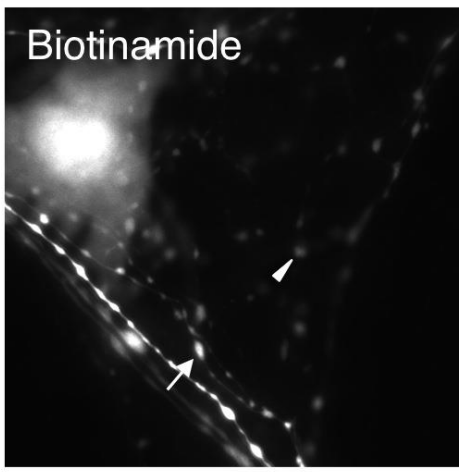


Fig5



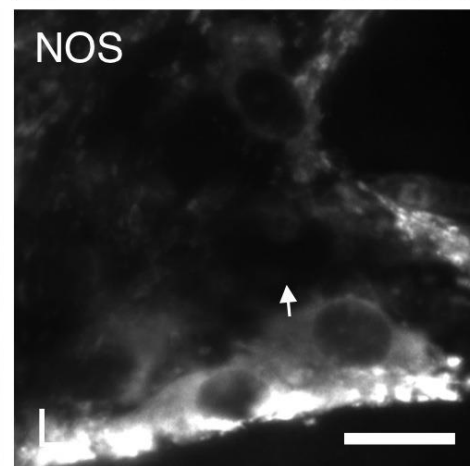
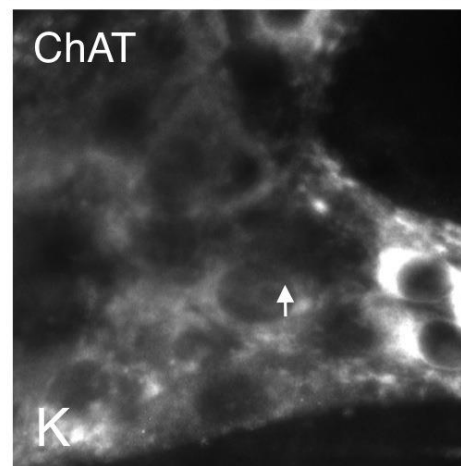
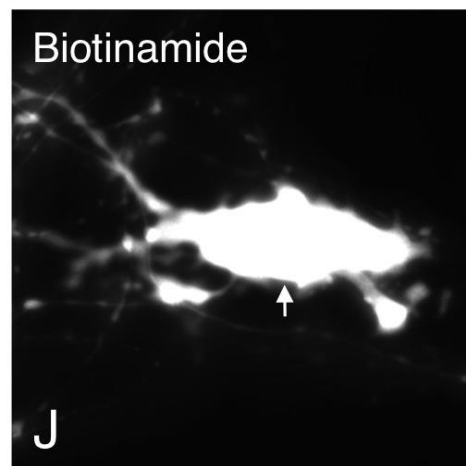
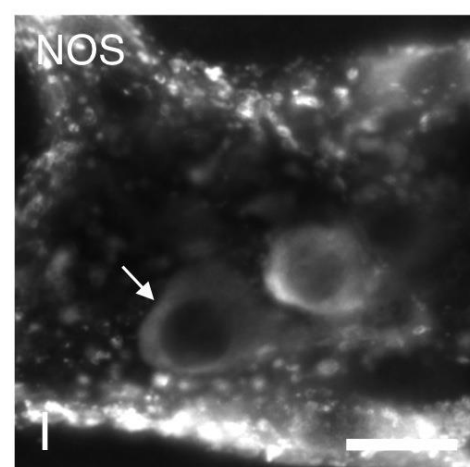
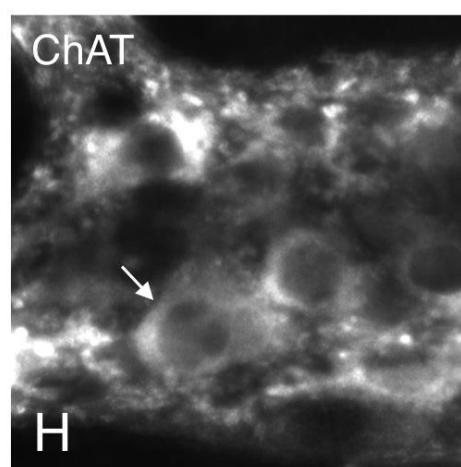
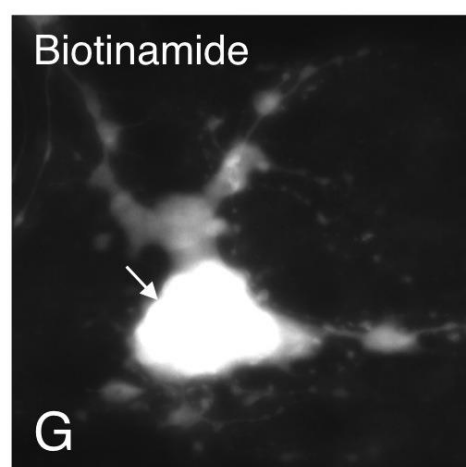
