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Distribution across tissue layers of extrinsic nerves innervating the mouse colorectum – An *in vitro* anterograde tracing study

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Abstract

Background—Anterograde *in vitro* tracing of the pelvic nerve (PN) and visualization in the horizontal plane in whole mount preparations has been fundamental in the analysis of distribution of peripheral nerves innervating the colorectum. Here we performed a similar analysis, but in cryostat sections of the mouse colorectum, allowing for a more direct visualization of nerve distribution in all tissue layers.

Methods—Colorectum with attached pelvic nerves (PN) was dissected from adult male BalbC mice. Presence of active afferents was certified by single fibre recording of fine PN fibres. This was followed by “bulk” (all fibres) anterograde tracing using biotinamide (BTA). Histo- and immuno-histochemical techniques were used for visualization of BTA-positive nerves, and evaluation of co-localization with calcitonin gene-related peptide (CGRP), respectively. Tissue was analyzed using confocal microscopy on transverse or longitudinal colorectum sections.

Key Results—Abundant BTA-positive nerves spanning all layers of the mouse colorectum and contacting myenteric plexus neurons, distributing within the muscle layer, penetrating deeper into the organ and contacting blood vessels, submucosal plexus neurons or even penetrating the mucosa, were regularly detected. Several traced axons co-localized CGRP, supporting their afferent nature. Finally, anterograde tracing of the PN also exposed abundant BTA-positive nerves in the major pelvic ganglion.

Conclusions & Inferences—We present the patterns of innervation of extrinsic axons across layers in the mouse colorectum, including the labile mucosal layer. The proposed approach could

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DISCLOSURE

None of the authors have any actual or potential conflict of interest.

AUTHOR CONTRIBUTION

PRB co-designed and co-wrote the manuscript with JHL and GFG; PRB performed PN fine fibres recording; PRB and JHL performed *in vitro* anterograde tracing; PRB performed histo- and immunohistochemistry; PRB performed imaging acquisition and analysis; GFG supervised analyzed data and the overall project.

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also be useful in the analysis of associations between morphology and physiology of peripheral nerves targeting the different layers of the colorectum.

Keywords

Anterograde; biotinamide; colorectum; *in vitro* tracing; peripheral nerves; primary afferents

Afferent and efferent activities in the colorectum are governed by both intrinsic and extrinsic neuronal systems [1–5]. The extrinsic innervation of the colorectum is provided by the pelvic (PN) and lumbar splanchnic nerves [4, 5], containing both sensory (primary afferents) and autonomic (sympathetic and parasympathetic) components. Specifically for the PN, lumbosacral (6th lumbar to the 2nd sacral) dorsal root ganglia (DRG) neurons provide the afferent component [4]. Afferent fibres in the PN are further subdivided into four functional classes, based on responses to mechanical stimulation of their receptive fields [6], including: 1) mucosal (responsive to light mucosal stroking and probing), muscular (responsive to circumferential stretch and probing), muscular-mucosal (responsive to stroking, stretch and probing) or serosal/vascular afferents (only responsive to probing), as described in rat [7], guinea pig [8–14] and mouse [15] colorectum. Responses to application of different chemical compounds further subdivide mechanically sensitive afferents [16–18]. The autonomic component in the PN is supplied by the postganglionic projections of sympathetic neurons in the lumbar sympathetic chain, or sympathetic and parasympathetic neurons present in the ‘mixed’ major pelvic ganglion (MPG) [19, 20]. Most information concerning the origin of the extrinsic innervation of the colorectum has been obtained through the use of retrograde tracers and careful analysis of their accumulation in DRG [21–24] and autonomic [25–27] neurons, several days after injection in the subserosal space.

