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http://onlinelibrary.wiley.com/

doi: 10.1111/j.1365-2982.2012.01979.x

Please cite this article as:

Hibberd, T. J., Zagorodnyuk, V. P., Spencer, N. J. and Brookes, S. J. H. (2012), Viscerofugal neurons recorded from guinea-pig colonic nerves after organ culture. Neurogastroenterology & Motility, 24: 1041–e548. d

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Viscerofugal neurons recorded from guinea-pig colonic nerves after organ culture

by

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Running Title: viscerofugal neurons in the guinea pig colon

Word count (excluding references and figure legends): 3989

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Background

Enteric viscerofugal neurons provide cholinergic synaptic inputs to prevertebral sympathetic neurons, forming reflex circuits that control motility and secretion. Extracellular recordings of identified viscerofugal neurons have not been reported.

Methods

Preparations of guinea pig distal colon were maintained in organotypic culture for 4-6 days (n=12), before biotinamide tracing, immunohistochemistry, or extracellular electrophysiological recordings from colonic nerves.

Results

After 4-6 days in organ culture, CGRP and TH immunoreactivity in enteric ganglia was depleted, and capsaicin-induced firing (0.4µM) was not detected, indicating that extrinsic sympathetic and sensory axons degenerate in organ culture. Neuroanatomical tracing of colonic nerves revealed that viscerofugal neurons persist and increase as a proportion of surviving axons. Extracellular recordings of colonic nerves revealed ongoing action potentials. Interestingly, synchronous bursts of action potentials were seen in 10 of 12 preparations; bursts were abolished by hexamethonium, which also reduced firing rate (400µM, $p<0.01$, n=7). DMPP (1,1-dimethyl-4-phenylpiperazinium, 10^{-4}M) evoked prolonged action potential discharge. Increased firing preceded both spontaneous and stretch-evoked contractions ($X^2=11.8$, df=1, $p<0.001$). Firing was also modestly increased during distensions that did not evoke reflex contractions. All single units (11/11) responded to von Frey hairs (100 - 300mg) in hexamethonium or Ca^{2+}-free solution.

Conclusions & Inferences

Action potentials recorded from colonic nerves in organ cultured preparations originated from viscerofugal neurons. They receive nicotinic input, which coordinates ongoing burst firing. Large bursts preceded spontaneous and reflex-evoked contractions, suggesting their synaptic

inputs may arise from enteric circuitry that also drives motility. Viscerofugal neurons were directly mechanosensitive to focal compression by von Frey hairs.

Keywords: afferent, enteric nervous system, organotypic culture, sensory, sympathetic, viscerofugal

Extrinsic reflex circuits from the intestine can bypass the central nervous system to modulate gut function (1). Viscerofugal neurons form the afferent arm of this circuit, with cell bodies in enteric ganglia and axons that project outside the gut through extrinsic nerve trunks and synapse onto prevertebral sympathetic neurons (2) . These, in turn, project back to the gut wall of the same, or more proximal regions, where they modulate gastrointestinal motility (3) and secretion (4). Localised gut distension activates this circuit, causing inhibition of gut contractility orally and locally (1). Characterizing activity of individual viscerofugal neurons would help us better understand and model gastrointestinal motility and secretion.

In isolated preparations of intestine with intact connections to decentralized prevertebral ganglia, converging synaptic inputs from viscerofugal neuron populations onto sympathetic neurons can be recorded (2). Luminal distension increases the frequency of nicotinic synaptic inputs to sympathetic neurons (2). Intestinal distension under synaptic blockade in the intestine reduces, but does not abolish synaptic input to sympathetic neurons – suggesting some viscerofugal neurons may be directly mechanosensory (5-7).

Intracellular recordings of retrogradely labelled viscerofugal cell bodies, showed they receive fast nicotinic excitatory synaptic inputs, suggesting they may function as interneurons (8). However, mechanical stimuli could not be tested during intracellular recording, preventing analysis of single viscerofugal neuron responses to mechanical stimuli. Extracellular recordings of viscerofugal neuron axons, located in colonic or mesenteric nerve trunks, would allow investigation of the effects of mechanical and pharmacological stimuli at a single neuron level. Many extracellular electrophysiological recordings from mesenteric nerve trunks have been made to study vagal and spinal afferent neurons innervating the intestines (9-14). Despite this, no neuronal firing activity has been identified which could be unequivocally attributed to viscerofugal neurons. It is not known whether viscerofugal axons contribute to extracellular recordings of nerve activity in colonic/mesenteric nerve trunks. We

hypothesized that: (a) selective ablation of severed extrinsic nerve fibers in colonic nerve trunks can be achieved in isolated gut preparations during organotypic culture, leaving viscerofugal axons intact; (b) that this would permit extracellular recordings from identified viscerofugal neurons, and; (c) individual viscerofugal neuron firing activity and responses to mechanical stimuli can be characterized.

Materials and Methods

Dissection and extracellular recording setup

Adult guinea pigs, weighing 200-350g, were killed by stunning and exsanguination as approved by the Animal Welfare Committee of Flinders University. Segments of distal colon (>20mm from the anus) and attached mesentery were removed and immediately placed into a Sylgard-lined petri dish (Dow Corning, Midland, MI) filled with oxygenated Krebs solution at room temperature. Krebs solution contained (mM): NaCl 118; KCl 4.7, $NaH_2PO_4 2H_2O$ 1; $NaHCO_3$ 25; $MgCl_2 6H_2O$ 1.2; D-Glucose 11; $CaCl_2.2H_2O$ 2.5; bubbled with 95%O_2 and 5%CO_2. Segments were cut open along the mesenteric border, pinned flat with the mucosa uppermost. In organ-cultured and acute control preparations, the mucosa and submucosa were removed by sharp dissection. Extrinsic nerve trunks (1-3 trunks per preparation, 3-10mm long) and a strand of connective tissue were dissected free from surrounding mesentery.

Organ-cultured preparations were maintained in sterile culture medium (Dulbecco's modified Eagle's [DME]/Han's F12, Sigma [1:1 ratio mix, supplemented with L-glutamine and 15 mM HEPES]; including 10% fetal bovine serum, 1.8 mM $CaCl_2$, 100 IU/ml penicillin, 100 g ml^{-1} streptomycin D, 2.5 g ml^{-1} amphotericin B, 20 g ml^{-1} gentamycin, Cytosystems, Castle Hill, NSW, Australia) and slowly agitated for 4-6 days in a humidified incubator (36°C, 5% CO_2 in air) (15). Culture medium was replaced every 24 hours. During electrophysiological recordings, preparations were superfused with Krebs solution (35°C). Acute control preparations were set up for recording or biotinamide tracing immediately after dissection.

