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A novel anterograde neuronal tracing technique to selectively label spinal afferent nerve endings that encode noxious and innocuous stimuli in visceral organs

Melinda Kyloh & Nick J. Spencer*

Discipline of Human Physiology and Centre for Neuroscience, Flinders University of South Australia, Adelaide, South Australia, Australia

***Corresponding author:**
Nick Spencer (Ph.D)
Department of Human Physiology,
School of Medicine, Flinders University
South Australia, Australia
e-mail: nicholas.spencer@flinders.edu.au

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Background

One major weakness in our understanding of pain perception from visceral organs is the lack of knowledge of the location, morphology and neurochemistry of all the different types of spinal afferent nerve endings, which detect noxious and innocuous stimuli. This is because we lack techniques to selectively label *only* spinal afferents. Our aim was to develop an anterograde tracing technique that labels *only* spinal afferent nerve endings in visceral organs, without also labeling all other classes of extrinsic afferent and efferent nerves.

Methods:
Mice were anesthetized with isoflurane and dextran-biotin injected, via glass micropipettes, into L6 and S1 dorsal root ganglia (DRG). Mice recovered for 7 days, were then euthanized and the colon removed.

Results:
Anterograde labeling revealed multiple unique classes of afferent endings that terminated within distinct anatomical layers of the colon and rectum. We characterized a particular class of intramuscular ending in the circular muscle (CM) layer of the colon that consists of multiple varicose axons that project circumferentially.

Conclusions:
We demonstrate a technique for selective anterograde labeling of spinal afferent nerve endings in visceral organs. This approach facilitates selective visualization of the precise morphology and location of the different classes of spinal afferent endings, without visual interference caused by indiscriminant labeling of other classes of afferent and efferent nerve axons which also innervate internal organs. We have used this new technique to identify and describe the details of a particular class of intramuscular spinal afferent ending in the CM layer of mouse large intestine.

Introduction

In the gastrointestinal (GI) tract, and other visceral organs, there is now extensive evidence that painful (noxious) and non-noxious stimuli are detected and transmitted by spinal afferent neurons to the central nervous system (1-3). Whilst the origin of the cell bodies of spinal afferents is clearly within the DRG (2-5), the location and morphology of the nerve endings of spinal afferents that innervate visceral organs, such as the GI-tract, is a major unanswered question. Previous studies have used anterograde tracing from mixed extrinsic nerve trunks, as they entered the gut wall (6-9). Using this approach, all extrinsic afferent and afferent nerve endings will be identified within the intestine, which will include labeling of sympathetic and parasympathetic efferents, spinal afferents, vagal afferents and intestinofugal neurons. We sought to develop a technique that *only* labeled spinal afferent endings within gut wall, but did not also label all other classes of extrinsic efferent or afferent axons. Selective anterograde labeling of vagal afferent nerve endings in the gut wall has been published in detail following neuronal tracer injections into nodose ganglia (10-13). But, a similar technique has been lacking for spinal afferents. What is known is that at least 5 different functional classes of spinal afferent innervate the large intestine (14); but, the sites of innervation and morphologies of these different classes of spinal afferent remain poorly understood.

Here, we demonstrate a reliable technique that can reveal the sites of innervation and morphology of the different classes of spinal afferent nerve endings in visceral organs. The major advantage of this technique is that it does not label all the other classes of extrinsic efferent or afferent nerve that also innervate these organs.

METHODS

Male & female C57BL/6 mice (30-60 days old) were anesthetized by isoflurane inhalation anesthetic (induced at 4%, maintained at 1.5% in oxygen). Whilst under anaesthesia, a 1.5cm long incision was made along the dorsal surface to expose the spine ~2-3 cm rostral to the anus. Only two DRGs were