The termination patterns of extrinsic nerves in the colorectum, on the other hand, have been more difficult to elucidate, partly because of the parallel contribution of a rich and complex neuropil produced by local intrinsic neurons [1, 19, 28, 29]. With some limitations, however, various immunohistochemical markers such as the calcitonin gene-related peptide (CGRP), tyrosine hydroxylase and the vesicular acetylcholine transporter (VACHT) or choline acetyltransferase (ChAT) have been used, respectively, for the identification of afferent, sympathetic or parasympathetic nerves innervating the colorectal wall in rodents [3, 4, 30–34]. More recently, the introduction of *in vitro* anterograde tracing of visceral nerves using biotinamide (BTA)[5, 13, 14, 25, 34–41] and its use in combination with electrophysiology has been fundamental for the detailed analysis of correlation between functional and anatomical characteristics of afferent nerves innervating visceral organs, including the colorectum (see [5]). However, while extremely useful, such characterization has been done mostly by visualization in whole mount preparations after removal of the mucosa and the circular muscle, limiting assessment across all layers of the colorectum. In fact, a detailed description of the anatomy of anterogradely traced extrinsic afferent endings in the different layers of the colorectum has yet to be reported [5].

In this study, we present results in mouse colorectum, analyzing its extrinsic innervation from a different perspective. “Bulk” tracing, followed by transverse or longitudinal tissue sectioning, was used to assess the general distribution of PN axons innervating the organ

across layers. In addition, immunohistochemical analysis using CGRP antiserum was employed to attempt identification of sensory extrinsic nerves. Finally, a few observations made on traced MPG are also provided.

EXPERIMENTAL PROCEDURES

Animal and tissue collection

Male BALB/c mice (n=10; Taconic, NJ, USA; 7–8 weeks old) were used. Research protocols were approved by the Institutional Animal Care and Use Committee (University of Pittsburgh) and were conducted in accordance to the ethical guidelines established by the International Association for the Study of Pain (IASP) for the use of animals in research.

Mice were euthanized by CO₂ inhalation (40 kPa) and the colorectum with associated neurovascular bundle containing the PN was dissected and transferred to ice-cold Krebs solution (in mM: 117.9 NaCl, 4.7 KCl, 25 NaHCO₃, 1.3 NaH₂PO₄, 1.2 MgSO₄ * 7H₂O, 2.5 CaCl₂, 11.1 D-glucose, 2 sodium butyrate, 20 sodium acetate, all purchased from Sigma, MO, USA; at ~32°C) bubbled with carbogen (95% O₂, 5% CO₂) as described previously (see [18, 42]). The L-type calcium channel antagonist nifedipine (4μM, Sigma; to block spontaneous contractions) and the prostaglandin synthesis inhibitor indomethacin (3μM, Sigma; to block synthesis of endogenous prostaglandins) were also added to the Krebs solution. The posterior wall of the colorectum was opened along the longitudinal axis, pinned flat, mucosal side up, in one chamber of a two chamber organ bath, and superfused with oxygenated Krebs solution (Figure 1A–C). The PN was placed into an adjacent recording chamber filled with light paraffin oil (Figure 1A–C).

PN recording

Under a dissection microscope, and after 60 min of adaptation to bath conditions, the PN sheath was carefully peeled back and the nerve trunk teased into 6–12 fine fibres from which activity was recorded (Figure 1D). Briefly, action potentials were differentially amplified, sampled at 20 kHz using and stored on a PC as previously described [15, 18]. Mechanical and chemical stimuli were applied to the colorectum to verify the presence of sensitive receptive fields (Figure 1E), and thus certify a healthy connection between the PN fibres and the organ being innervated.

Anterograde tracing

After electrophysiological testing, “bulk” tracing was employed based on previous publications [13, 40]. Briefly, all split fine PN fibres were washed three times (5 min each) in intracellular solution (in mM: monopotassium glutamate, 150; MgCl₂, 7; Glucose, 5; EGTA, 1; HEPES, 20; Disodium Adenosine Triphosphate, 5; plus saponin, 0.02%; DMSO, 1%; streptomycin, 50 μg/ml; penicillin, 50 UI/ml; Sigma). This was followed by exposure to a 7.5% biotinamide (BTA) solution (Molecular Probes, OR, USA; dissolved in intracellular solution). Perfusion of the organ chamber (Krebs solution, ~32°C) continued for 2–4 hr from the start of BTA incubation, after which it was replaced with extracellular solution (500 ml of DMEM/F12 (Invitrogen, NY, USA) containing 10% fetal bovine albumin, 0.75 mM CaCl₂, 2 mM sodium butyrate, 20 mM sodium acetate, 50 μg/ml streptomycin, 50UI/ml

penicillin and 4 μ M nifedipine (Sigma)). The extracellular solution, refreshed half-way through tracing (18–24 hr), was re-circulated from a continuously oxygenated reservoir and maintained between 33–35°C (pH 7.4). Colorectal tissue was analyzed further (see below) only when: 1) active afferents were present in the tested filaments; 2) PN fibres remained in contact with BTA during the incubation period; 3) tissue preservation was acceptable.