Dissected nerve trunks and connective tissue were pulled into a paraffin oil-filled chamber (1mL volume) under a coverslip and sealed with silicon grease (Ajax Chemicals, Sydney,

Australia) as described previously (16). Differential extracellular recordings were made between a nerve trunk and the connective tissue strand using 100µm Pt/Ir electrodes. Signals were amplified (ISO80; WPI, Sarasota, FL, USA) and recorded at 20kHz (MacLab16sp, LabChart 7, ADInstruments, Castle Hill, NSW, Australia); single units were discriminated by amplitude, duration and spike shape using Spike Histogram and Scope View software (ADInstruments). In some preparations, a 10mm array of hooks (Biomedical Engineering, Flinders Medical Centre, Bedford Park South Australia) connected the preparation to an isotonic transducer (Harvard Bioscience, model 52-9511, S. Natick, MA, USA), allowing distending loads to be applied in the circumferential axis while measuring changes in length. Sensitivity to transient focal tissue compression was assessed with calibrated von Frey hairs (100-300mg). Ca^{2+}-free Krebs solution (6mM Mg^{2+}, 1 mM ethylenediaminetetraacetic acid [EDTA]) was used to differentiate direct and indirect effects.

Biotinamide labelling

A bubble of biotinamide solution (5% biotinamide (N-[2-aminoethyl] biotinamide hydrobromide), dissolved in artificial intracellular solution (150 mmol L^{-1} monopotassium L-glutamic acid, 7 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} glucose, 1 mmol L^{-1} ethylene glycolbis(ß-aminoethyl ether)-N,N,N=,N=-tetraacetic acid, 20 mmol L^{-1} HEPES buffer, 5 mmol L^{-1} disodium adenosine triphosphate, 0.02% saponin, 1% dimethyl sulfoxide, 100 IU mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 20g mL^{-1} gentamycin sulphate) was placed on a dissected nerve trunk and the main chamber was filled with sterile culture medium (17). Preparations were incubated overnight (12-16 hours; 36°C, 5% CO_2 in air). After incubation, preparations were fixed overnight in Zamboni's fixative (15% saturated picric acid, 2% formaldehyde in 0.1 M phosphate buffer, pH 7.0). Preparations were cleared in DMSO (3 x 10 minute washes) then washed in 0.1M phosphate-buffered saline (0.15 M NaCl, pH 7.2; 3 x 10

minute washes) followed by incubation for 3 hours in 3-1-*O*-(2-cyanoethyl)-(*N,N*-diisopropyl)indo-carbocyanine (CY3) conjugated streptavidin. Preparations were then washed with PBS (3 x 10 minute) and equilibrated in a series of carbonate-buffered glycerol solutions (50, 70 and 100% solutions; 3 x 10 minutes) prior to mounting on glass slides in buffered glycerol (pH 8.6).

Image analysis

Biotinamide-labelled nerves were viewed and analysed on an Olympus IX71 epifluorescence microscope fitted with an appropriate dichroic mirror and filter. Images were captured with a Roper Scientific Photometrics digital camera operating with a HP Compaq dc7100 CMT computer with a Microsoft Windows XP operating system, running AnalySIS 5.0 software (build 1153). Figures 1G & 1H were acquired with a Leica SP5 scanning confocal microscope (Leica Microsystems, Mannheim, Germany). CY3 fluorophores were excited with 561nm laser light. Emitted photons (565-645nm) were captured with a photon multiplier tube, pinhole set to 1 Airy unit. Laser power, photon multiplier tube gain and offset were adjusted as required. Z-stacks were taken with a 63x oil-immersion lens at 0.5µm slices through the Z-axis. Brightness and contrast adjustments, cropping, pseudocolouring and photomontages of biotinamide labelled preparations were performed using Adobe Photoshop (CS1, Adobe Systems Inc, San Jose, CA).

Quantification of viscerofugal axons in nerve trunks

In organ-cultured and acute preparations, biotinamide-labelled viscerofugal cell bodies, as well as all biotinamide-filled axons were visualized on an epifluorescence microscope and counted to obtain the percentage of viscerofugal axons among all labelled axons. Axons were

counted where bundles of mesenteric nerves spread out as they enter the myenteric plexus, taking care to focus throughout the depth of each trunk.

Immunohistochemistry

Preparations were incubated with primary antibodies for 16–72 hours at room temperature, rinsed with phosphate-buffered saline and incubated with secondary antibodies for 2–4 hours, mounted and analysed as described above. Primary Antibodies were as follows: Rabbit anti-CGRP (Peninsula, cat. no. IHC6006) used at 1:1600, Mouse anti-TH (Diasorin, cat. no. 021048) used at 1:600. Secondary Antibodies: CY3 – Donkey anti-rabbit (Jackson, cat. no. 74548) used at 1:400, CY5 – Donkey anti-mouse (Jackson, cat. no. 86275) used at 1:100.

Drugs

Stock solutions of drugs were made as follows: 10^{-2}M nicardipine hydrochloride in water (Sigma; N7510), 10^{-3}M hyoscine hydrobromide in water (Sigma; S0929), 10^{-1}M hexamethonium chloride in water (Sigma; H2138), 10^{-1}M 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) in water (Sigma; D5891), $3x10^{-4}$M tetrodotoxin (TTX) in water (Alomone; T-500), 10^{-2}M N-Vanillylnonanamide (synthetic capsaicin) in ethanol (Sigma; V9130). All drugs were kept refrigerated and diluted to working concentrations in Krebs solution, shortly before use.

Statistical analysis

Statistical analysis was performed by Student's two-tailed t-test for paired or unpaired data or by repeated measures analysis of variance using Prism v.5 software (GraphPad Software, Inc., San Diego, CA, USA). Differences were considered significant if $P<0.05$. Results are

expressed as mean ± standard deviation except where otherwise stated. The number of animals used in each set of experiments is indicated by lower case “n”.