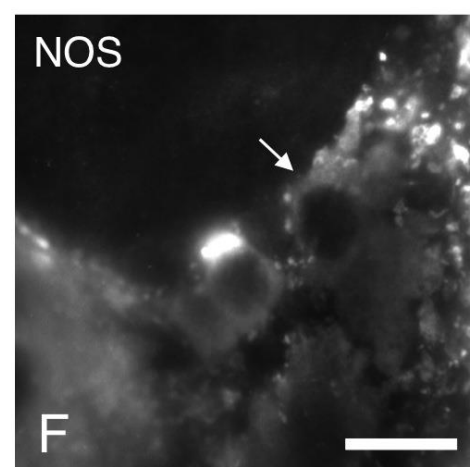
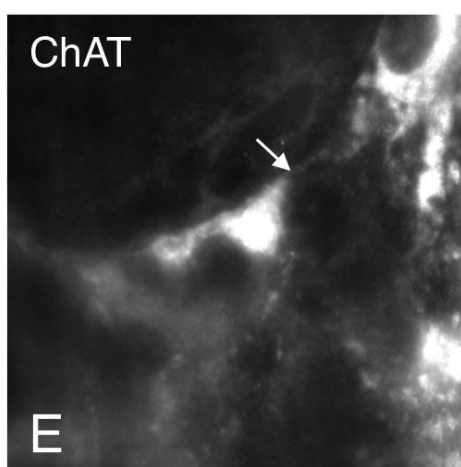
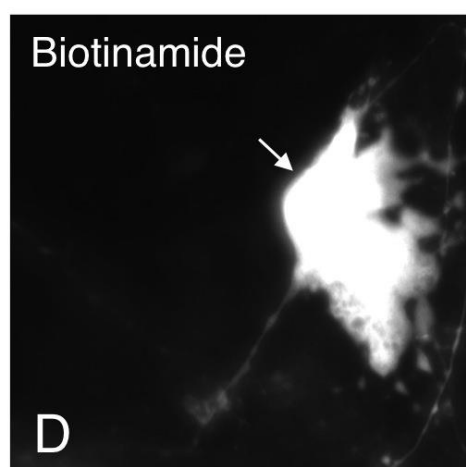
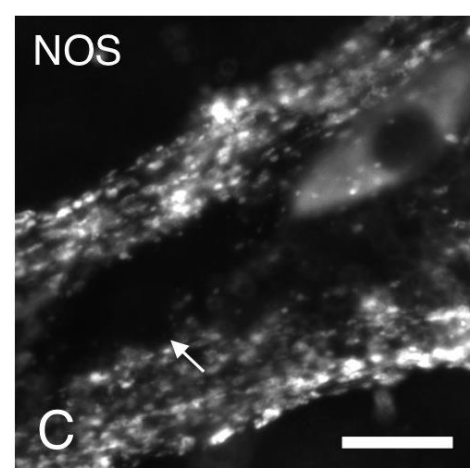
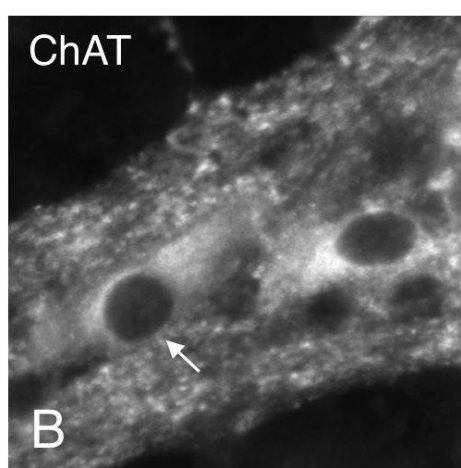
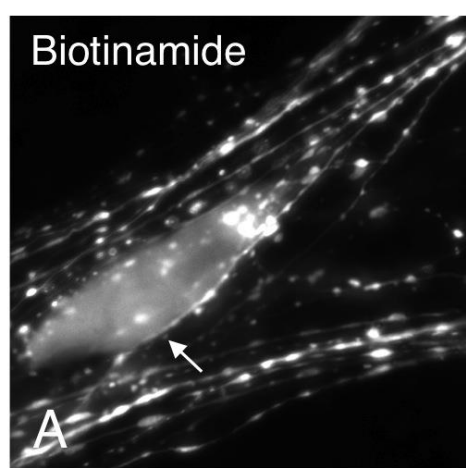
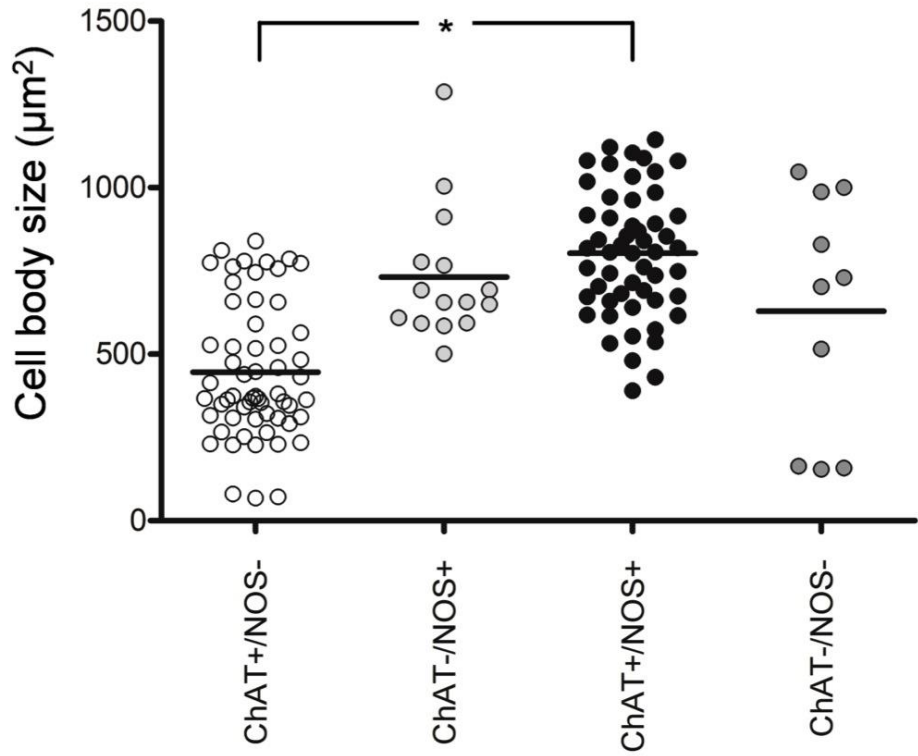