Histochemistry, immunohistochemistry

Following tracing, tissue was carefully removed from the organ bath, pinned flat to a Sylgard (Dow Corning, Melbourne, Australia) mould and immersed in a mixture of 4% paraformaldehyde and 0.2% picric acid dissolved in 0.16 M phosphate buffer (pH 6.9) at 4°C for 24 hrs [43, 44]. This was followed by successive 24 hr incubations in 10 and 20% sucrose in phosphate-buffered saline (PBS; pH 7.4; 4°C) containing 0.01% sodium azide and 0.02% bacitracin (both from Sigma, St. Louis, MO, USA). After embedding in Tissue-Tek O.C.T. compound (Sakura, CA, USA) and deep freezing on dry ice, tissue was cut in longitudinal or transverse sections in a cryostat (Leica, Heidelberg, Germany) at 30–40 μ m thicknesses and mounted onto SuperFrost slides.

For visualization of BTA, tissue sections were washed twice in PBS, incubated during 2 hr at room temperature (RT) in Streptavidin-Alexa Fluor® 488 conjugate (1:1,000–2,000; Molecular Probes) and washed twice in PBS.

After histochemical detection of BTA, a few sections were further incubated for 10 min at RT with propidium iodide diluted in Tris-NaCl-Tween buffer (TNT; 0.0001%; Sigma), washed three times in TNT (see below) and twice in PBS. Other sections were further processed using the tyramide signal amplification technique (TSA™ Plus, Perkin Elmer, Boston, MA), where sections were incubated with a rabbit calcitonin gene-related peptide antiserum (1:10,000–20,000, CGRP; Sigma) diluted in 0.01 M PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin. After 24 hr incubation at 4°C, sections were washed in TNT buffer (0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.05% Tween 20) for 10 min, incubated with TNB buffer (kit; 0.1 M Tris-HCl, pH 7.5; 0.15 M NaCl; 0.5% Dupont Blocking Reagent; Perkin Elmer) for 30 minutes at RT, and incubated for 1 hr at RT with a donkey anti-rabbit/horseradish peroxidase (HRP) conjugate (Jackson ImmunoResearch, PA, USA) diluted 1:200 in TNB buffer. This was followed by two washes in TNT buffer, incubation in a biotinyl tyramide-tetramethylrhodamine (BTTMR) conjugate kit (NEN) diluted 1:1,500 in amplification diluent kit (NEN) for 30 minutes at RT and two final washes in PBS. Nonspecific staining by secondary antibody was tested in some sections by omitting the primary antibody. CGRP antiserum was previously described in detail [45].

Microscopic analysis

After coverslipping using 2.5 % DABCO in glycerol (Sigma), sections were examined on a Fluoview FV 1000 confocal microscope (Olympus, Tokyo, Japan). Optical sections (1–2 μ m thick) were taken through tissue sections containing BTAp positive nerves, merged and uploaded in Adobe Photoshop CS3 software (Adobe Systems Inc., CA, USA) for optimization of their resolution, brightness and contrast. Subsequently, and when needed, careful assembly of composite photographs following various histological references (e.g.

blood vessels, surface landmarks) was performed before final merging and global analysis of the distribution of traced nerves across the colorectal wall.

RESULTS

“Bulk” tracing of fine PN fibres was successful in 9 mice. This normally resulted in abundant BTA-positive nerves throughout the colorectal wall (Figures 2–6).