Results

Rapid biotinamide filling of colonic nerves in acute and organ-cultured preparations

Flat sheet preparations of guinea-pig distal colon (1-2cm in length, mucosa and submucosa removed) were maintained in organotypic culture for 4-5 days to determine whether severed axons of spinal afferent and sympathetic efferent neurons would degenerate during this period. Biotinamide filling of colonic nerve trunks in acute preparations (freshly removed from animal, n=6) revealed viscerofugal cell bodies and dense labelling of fine branching varicose fibers of spinal afferent and sympathetic neurons, see figure 1E (17). The same protocol was then applied to 6 preparations after culture (4-5 days). In these preparations, the density of biotinamide-labelled fibers was considerably reduced, and most remaining fibers could be traced to viscerofugal neuron cell bodies (figure 1F). This suggested that viscerofugal neurons persisted in culture, while extrinsic nerve fibers had degenerated. To quantify this effect, biotinamide labelled axons, and viscerofugal cell bodies, were counted (see methods) to obtain the proportion of axons that belonged to viscerofugal neurons. In acute preparations (n=6), viscerofugal axons were a minority, comprising 8.6±4.2% of all filled axons within labelled nerve trunks. After culture, the proportion of viscerofugal axons of labelled axons in colonic nerve trunks increased to a majority of 67.1±13.8% (n=6, $p<0.001$), confirming that viscerofugal neurons and their axons persisted in organ culture and were considerably enriched as a proportion of all surviving axons.

Immunohistochemistry

The most likely explanation for the increased proportion of biotinamide-labelled viscerofugal neuron axons after organ culture was that spinal afferent neurons and symapthetic neurons degenerated, while intrinsic neurons, including viscerofugal neurons, persisted (15). To test this, preparations were immunohistochemically labelled for common neurochemical markers of sympathetic and spinal sensory axons: tyrosine hydroxylase (TH), and calcitonin gene-related peptide (CGRP), respectively. In control preparations, fixed shortly after removal from the animal, a dense network of TH-immunoreactive varicose branching axons was visualised within myenteric ganglia (figure 1C). There was also an abundance of CGRP-immunoreactive varicose axons (figure 1A), previously shown to belong to spinal afferent neurons (18). In organ-cultured preparations, TH-immunoreactive axons were nearly abolished (figure 1D). Likewise, CGRP-immunoreactivity was greatly diminished in organ-cultured preparations (figure 1B); except for occasional intrinsic CGRP-immunoreactive cell bodies seen in 2 out of 4 preparations. In organ-cultured preparations, no colocalization of biotinamide-labelled axons with either CGRP or TH-immunoreactivity was evident. These results suggest that 5 days in organ culture was sufficient to cause nearly-complete degeneration of extrinsic axons in these preparations.

Cell body morphology

The degeneration of extrinsic axons in organ culture allowed examination of the detailed morphology of biotinamide-labelled viscerofugal nerve cell bodies, without the complication of a dense network of labelled axons surrounding the cell. A total of 78 retrogradely labelled cell bodies in organ-cultured preparations were examined in this way. In 36 cells, their axons could be unequivocally distinguished from other labelled axons-of-passage. All of these cells were uni-axonal (examples in figure 1G & 1H) and either had lamellar dendritic Dogiel type I

morphology or a few short filamentous and/or lamellar dendrites typical of “small simple cell” Dogiel type I morphology (19). No cells had two axons emerging from the cell body; thus none had Dogiel type II morphology. The remaining 42 cells could not be unequivocally classified due to the proximity of processes from other neurons or due to faint labelling. It is worth noting that viscerofugal neuron cell bodies were always labelled by biotinamide applied to colonic nerves after organ culture (average: 27±22 per preparation). Variability between preparations in the number of retrogradely traced cell bodies (range 4-61) probably reflects an uneven distribution between extrinsic nerve trunks (20).

Electrophysiology

Extracellular recordings of colonic nerve trunks were made from 12 preparations maintained for 5-6 days in organ culture (n=8). Spontaneous action potentials were recorded in all preparations, from which 14 single units could be readily discriminated by spike amplitude and duration (mean firing rate 4.0±1.9Hz, 14 units, n=7). Units in 10 of 12 preparations showed regular spontaneous bursts of firing (inter-burst interval 2.4±0.2s, burst duration 190±6ms). Bursts of firing involved several single units, distinguishable by spike amplitude and duration, suggesting synchronization of firing in pools of viscerofugal neurons. Single units fired an average of 5.4±2.3 action potentials within a burst (mean instantaneous frequency 34±15Hz). In 4 units from 4 preparations, firing was regular without bursts (4/12 preparations). Typical examples are shown in figure 2A. All firing was abolished by tetrodotoxin applied in the organ bath (1μM, 3/3 preparations tested).

Capsaicin activates 85% of medium-high threshold spinal afferent neurons innervating the gut wall (13). Capsaicin (0.4μM) reliably evoked robust firing responses from many units in colonic nerves of freshly dissected, control preparations (5/5 preparations). In contrast, the

same concentration of capsaicin evoked no change in firing in organ-cultured preparations (8/8 tested; figure 2C), consistent with degeneration of severed axons of extrinsic sensory neurons during 5-6 days organ culture.

Viscerofugal neurons in the guinea-pig colon have been reported to express nicotinic receptors (2, 8, 21). Application of the nicotinic receptor agonist, DMPP (10^{-4}M) in the recording chamber increased firing up to 50Hz in colonic nerves from organ-cultured preparations (mean 37.9±8.6Hz; 10 units, n=6, figure 2B). Responses persisted when smooth muscle was paralysed with nicardipine (1µM) and hyoscine (1µM), indicating that increases in firing rate was not a result of muscle contraction. All responses were blocked by pre-administration of 400µM hexamethonium (12 units, n=7)

The nicotinic receptor antagonist, hexamethonium (400µM) significantly reduced, but did not abolish, spontaneous firing in 7 organ-cultured preparations (12 units, n=7, $p<0.01$; figure 3*A* & 3*B*). Hexamethonium abolished bursts of firing activity (5/5 preparations; example figure 3C), suggesting that nicotinic receptors were required to synchronize and drive multi-unit burst-firing.