exposed (at L6 & S1) and both were injected with 100-200nL of biotinylated dextran amine (BDA, 10-20% soln.; MW 10,000) (Cat # D1956, Molecular Probes, Eugene, Oregan, USA). BDA was injected into DRGs via glass micropipettes (tip diameters: 5µm) using a nitrogen driven spritz system (Biomedical Engineering, Flinders University) that applied brief pulses of high pressure to the electrode. The skeletal muscle around the spine was then sutured with 4.0 suture (Dynek, Australia) and the wound site and incision closed with fine suture. The skin was closed using 7mm wound clips (Fine Science Tools, Canada) and animals allowed to recover for 7 days. The welfare of mice was monitored closely twice daily and any mice that showed signs of severe pain or discomfort were euthanized. At 7 days post-operative, all mice were euthanized by isoflurane inhalation overdose, followed by cervical dislocation. The entire procedure was approved by the Animal Welfare Committee at Flinders University. After euthanasia, the entire colon (full thickness) and bladder were removed placed into a Petri dish, and an incision made in the longitudinal axis. The preparations were opened as a sheet, then pinned under circumferential stretch and immediately fixed in 4% paraformaldehyde for 2 hours, followed by three 10 minute washes in phosphate buffered saline (PBS). After fixation, preparations were incubated for 2 hours in Cy3-conjugated Streptavidin (Cat # 016 160 084, Jackson Immuno Research Laboratories Inc, West Grove, PA, USA). Preparations were then washed again 3 times 10 minutes in PBS and mounted in buffered glycerol. Images were captured on a Nikon i50 fluorescence micrcoscope using Cy3 fluorescence filters.

RESULTS

Seven days after injection of BDA, mice were euthanized and the entire colon and bladder were removed, fixed and processed (see methods). In each animal, 3-14 individual axons were labeled at different sites around the circumference of the distal colon and rectum (N=4). Labeled axons entered the intestine through the serosa and ramified extensively through many rows of myenteric ganglia, projecting up to 17.5 mm orally (mean: 13.6 ± 0.8mm; 11 axons, N=4) and up to 13.5mm anally (mean: 7.8 ± 1mm; 6 axons, N=4) along the distal colon and rectum. The endings of single axons were identified in distinct anatomical layers of the large intestine and rectum (N=4). One distinct class of ending was identified that ramified extensively within the circular muscle layer from a single parent axon

(Fig. 1F). To confirm that these nerve endings terminated within the circular muscle, we removed the mucosa and submucosal plexus and it was clear that the intramuscular endings were present and clearly oriented parallel to, and within, the CM layer. We refer to an intramuscular “ending” as a single axon that branches in the CM layer with multiple varicose processes. On average, a single labeled intramuscular axon subdivided in the CM into a mean of 14 ± 3 varicose processes (8 axons; N=4; Fig.1F & Fig. 2). The diameter of the individual intramuscular processes were in the order of 1µm. The maximum mean projection length of these intramuscular processes was 830 ± 230µm around the circumferential axis of the colon and 110 ± 10µm in the rostral to caudal (oral to anal) axis of the colon (8 endings, N=4). The characteristics of this class of intramuscular ending are described in Table 1. Injection of saline into DRGs with normal post-fixation, followed by Cy3-conjugated streptavidin never revealed any labeling; and similarly injection of neuronal tracer into DRGs without Cy3-conjugated streptavidin did not reveal any fluorescent labeling. To confirm that labeled nerve endings in the colon arose from injection of BDA into DRG, we injected the same quantity of BDA outside the DRG into the surrounding space; and allowed animals to recover for 7 days. This procedure did not label any nerve endings or axons in the colon (N=2).

DISCUSSION

To date, there has been no technique available to selectively label only spinal afferent nerve endings in visceral organs, such as the GI-tract. This has severely hindered our progress in identifying all the different types of spinal afferent nerve endings that innervate the viscera. We present a reliable new anterograde tracing technique that facilitates selective labeling of only spinal afferent nerve endings in different visceral organs of mice. The major advantage of this new approach is that it does not indiscriminately label all classes of extrinsic efferent and afferent fibres that run alongside spinal afferents. Also, this new technique does not involve any culturing of tissue and therefore optimal viability of labeled organs is retained, enabling these tissues further exposure to primary antibodies for immunohistochemical characterization. We are aware of only one study that has injected neuronal tracers in DRG. This study was performed in rats, where nerve fibres were labeled in the pancreas (15).

Here, we have characterized a class of intramuscular spinal afferent ending that terminates within the CM. This class of ending projects circumferentially around the colon, branches extensively within the CM; and consists of multiple varicose processes. These afferent nerve endings arise from a single parent axon (see Fig.1F) that emanates from the myenteric plexus. This class of intramuscular afferent ending is analogous to the intramuscular arrays have been described in the stomach (16) and intramuscular endings in the guinea-pig rectum (17). The functional role of this class of intramuscular afferent is, at present, not clear in the mouse colon. This new technique will be useful in identifying the different classes of spinal afferent endings in visceral organs.