Traced pelvic nerve fibres are detected in all layers of the colorectum and their morphology varies between layers

Thick BTA-positive fibres of passage were detected in the proximity of the colorectum (Figures 2A–D), as well as rare, thinner traced nerves in association with the mesentery (Figure 2A). After penetrating the organ through its serosal surface (Figures 2C, D) and traversing the longitudinal muscle layers, the thick BTA-positive fibres of passage often extended projections variable in thickness and length within the myenteric plexus, covering distances as long as 500 μ m in the longitudinal plane of the colorectum (Figures 2C–E). These fibres of passage were often found running and intertwining in close association with myenteric plexus neurons (Figure 3A–C). Occasionally, they were also seen terminating in the form of large, dilated varicosities (Figures 4A, B).

Thinner BTA-positive fibres of passage (Figure 2C, E) or what appeared to be individual axons (Figure 2G) were commonly observed within the muscular layers penetrating and traversing completely through the circular muscle layer, reaching the submucosal layer and abruptly changing direction to run in between the circular muscle layer and the submucosa, to then cover variable distances. Such fibres of passage, normally associated with myenteric plexus neurons, could also be seen penetrating deeper into the submucosal layer (Figure 3D–F). Two additional distribution patterns were seen within the muscular layers, including intramuscular varicose nerve endings expanding in the longitudinal plane within the circular muscle layer (Figure 4C), and nerves dividing and apparently targeting both the muscular and submucosal layers (Figure 4D).

In the submucosal layer itself, a number of BTA-positive nerve endings were detected in close association with blood vessels (Figures 2E, F; 3G–L), and occasionally also contacting submucosal plexus neurons (Figures 3M–O). A number of fine BTA-traced nerves were also detected in the mucosal layer of the colorectum (Figures 2B; 4E–H), often associated with BTA-positive nerves located in the submucosal layer (Figures 2B; 4E). Traced nerves in the mucosa were normally seen as either fibres of passage (Figures 2B; 4E, G, H) or free nerve endings distributed transversally or longitudinally to the mucosal surface (Figures 2B; 4F). Some of the BTA-traced mucosal fibres of passage travelled parallel to the mucosal surface a few hundred micrometers before terminating (Figure 4G, H).

“Bulk” tracing of the pelvic nerve exposes a discrete number of viscerofugal neurons

BTA-positive cell bodies located in the myenteric plexus were occasionally observed in 3 of the 9 mice tested (Figures 4A, B; 5).

Colocalization with CGRP reveals a number of BTA-positive afferents innervating the mouse colorectum

Colocalization analysis exposed a considerable number of BTA-positive nerves containing CGRP. Thus, the peptide was detected in individual axons in fibres of passage penetrating the colorectum (Figure 6A–C) or within the myenteric plexus (Figure 6D–F), and in the submucosal layer (Figure 6G–O), sometimes in association with blood vessels (Figure 6J–L). In the mucosal layer, both BTA-positive and CGRP-immunoreactive (IR) single nerves were also found (Figure 6P–R). Additional CGRP-IR or BTA-positive nerves representing different subpopulations were also detected (Figure 6).

Pelvic nerve tracing exposes BTA-positive nerves, perineuronal baskets and occasional neuron profiles in the MPG

Abundant BTA-positive fibers of passage were found within the MPG (Fig. 7A–I). These were also accompanied by presence of a number of perineuronal baskets of varicose endings around MPG neuron profiles (Fig. 7A–F). Finally, occasional BTA-positive MPG neuron profiles were also detected (Figures 7A–C, G–I).

DISCUSSION

A number of previously published studies have presented a detailed account of the distribution of *in vitro* [13, 25, 34, 41] and *in vivo* [46] anterogradely traced PN axons innervating the rodent colorectum, mainly through analysis of whole mount preparations, and focusing in the muscle layers. In the present study, we analyzed the distribution of anterogradely traced PN axons in sections across the transverse or longitudinal planes of the colorectum. We thus provide a comprehensive description of the distribution of labeled extrinsic axons across all colorectal layers, including previously undescribed distributions in the submucosal and mucosal layers.