Spontaneous contractions

Preparations kept in organ culture for 5-6 days showed irregular spontaneous contractions of the circular smooth muscle (Δlength 2.2±1.4mm, 23 contractions, mean frequency 0.8±0.2 per min during active periods, n=5). Large bursts of viscerofugal neuron firing preceded the onset of all spontaneous contractions by 2.3±2.1s, (23/23 contractions, 7 units, n=5). These bursts were significantly longer than regular bursts (burst duration 1.91±0.3s, $p<0.001$ paired t-test; figure 4*A*). Overall, average firing rate in the 5 seconds preceding spontaneous contractions

(11.5±5.0Hz) was significantly greater than both mean basal firing (5.2±3.2Hz) and mean firing during contractions (4.0±3.0Hz, 7 units, n=5, $p<0.001$ 1 way ANOVA). This suggests that viscerofugal neurons are activated, prior to contraction, by enteric neuronal circuits that subsequently cause spontaneous smooth muscle contractions.

Circumferential stretch

The effects of circumferential stretch on firing in organ-cultured preparations were examined (1-3g, 8 preparations, 9 units, n=6). In some cases (10/35 stretches), distension evoked reflex contractions of the circular muscle. On these occasions, large bursts of firing preceded contractions (by 2.75±0.52s) (figure 4*B*). There was a significant association between these large bursts of firing (defined by being more than 1s long with firing rates exceeding 10Hz) and stretch-evoked contractions ($X^2=11.8$, df=1, $p<0.001$, using Yates' correction for continuity). A summary of the effect of distension is shown in table 1 and a typical example is shown in figure 4*B*. Firing was also increased when distension failed to evoke a measureable reflex response. Overall (including all stretches, regardless of whether a contraction was evoked), stretch caused a significant load-dependent increase in firing rate of viscerofugal neurons (9 units, n=6, $p<0.05$, 1 way ANOVA; figure 4C).

Focal tissue compression

In a further series of experiments, single viscerofugal units were assessed for sensitivity to focal tissue compression by von Frey hairs (100-300mg). In 5 preparations (n=5), 11 of 11 recorded single units promptly discharged a train of action potentials to focal probing of a single site of about 200µm diameter on the preparation in normal Krebs (11/11 units tested), which persisted in 400µM hexamethonium (4/4 units tested, n=1) or Ca^{2+}free Krebs (7/7 units

tested, n=4). Thus, probing resulted in direct mechanosensory firing responses, probably transduced at a single cell body (typical firing response shown in figure 4D).

Discussion

In this study, we have developed a preparation to preserve functional viscerofugal neurons but selectively degenerate CGRP positive spinal nerve trunks that innervate the distal colon of guinea-pigs. Several lines of evidence indicate that viscerofugal neurons were recorded in this study. First, viscerofugal cell bodies were always revealed by biotinamide applied to colonic nerves in both cultured and acute preparations. After organ culture, viscerofugal axons constituted the majority of all surviving axons in colonic nerve trunks. The common markers for extrinsic spinal afferent neurons and sympathetic efferent neurons (CGRP and TH, respectively) were almost entirely depleted by 5 days organ culture apart from a few intrinsic neurons containing CGRP. This strongly implicates degeneration of severed extrinsic fibers, including spinal afferent neurons. Correspondingly, the absence of responses to capsaicin, which activates spinal afferent neurons (22), provides further support that extrinsic mechanosensitive nerves were depleted. Sensitivity to nicotinic receptor agonists, regardless of the contractile state of the gut, is consistent with previous reports of viscerofugal neuron pharmacology (2), which have been shown immunohistochemically to express nicotinic receptors (21). Finally, the punctate mechanosensitive sites revealed by von Frey hairs in this project are consistent with responses by isolated viscerofugal neurons but not with the extensive fields of innervation of extrinsic sensory neurons in the gut wall. Based on these data, it is reasonable to conclude that viscerofugal neurons were the source of the action potentials recorded from colonic nerve trunks in cultured preparations.

Viscerofugal neuron firing and motor activity

Burst firing patterns in viscerofugal neurons has not previously been reported in any electrophysiological studies. Previous extracellular recordings in the small intestine of enteric neurons, in vitro, identified "burst" and "single spike" units (23). Burst-type units were sub-classified into "steady" and "erratic" bursters based on the variability of their inter-burst interval (24). The activity of the latter type was blocked in Ca^{2+}-free solution while the former was not (24). From the present study, viscerofugal neurons are similar to 'erratic bursters' with inter-burst interval and the number of action potentials within each burst similar to Wood's report (2.9±1.4s and 4.2±1.4 action potentials per burst (25), and 2.4±0.2s inter-burst interval and 5.4±2.3 actions potentials in the present study).

In vitro, the guinea pig colon demonstrates irregular ongoing activation of motor pathways to both circular and longitudinal muscle layers (26), driven by myenteric neuronal circuitry which is largely hexamethonium-sensitive (27). This activity cycles at 2 – 5 s intervals. We speculate that burst firing of viscerofugal neurons may reflect synaptic input from the same motor circuits. This would also explain why viscerofugal neuron firing typically increased before spontaneous contractions of smooth muscle. It may also explain why the peak response to distension was reached after the onset of stretch just prior to a reflex contraction evoked by the stretch. Compatible with this, Miller and Szurszewski (2002 (28)) showed that fast synaptic inputs (from viscerofugal neurons) to sympathetic ganglion neurons peaked prior to phasic contractions of the intestine with reduced firing at the peak of contraction (when the intestine was empty and the circumference was shortened). The amplitude of viscerofugal neuron responses to stretches that did not evoke reflex contractions was modest, rarely doubling above the basal firing rate. This modest response amplitude is comparable to the small increase in frequency of synaptic fast potentials recorded in inferior mesenteric ganglion of the guinea pig in response to colonic distension (5, 6, 29-31). With the largest

stimulus applied, the peak firing was less than 8Hz, compared to basal ongoing firing close to 4 Hz in the absence of distension.

Dual roles of viscerofugal neurons

Distension activates robust cholinergic pathways from the gut to sympathetic pre-vertebral ganglion neurons, mediated via viscerofugal neurons. When nicotinic receptors in the gut wall are pharmacologically blocked, distension-evoked viscerofugal synaptic input to sympathetic neurons is significantly depressed (2). This suggests that viscerofugal neurons may be synaptically driven via cholinergic pathways, similar to many other classes of enteric neurons (32-35). However, some viscerofugal output from the gut persists, even when synaptic transmission in the gut is entirely blocked by Ca^{2+}-depleted solution (5-7). This has been interpreted as evidence that viscerofugal neurons may also be directly mechanosensitive. However, it has also been suggested that fast cholinergic synaptic inputs in prevertebral ganglia may also arise from collaterals of spinal afferent neurons (36). The present study suggests that viscerofugal neuron cell bodies are directly mechanosensory. When tested, direct, localised responses to von Frey hairs (100-300mg) occurred in all viscerofugal units identified in our recordings at a single restricted site on the preparation. The observation that some enteric neurons are both mechanosensitive and receive synaptic input is not unprecedented. Recordings using voltage-sensitive dyes suggest that many enteric interneurons and motor neurons with fast synaptic inputs (S/Type I cells) may function as rapidly adapting mechanosensors to physiological stimuli (37). In addition, slowly-adapting Dogiel type 1 “sensory interneurons” in the guinea pig distal colon are both mechanically and synaptically activated by ongoing distension of the gut wall (38).

