**Measurement of strains experienced by viscerofugal nerve cell bodies during mechanosensitive firing using Digital Image Correlation**

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**Running Head:** DIC analysis of strain-sensitivity in viscerofugal neurons

**Word count** (excluding references and figure legends): 5124

AUTHOR CONTRIBUTIONS

GP and TR carried out the DIC analysis and interpretation

TJH made the recordings of viscerofugal neurons

TJH, SJHB and MT planned the study, designed experiments and assisted with analysis and interpretation.

All authors contributed to the preparation of the manuscript.

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ABSTRACT

Mechanosensory neurons detect physical events in the local environments of the tissues that they innervate. Studies of mechanosensitivity of neurons or nerve endings in the gut have related their firing to strain, wall tension or pressure. Digital Image Correlation (DIC) is a technique from materials engineering that can be adapted to measure the local physical environments of afferent neurons at high resolution. Flat sheet preparations of guinea pig distal colon were set up with arrays of tissue markers, in vitro. Firing of single viscerofugal neurons was identified in extracellular colonic nerve recordings. The locations of viscerofugal nerve cell bodies were inferred by mapping firing responses to focal application of the nicotinic receptor agonist, DMPP. Mechanosensory firing was recorded during load-evoked uni-axial or bi-axial distensions. Distension caused movement of surface markers which was captured using video imaging. DIC tracked the markers, interpolating the mechanical state of the gut at the location of the viscerofugal nerve cell body. This technique revealed heterogeneous load-evoked strain within preparations. Local strains at viscerofugal nerve cell bodies were usually smaller than global strain measurements and correlated more closely with mechanosensitive firing. Both circumferential and longitudinal strain activated viscerofugal neurons. Simultaneous loading in circumferential and longitudinal axes, caused the highest levels of viscerofugal neuron firing.Multiaxial strains, reflecting tissue shearing and changing area, linearly correlated with mechanosensory firing of viscerofugal neurons. Viscerofugal neurons were mechanically sensitive to both local circumferential and local longitudinal gut strain, and appear to lack directionality in their stretch sensitivity.

**Keywords:** mechanosensory, afferent, strain, biomechanics, viscerofugal, digital image correlation

NEW AND NOTEWORTHY

High-resolution, non-contact optical measurement of gut strain (digital image correlation) was combined with neurophysiological recordings of viscerofugal neurons, for the first time. Isotonic circumferential and longitudinal distensions activated viscerofugal neurons. Distensions evoked heterogeneous strain in gut preparations. Local strain measured at viscerofugal nerve cell bodies correlated closely with stretch-evoked firing. Accounting for strain in multiple axes increased the correlation with viscerofugal neuron firing. Digital image correlation can be usefully combined with gastrointestinal neurophysiology.

Mechanosensitivity has been demonstrated in several types of enteric neurons ([23](#_ENREF_23), [24](#_ENREF_24), [33](#_ENREF_33), [36](#_ENREF_36)). These include the tension-sensitive enteric primary afferent neurons ([19](#_ENREF_19)); rapidly adapting mechanosensitive enteric neurons ([25](#_ENREF_25), [26](#_ENREF_26)); length-sensitive S-neurons ([38](#_ENREF_38)); and enteric viscerofugal neurons ([11](#_ENREF_11), [12](#_ENREF_12), [34](#_ENREF_34)). Mechanosensitive neurons are activated by deformation of the local tissue that surrounds their mechanosensitive transduction sites ([12](#_ENREF_12), [26](#_ENREF_26)). Deformation is transduced into ionic currents via mechanosensitive ion channels, which in turn drive action potential discharge ([5](#_ENREF_5)). Thus, information about the local mechanical environment at transduction sites is encoded in firing frequency. The firing behaviour of many types of mechanosensitive neurons has been recorded in detail using conventional electrophysiological techniques. However, high resolution measurements of the mechanical state of the receptive field have been challenging. In studies of intact, tubular preparations of gut, gross intraluminal volume and/or pressure have typically been recorded. In flat sheet preparations, average length/strain or tension across an entire preparation have been recorded, but these average measures may not reflect the local mechanical conditions within the receptive field ([22](#_ENREF_22)).