Before discussing our findings, some methodological issues deserve attention: 1) To maintain the integrity of the colorectum, we kept oxygenation, pH and bath temperature constant and adequate. Presence of nerve endings in the mucosal layer (probably the most labile in the colorectum) after “bulk” tracing suggests that such care prevented tissue degradation. 2) We occasionally detected some large BTA-positive swellings within the myenteric plexus. These could be swelling artifacts due to damage during an experiment, actual varicose nerve endings, or the cell bodies of viscerofugal neurons; their true nature remains to be established. 3) In some samples, artifactual separation between the muscle and submucosal/mucosal layers was observed. If such an artifact occurs during *in vitro* tissue manipulation or later longitudinal cryosectioning, it cannot be confirmed. However, regular observation of traced nerves in the submucosal/mucosal layers suggests the latter scenario. 4) Visualizing BTA-positive nerves is not sufficient to distinguish them as being sensory or autonomic. We thus included use of an antibody against CGRP, normally utilized for the identification of afferent nerves in the mouse colorectum (see [45, 47]), for exposing extrinsic colorectal sensory nerves. 5) A number of BTA-positive nerves did not exhibit CGRP-colocalization. While many of these traced nerves are most certainly autonomic, a

proportion could still derive from colorectal DRG neurons shown to lack the neuropeptide [4].

“Bulk” tracing exposed a few thick and intensely BTA-positive fibres of passage in the surface of the colorectum, often penetrating the organ in a sharp angle and traversing the longitudinal muscle layer. Rarely were these thick fibres of passage accompanied by thinner nerves distributing within the surrounding colorectal mesentery. Such scarcity in the mesentery correlates with electrophysiological analysis of mouse PN afferents, showing absence of receptive fields in the colorectal mesentery, in contrast to their abundance in recordings from the lumbar splanchnic nerve [15, 48]. However, whether the mesenteric BTA-positive nerves seen here are afferent or autonomic, it remains to be established.

After traversing the longitudinal muscle layer, the thick BTA-positive fibres of passage travelled within the myenteric plexus for several hundred micrometers, often approaching and seemingly wrapping around myenteric neurons, before eventually terminating in large varicose axonal endings. Such nerve distribution and termination correlates with findings by Spencer and cols. [40] who showed, using combined single fibre recording/anterograde tracing in mouse whole mount rectum preparations, similar large varicose myenteric nerve endings corresponding to capsaicin sensitive, vesicular glutamate transporter type 2 (VGLUT₂)-and transient receptor potential cation channel subfamily V member 1 (TRPV1)-expressing, slowly-adapting mechanoreceptors. More recently, afferent nerves distributing within the myenteric plexus and producing smaller (2.5 µm in diameter) perineuronal globular clusters were exposed in the mouse colon after transfection of lumbar splanchnic DRGs using an adeno-associated virus driving the over-expression of green fluorescent protein (GFP) [49]. Alternatively, BTA-positive nerves targeting myenteric plexus neurons could also derive from sympathetic and parasympathetic sources (see [1, 50–52]), and participate in controlling enteric motor activity (see [3, 35]). In fact, BTA-positive nerves innervating the myenteric plexus and coexpressing TH, choline acetyltransferase [35] or vesicular acetylcholine transporter [34] have been described in guinea pig small intestine [35] and rectum [34].

“Bulk” tracing of the PN also exposed a discrete number of viscerofugal neurons in the midst of the myenteric plexus, further supporting previous studies in guinea pig, rat, dog and pig ([53–57]).

A number of myenteric plexus-associated BTA-positive axons were visualized traversing the circular muscle before reaching the submucosal layer, abruptly changing direction and covering variable distances parallel to the myenteric plexus. If these ‘traversing’ axons were afferent, it could be speculated that they are capable of sensory transduction in more than one layer. Interestingly, afferent nerves functionally classed as muscular-mucosal have been identified in mouse colorectum [15] and bladder [42]. The morphology of these nerves, and its correlation to function, is not completely understood. However, it has been recently proposed that muscular-mucosal afferents terminate exclusively within the submucosal layer [5, 38]. This is supported by a study in guinea pig bladder, showing that the experimental removal of the urothelium/submucosa alters the ability of mucosal and muscular-mucosal

afferents to respond to mucosal stroking [58]. However, a proportion of bladder muscular-mucosal afferent nerves retain some stretch sensitivity after urothelial removal [38, 58].