Subtypes of viscerofugal neurons

Retrograde tracing studies, using biotinamide or fast blue, or intracellular dye filling with biocytin, suggest that viscerofugal neurons are uniaxonal with smooth or irregular shaped cell bodies and short lamellar or filamentous dendrites (8, 17, 39-45). A study using DiI and intracellular filling with Lucifer Yellow in guinea pig colon revealed a subset (30%) of multiaxonal viscerofugal neurons, although the majority were uniaxonal (21). In the present study, degeneration of sympathetic and sensory fibers in organ culture allowed observation of large numbers of biotinamide-filled viscerofugal neurons uncomplicated by surrounding nerve fibers. All appeared to be uni-axonal and we were not able to distinguish clear morphological subtypes. The discrepancy in morphological identification may be related to the type of tracer used (DiI labelling tends to be punctate and lucifer yellow gives less complete fills that biotin derivatives) (46), or differences between strains of guinea pigs.

Conclusion

The present study has demonstrated that action potentials of enteric viscerofugal neurons can be recorded from colonic nerves in organ-cultured tissue. Viscerofugal neurons appear to function as both mechanosensory neurons and interneurons. Future studies in acute preparations are warranted to identify and characterize the optimal mechanical stimuli for viscerofugal activation, their proximal enteric neuronal inputs, and to determine whether all, or just a subset of viscerofugal neurons are capable directly transducing mechanical stimuli.

Authors' contributions

Experiments were planned and designed by SJHB and TJH, with advice and assistance from NJS and VPZ and carried out primarily by TJH. Data analysis, presentation and writing was carried out by all four authors.

Acknowledgments, funding, and disclosures

We would like to acknowledge the assistance of Ms Bao Nan Chen with many aspects of this project, Dr. Jennifer Clark for assistance with confocal microscopy, and Mr. Dale Sharrad for assistance with immunohistochemistry. TJH was supported by a Flinders University Research Scholarship. This work was also supported, in part, by NHMRC project grant #595969 to SJHB and NJS. No competing interests declared.

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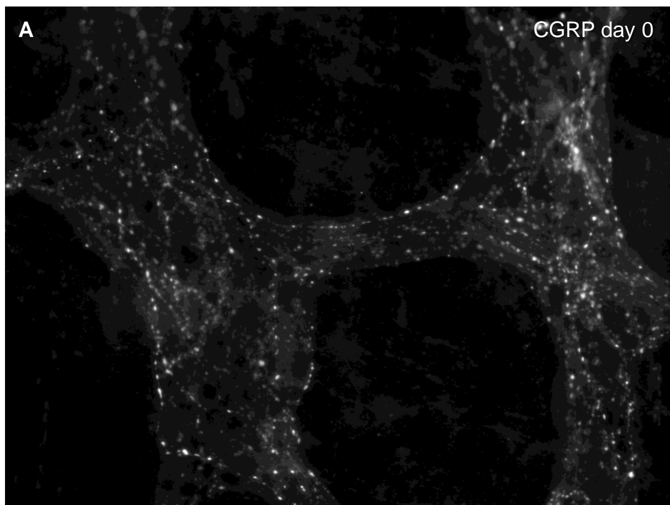
A
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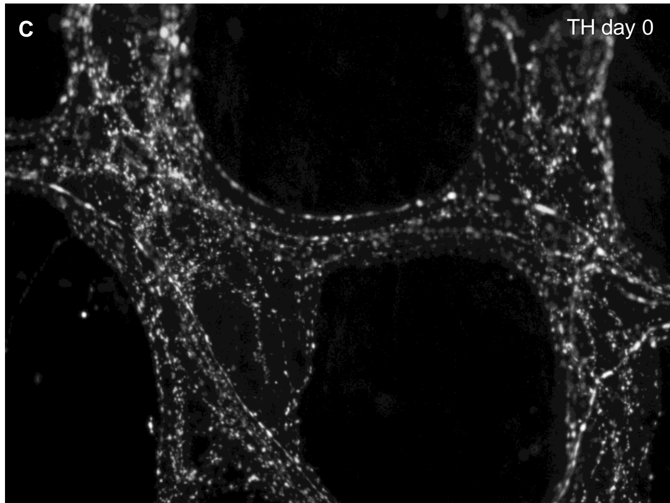
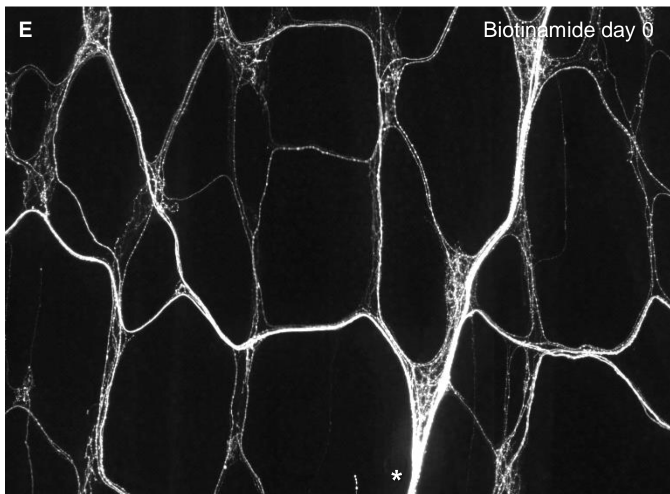
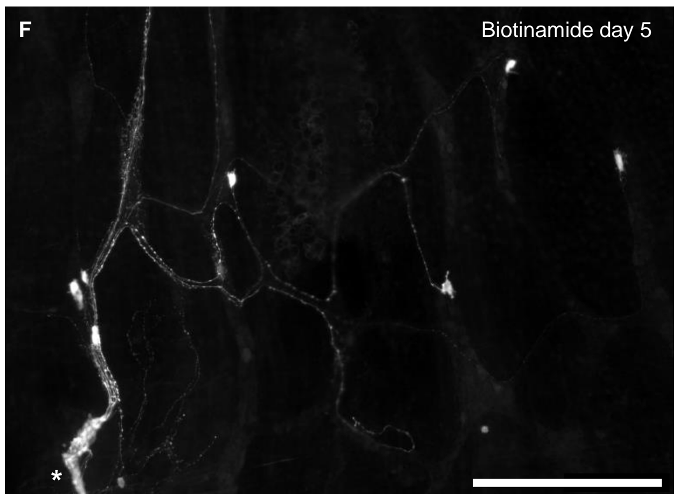
B
CGRP day 5