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TABLE 1 Characteristics of intramuscular spinal afferent nerve endings in circular muscle of mouse colon

	Physical extent of intramuscular axons circumferential axis) (µm)	Physical extent of Intramuscular axons (rostral -caudal axis) (µm)	Size of intramuscular Varicosities (µm2)	Width of intramuscular Varicosities (µm)	length of intramuscular Varicosities (µm)
Mean	827	112	8	2	4
SEM	230	20	1	1	1
range	70-1760	60-170	1 – 22	1-4	2-9
N	8 endings 4 animals	8 endings 4 animals	113 Varicosities 4 animals	117 Varicosities 4 animals	117 Varicosities 4 animals

Figure Legends

Figure 1

Schematic of the site of injection of dextran biotin into L6 S1 DRG facilitating anterograde labeling. B, Shows a photomicrograph of the exposed spinal cord and L6 DRG. C, Anterogradely labeled single spinal afferent that courses through myenteric ganglia. Discrete nerve endings are clearly resolved. D, A single nerve axon branches into an intramuscular varicose afferent ending that lies parallel to the CM layer. E & F, shows an enlarged micrograph of the same intramuscular ending shown in panel D. F, the red arrow shows the parent axon of the intramuscular ending prior to branching in the CM layer.

Figure 2

A, shows an example of an intramuscular varicose spinal afferent ending in the CM layer that arises from a single axon in the myenteric plexus. B, shows an enlarged portion of the intramuscular ending represented at arrow b in panel A. C, shows an enlarged portion of the intramuscular ending represented by the arrow c in panel A.

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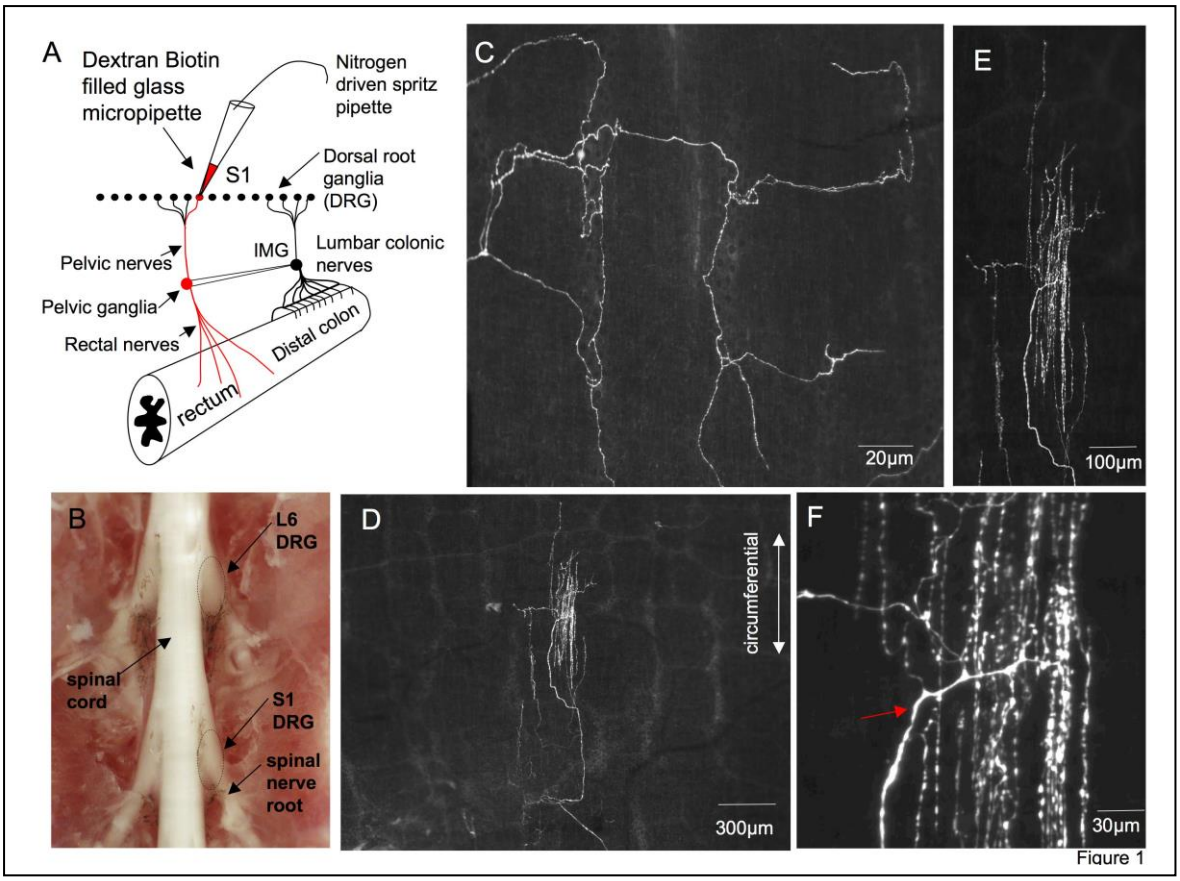
A
Dextran Biotin filled glass micropipette
Nitrogen driven spritz pipette
S1
Dorsal root ganglia (DRG)
IMG
Lumbar colonic nerves
Pelvic nerves
Pelvic ganglia
Rectal nerves
Distal colon
rectum
B
L6 DRG
spinal cord
S1 DRG
spinal nerve root
C
20µm
D
circumferential
300µm
E
100µm
F
30µm