We failed to identify traced nerves within the longitudinal muscle layer, even though they were previously documented in mouse [40, 41] and guinea pig [39]. It is possible that sectioning through the colorectum limited our ability to visualize thin, varicose nerve projections present in the longitudinal muscle layer [39]. In contrast, we found abundant traced nerves within the circular muscle layer, sometimes exhibiting distributions compatible with recently described intramuscular arrays [46]. In fact, intramuscular afferent endings in the circular muscle, distributing along the circumference of the colon for several hundred micrometers, and presenting with multiple varicose processes were recently described by Spencer and colleagues [46] after injection of dextran-biotin directly into DRGs. Similar arrays have been described in the mouse [40, 41], and guinea pig rectum [13] and internal anal sphincter [39] whole mounts, and characterized as the primary transduction site of low threshold, slowly adapting mechanoreceptors. However, it should be noted that the circular muscle is heavily innervated by sympathetic nerves [1]; thus, some traced axons in the circular muscle may have functions other than sensory.

While BTA-positive nerves reaching submucosal plexus neurons were occasionally detected, traced nerves contacting blood vessels, often coexpressing CGRP, were frequently observed. It is known that mesenteric and submucosal arteries are the target of sympathetic innervation, as shown in human, guinea pig and rat (see [59]). Thus, some blood vessel-related BTA-positive nerves identified here could derive from lumbar sympathetic chain neurons. Conversely, mechanoreceptive afferent neurons are the likely source of blood vessel-related BTA-positive/CGRP-IR axons shown here. Such afferents, possibly involved in nociception [5], are sensitive to distention and contraction of the gut wall [60], blunt probing or compression of the gut surface [15, 61], capsaicin [62], and express CGRP and TRPV1 [63]. Alternatively, afferent fibres innervating blood vessels could also serve as “ischemia-sensing” chemosensitive fibers. Finally, while the debate is still ongoing, it is possible that such vascular afferents were those originally classified as serosal, based on electrophysiological assessment. However, functionally classified serosal afferents have never been found in morphological association with the colorectal serosa [13, 14]. Accordingly, no CGRP- and/or VGLUT₂-IR [64] or BTA-positive (present study) nerves are detected in the colorectal serosa or adventitia.

To date, the morphology of colorectal mucosal afferent endings and their association with mapped receptive fields remains obscure [5]. One of the objectives of this study was to determine whether “bulk” PN tracing and the specific tracing and histological protocols carried out would result in the visualization of traced colorectal mucosal nerve endings. To our knowledge, this is the first time that extrinsic nerves penetrating and terminating in the mucosal layer have been traced *in vitro* and visualized in the rodent colorectum. These mucosal nerve terminals could be sympathetic (see [47]) and participate in a number of functions, including blood flow, fluid transport, mucous secretion and inflammation (see [65, 66]). Conversely, mucosal BTA-traced nerves could be sensory, as mucosal afferents have been electrophysiologically demonstrated in mouse [15] and rat [8, 67], and we here show presence of the afferent nerve marker CGRP in some BTA-positive mucosal axons. In

support, transfection of GFP into lumbar splanchnic DRGs results in a similar mucosal distribution of GFP-positive fibres in mouse colorectum [49].

Abundant traced fibres of passage, peri-neuronal baskets of varicose endings, and occasional BTA-positive neurons were also observed in the MPG. Perineuronal baskets derived from parasympathetic preganglionic neurons have been previously described in rat [68] and mouse [69] MPG, as shown by their content of cholinergic markers [20, 69]. However, afferent neurons appear also to be their source, as recently shown in mouse by their content of VGLUT₂ [70]. Finally, the occasional BTA-positive neurons may belong to the small population of cholinergic or noradrenergic MPG neurons (<1%) known to project axons through the pelvic and hypogastric nerves in the rat [71]. To our knowledge, this is the first time they are shown in mouse.

In conclusion, we presented a comprehensive description of the distribution in layers of *in vitro* anterogradely traced PN axons innervating the mouse colorectum, including the submucosal and mucosal layers. Future tracing of single thin PN fibres may be informative for the correlation between the morphology and the electrophysiology of single afferent fibres terminating in different colorectal tissue layers.