C
TH day 0

D
TH day 5


E
Biotinamide day 0
*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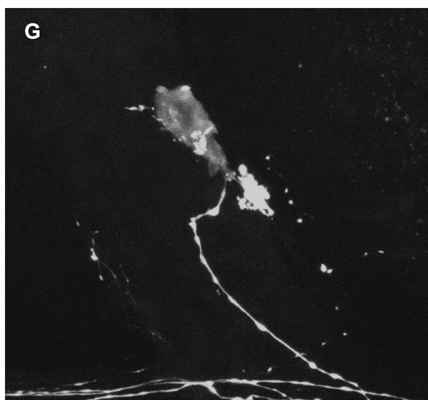
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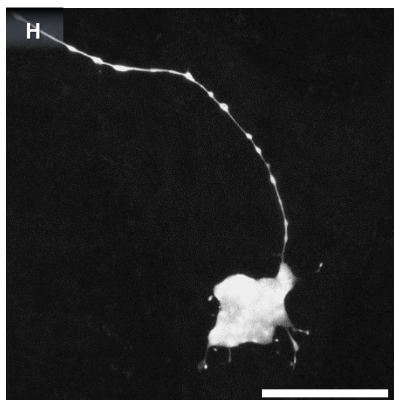
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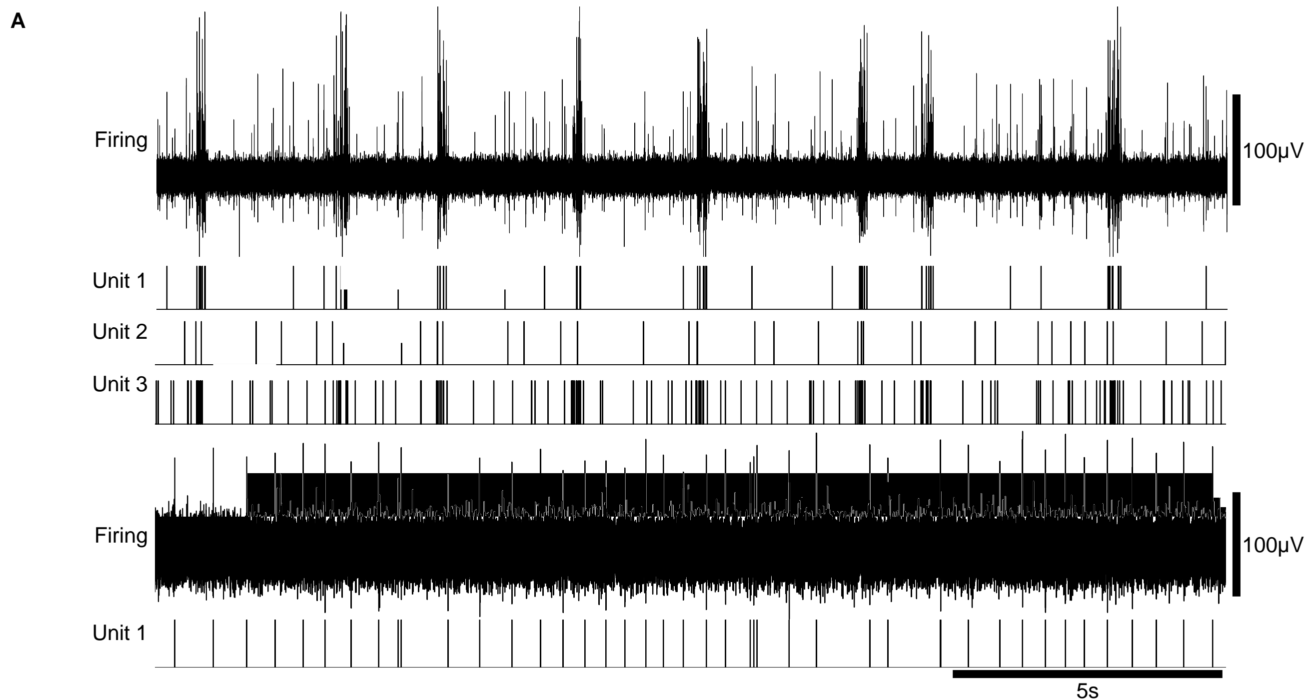
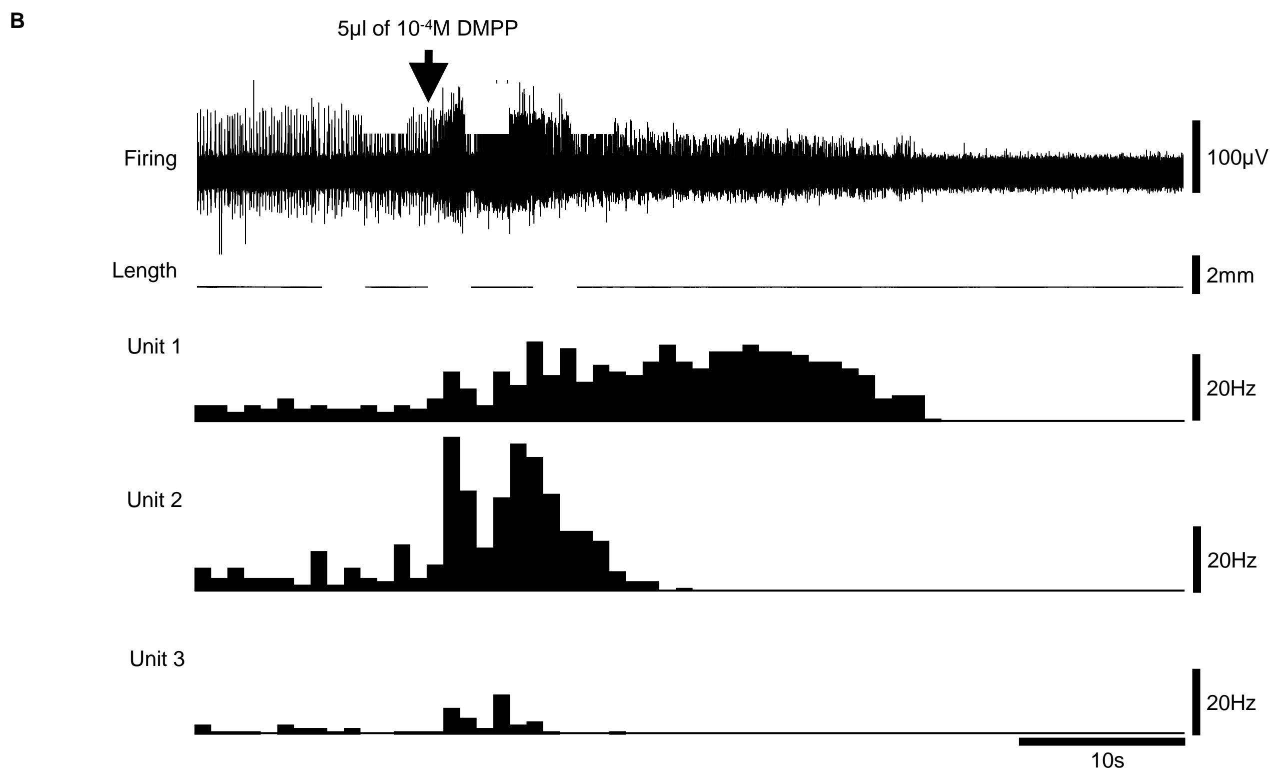
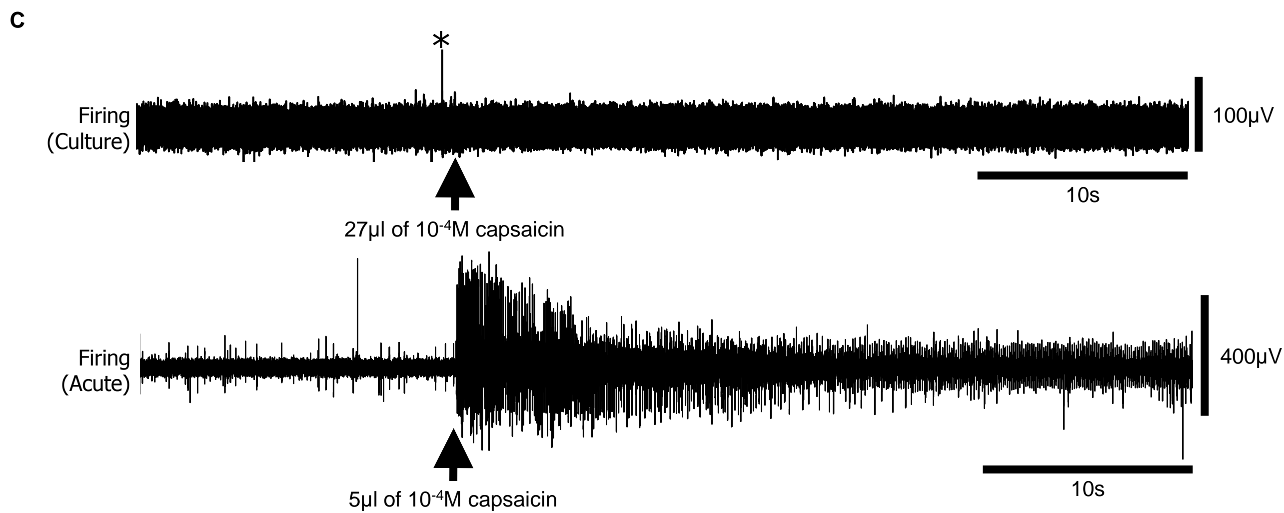
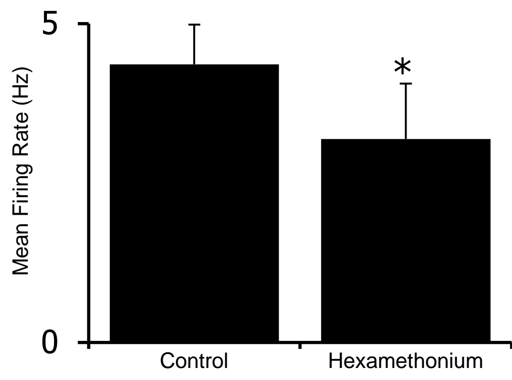