Figure 1

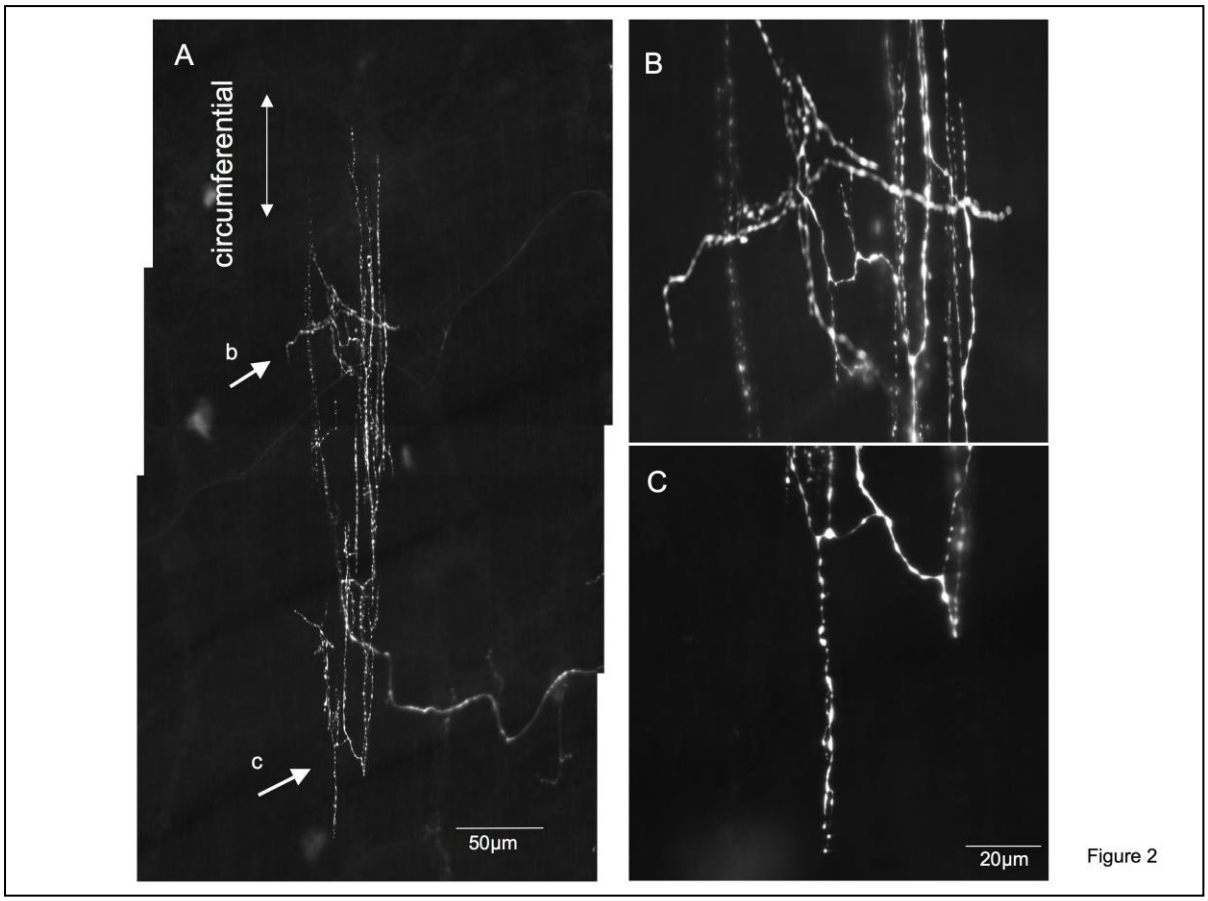

Figure 2