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Key Messages

- Afferent and autonomic nerves innervate the colorectum, and their morphology and distribution across layers, in correlation with their function, has only recently started to be elucidated. However, most analyses so far have been limited to the colorectal muscle layers and the myenteric plexus in whole mount preparations.
- The goals of this study were to reveal the termination patterns of anterogradely traced pelvic nerve (PN) axons innervating the mouse colorectum, across tissue layers, including the labile mucosal layer, and to prompt future histological/ electrophysiological correlation analysis.
- After biotinamide-antegrade tracing of the PN innervating the colorectum of male BalbC mice, histological and immunohistochemical techniques were employed for visualization of traced nerves and evaluation of co-localization with calcitonin gene-related peptide (CGRP), respectively. Transverse or longitudinal sections of the tissue were analyzed using confocal microscopy.
- Various distribution patterns of BTA-traced nerves were observed across layers in the mouse colorectum. Tissue layer-specific morphologies were detected. Nerves reaching and penetrating the mucosa were also observed. A number of traced nerves co-localized CGRP, supporting afferent nature. Anterograde tracing also exposed BTA-positive nerves and neurons in the major pelvic ganglion.

Figure 1.

Tissue preparation and fibre recording. (A) Mouse pelvic cavity showing partially dissected organs during collection of tissue for *in vitro* PN recording and anterograde tracing. (B) PN (black arrow) tributaries (white arrows) to the colorectum are dissected and shown, penetrating the organ at an angle. (C) Colorectum opened and pinned flat mucosal side up in a Sylgard®-lined organ chamber perfused with 32°C Krebs solution. The paraffin-filled recording chamber is isolated from the organ chamber by a removable plastic gate with a mouse-hole through which the PN and accompanying sciatic nerve passes. A PN fine fibre is

shown (arrow), previous to its attachment to the recording electrode (white double arrowhead). For illustration purposes, the location of a number of receptive fields tested during the experiment is shown by probing using a carbon-coated von Frey hair (black double arrowheads). (D) The PN after splitting into fine fibres (dotted line shows area covered by BTA). (E) Examples of electrophysiological recording from three different fine fibers (1–3), showing the response patterns to blunt stroking (using a camel's hair brush), 400 mg probing using a von Frey filament, gross stretching, 10 mg stroking using a calibrated taklon brush, or exposure to an inflammatory soup (see [18]).

Figure 2.

Distribution of BTA-positive nerves terminating in different layers of the mouse colorectum. Confocal fluorescence photomicrographs of longitudinal (A, C–G) or transverse (B) sections of the colorectum incubated with streptavidin, alexa fluor 488. (A) Thick and intensely stained BTA-positive nerve fibres of passage are seen in the proximity of the colorectal surface (white arrows), along with thinner traced nerves associated with the mesentery (arrowheads). A fine BTA-positive nerve fibre in the underlying myenteric plexus is also detected (black arrows). (B) Abundant BTA-positive nerves are detected in the submucosal

(white double arrowheads) and mucosal layers (black double arrowheads), as well as in relation to blood vessels (black arrowhead; asterisks indicate the lumen of blood vessels). (C–G) Various examples of BTA-positive nerves distributing within the myenteric plexus (black arrows in C, D, E) or traversing the circular muscle layer (double arrows in C, E, F). Note presence of thick (double arrow in E) and thin (double arrow in G; shown at higher magnification in G) traversing BTA-positive nerves changing direction in an abrupt angle, and extending for several micrometers with an orientation parallel to the myenteric plexus within the submucosal layer. Additional BTA-positive nerves innervating blood vessels (black arrowhead in E, F; asterisks show blood vessels) or traveling within the submucosal plexus underneath the mucosa (white double arrowhead in G). Scale bars: 50 μm (A–E, G); 20 μm (F, inset in G).

Figure 3.