A
Firing
100μV
Unit 1
Unit 2
Unit 3
Firing
100μV
Unit 1
5s


B
5μl of 10-4M DMPP
Firing
100μV
Length
2mm
Unit 1
20Hz
Unit 2
20Hz
Unit 3
20Hz
10s


C
*
Firing
(Culture)
100μV
10s
27μl of 10-4M capsaicin
Firing
(Acute)
400μV
5μl of 10-4M capsaicin
10s

A



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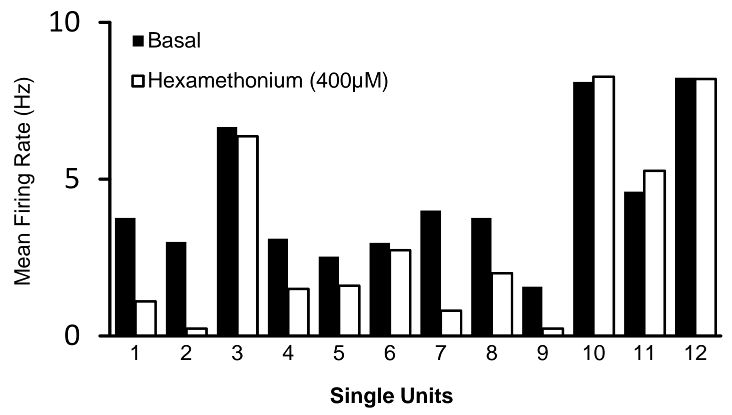