Fig7

A



B

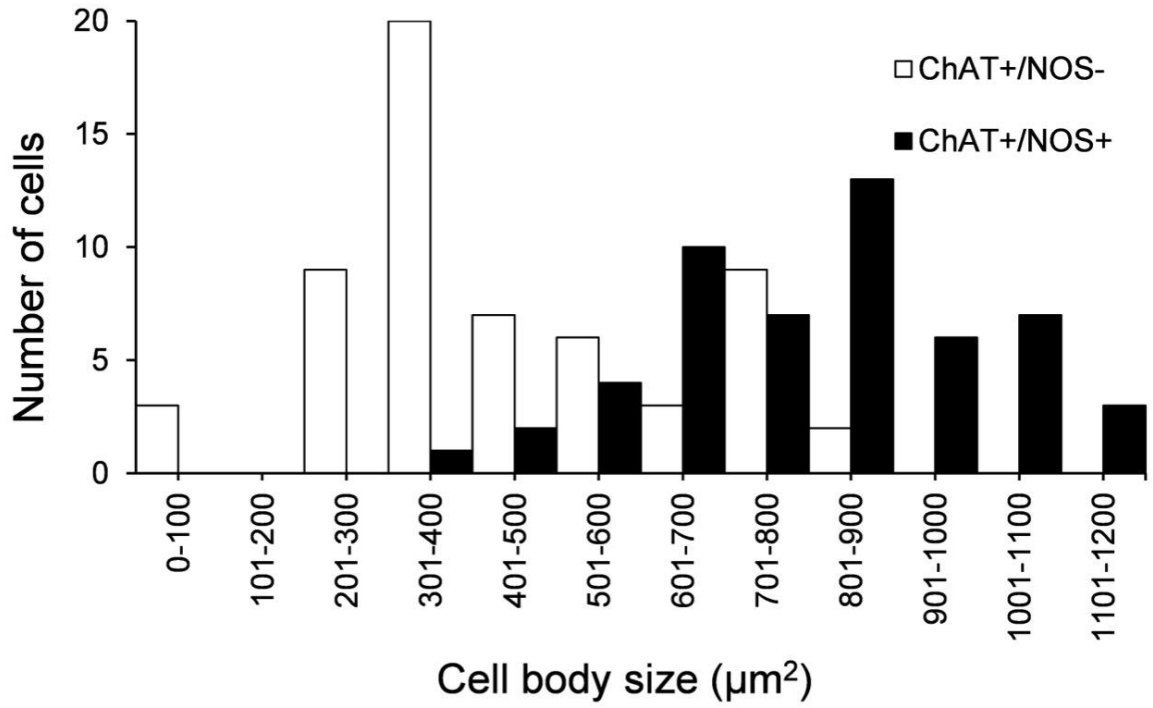


Fig8