Identification of BTA-positive colorectal nerves in association to neurons in the myenteric and submucosal plexuses, or to blood vessels. Confocal immunofluorescence photomicrographs of longitudinal sections of the colorectum incubated with streptavidin, alexa fluor 488 (A, D, G, J, M), co-incubated with propidium iodide (B, E, H, K, N) (C, F, I, L, O show merged photomicrographs). (A–F) BTA-positive nerves (black arrows in A, D) are shown in close relationship with myenteric plexus neurons (white arrows in B, E). A nerve bundle previously associated with the myenteric plexus traverses the circular muscle

layer (double arrow in D) before dividing into two fibres penetrating the submucosal layer (double arrowheads in D). (G–I; shown at higher magnification in J–L) BTA-positive nerves (black arrowheads in G, J) are seen in close association with blood vessels (arrows in H, K). (M–O) A BTA-positive nerve (double arrowheads) reaching submucosal plexus neurons is shown. Scale bars: 10 μm (C=A, B; F=D, E); 20 μm (L=J, K; O=M, N); 50 μm (I=G, H).

Figure 4.

Distribution in the myenteric plexus, the circular muscle and mucosal layers, of BTA-positive nerves terminating in the mouse colorectum. Confocal fluorescence photomicrographs of longitudinal (A–C, E–H) or transverse (D) sections of the colorectum incubated with streptavidin, alexa fluor 488. (A; shown at higher magnification in B) Intensely stained BTA-positive varicose nerve endings (black arrows) are seen terminating in the myenteric plexus (black asterisks). A presumable viscerofugal neuron is also observed (white asterisk) (C) An apparently single BTA-positive axon (double arrows) penetrates and

subdivides into multiple varicose nerve endings (arrowheads) within the circular muscle. (D) What appears to be a single BTA-positive axon is seen dividing into a projection towards the circular muscle layer (double arrow) and the submucosal layer (double arrowhead). (E–H) Several BTA-positive nerves, often of small calibre, are detected in the mucosal layer (black double arrowhead), often associated with traced nerves in the submucosal layer (white double arrows in E). Occasionally, these nerves could be seen extending for several micrometers before terminating (black arrowheads in G; shown at higher magnification in H). Scale bars: 20 μm (A, C–H); 10 μm (B).

Figure 5.

Viscerofugal neurons are detected in the myenteric plexus after “bulk” tracing of the PN nerve. Confocal fluorescence photomicrographs of longitudinal sections of the colorectum incubated with streptavidin, alexa fluor 488 (C and D, E show magnified views of A and B, respectively). (A–E) A discrete number of viscerofugal neurons are detected in the myenteric plexus (arrowheads). Traced fibres of passage are also observed (double arrowheads). Scale bars: 100 μ m (A); 50 μ m (B); 25 μ m (C; D=E).

Figure 6.

Several traced PN colorectal nerves coexpress with CGRP. Confocal immunofluorescence photomicrographs of longitudinal sections of the colorectum incubated with streptavidin, alexa fluor 488 (A, D, G, J, M, P) and co-incubated with a CGRP antiserum (B, E, H, K, N, Q) for the identification of afferent nerves (C, F, I, L, O, R show merged photomicrographs). (A–R) Several BTA-positive nerves colocalizing CGRP (double arrows) are detected in the myenteric plexus (A–C; shown at higher magnification in D–F), the submucosal layer (G–I, J–L; the latter shown at higher magnification in M–O), in association to blood vessels in the

submucosal layer (J–L), and in the mucosal layer (P–R). Several other BTA-(arrowheads) or CGRP-only nerves (arrows) are seen throughout all colorectal layers. Scale bars: 50 μm (C=A, B; L=J, K); 20 μm (F=D, E; I=G, H; O=M, N; R=P, Q).

Figure 7.

Traced fibres of passage, perineuronal baskets of varicose endings and neuron profiles are detected in the MPG. Confocal immunofluorescence photomicrographs of the MPG incubated with streptavidin, alexa fluor 488 (A, D, G) and co-incubated with a propidium iodide (B, E, H) (C, F, I show merged photomicrographs). (A–I) Abundant BTA-positive fibres of passage are detected in the MPG (arrowheads). BTA-positive perineuronal baskets of varicose endings (black arrowheads) engulfing propidium iodide-positive neuron profiles (arrows) are also often detected. Occasional BTA-positive MPG neuron profiles are observed (double arrowheads in A–C, G–I). Scale bars: 50 μ m (C=A, B); 20 μ m (F=D, E; I=G, H).