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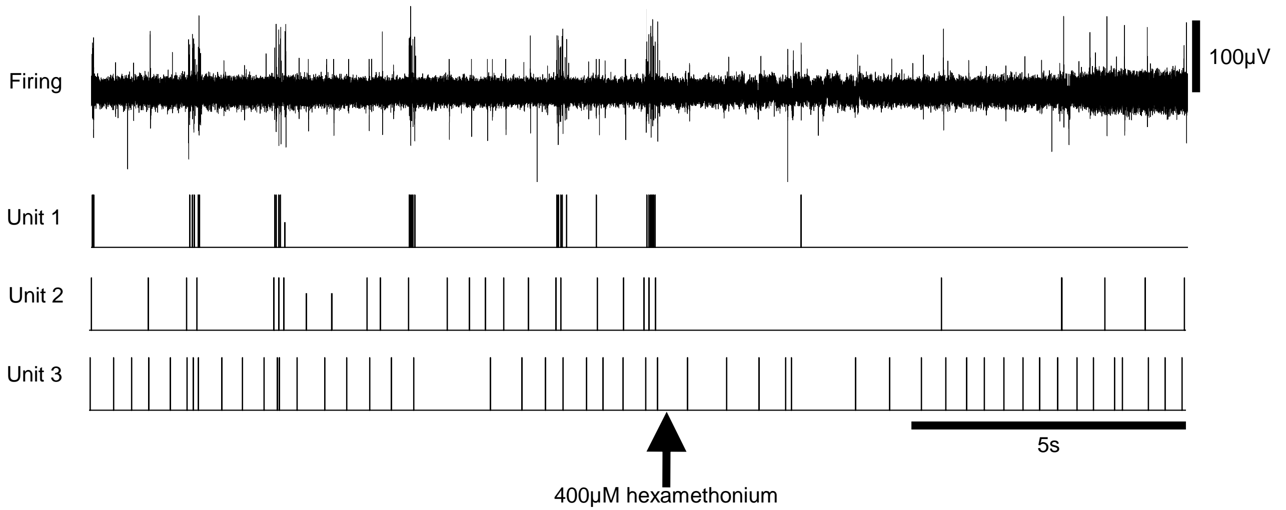

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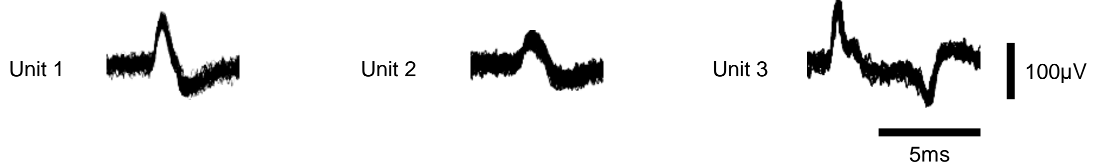


A

Firing

100μV

Length

1mm

Unit 1

20Hz

Unit 2

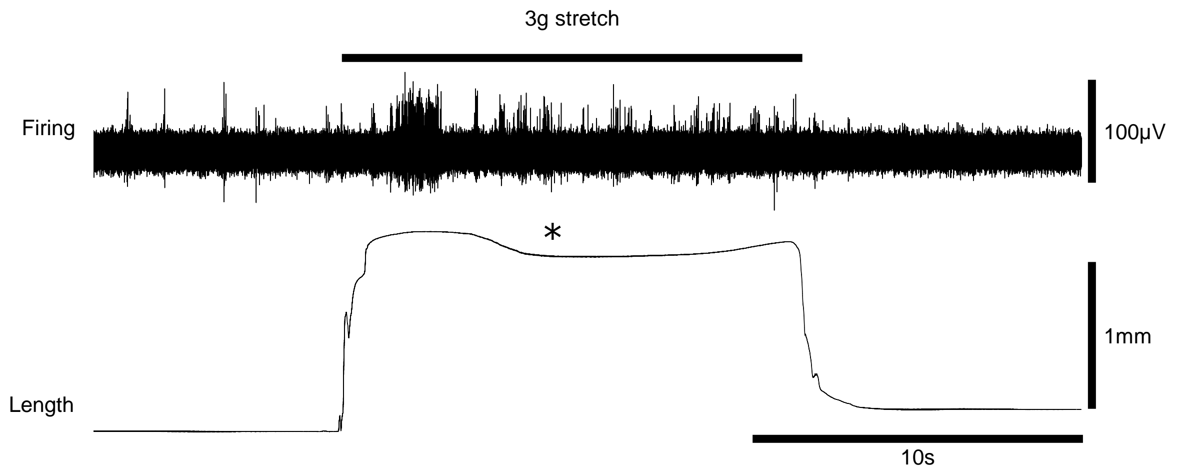
20Hz

Unit 3

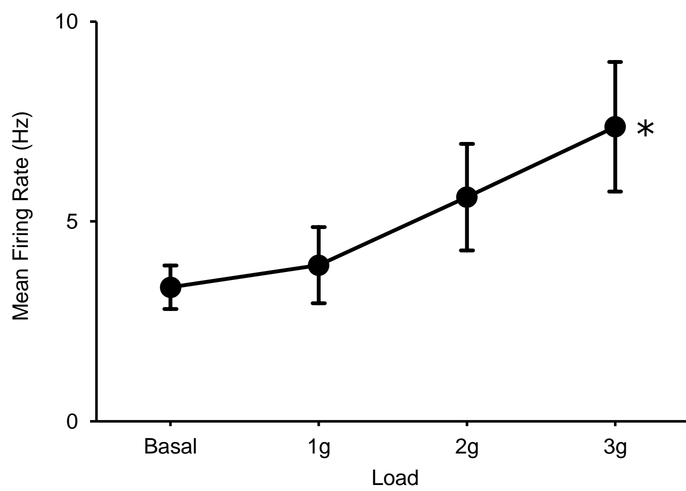
20Hz

10s

B



C



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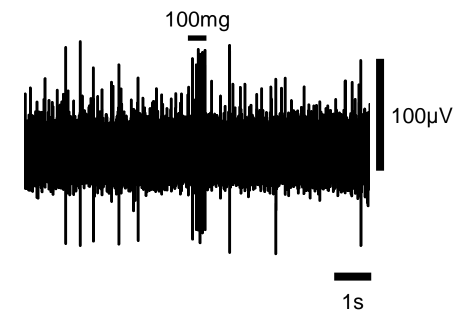

Tables

Table 1 – The effect of circumferential stretch on evoked contractions of the circular muscle and occurrence of 'large burst' firing.

Stretches	'Large burst' firing (+)	'Large burst' firing (−)
Contraction (+)	8	3
Contraction (−)	2	21