Digital image correlation (DIC) is a non-contact optical technique that allows full field estimation of deformations and strains of a structure subjected to load. DIC measurements are based on a set of images of a specimen in an undeformed state (the reference state), and in deformed states. The reference image is divided into regions. A matching algorithm is used to track features or textures of the reference image in the subsequent deformed images. The displacement can then be calculated, followed by the strains. The technique may be applied using any imaging system and at any length scale. This technique enables the mechanical state of areas within samples, including biological tissues, to be determined with high precision ([30](#_ENREF_30), [31](#_ENREF_31)). We tested whether the DIC method could be adapted for flat sheet preparations of gut, during neurophysiological recordings from mechanosensitive enteric neurons. Behaviour of individual mechanosensitive enteric neurons could thus be related to their local mechanical environments within receptive fields, rather than to lower resolution, parameters averaged across larger areas of the preparation.

Enteric viscerofugal neurons have nerve cell bodies in myenteric ganglia and their axons exit the gut, synapsing with prevertebral sympathetic neurons ([40](#_ENREF_40)). They are involved in extrinsic reflex control of motility ([41](#_ENREF_41)) and secretion ([32](#_ENREF_32)). Their cell bodies typically have simple morphology, with absent or very short dendritic processes, and are directly mechanosensitive ([12](#_ENREF_12)). Their spatially restricted receptive fields make them ideal for high resolution analysis of mechanosensitivity.

In this study, we recorded discharge of single enteric viscerofugal neurons using conventional extracellular recording techniques and mapped the locations of their nerve cell bodies, using a method previously shown to be very reliable ([12](#_ENREF_12)). Synaptic activation of viscerofugal neurons was prevented by use of low [Ca2+], high [Mg2+] solution ([34](#_ENREF_34)), which also abolished muscle contractility. Mechanically-evoked firing was recorded during circumferential and longitudinal gut distensions by applied, fixed loads. DIC was used to determine the local physical conditions acting on viscerofugal nerve cell bodies.

MATERIALS AND METHODS

*Dissection*

Adult guinea pigs (Dunkin Hartley) of either sex, weighing 200-350g, were euthanized 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. The Krebs solution contained (in mM concentrations): NaCl 118; KCl 4.7, NaH2PO4 1; NaHCO3 25; MgCl2 1.2; D-Glucose 11; CaCl2 2.5; bubbled with 95% O2 and 5% CO2. Segments were cut open along the mesenteric border, pinned flat with the mucosa uppermost. 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.

*Extracellular recording setup*

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 ([42](#_ENREF_42)). Conventional differential extracellular recordings were made between extrinsic nerve trunks and connective tissue using 100µm Platinum/Iridium 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 shape using Spike Histogram software (ADInstruments). Longitudinal and circumferential tissue length was measured using two calibrated isotonic transducers (Harvard Bioscience, model 52-9511, S. Natick, MA, USA) coupled to preparations via a pulley and an array of hooks, 5-10mm wide. Distending loads were applied as weights attached to the arm of an isotonic transducer, which recorded from either the longitudinal or circumferential axis (figure 1). Each stretch was maintained for approximately 30 seconds. The smooth muscle was paralysed and all synaptic transmission was abolished by superfusion of oxygenated, calcium-free Krebs solution. Importantly, calcium-free Krebs solution (with raised [Mg++]) does not change the basal firing of viscerofugal neurons or their mechanosensitive firing responses to gut distension ([12](#_ENREF_12)), but abolishes activation via synaptic inputs from other enteric neurons ([34](#_ENREF_34)). Calcium-free Krebs solution was continuously superfused at a rate of ~1.6ml/min, (35°C) and contained (in mM): NaCl 115; KCl 4.7, NaH2PO4 1; NaHCO3 25; MgCl2 6; D-Glucose 11; EDTA 1. A schematic of the experimental set up is shown in figure 1.

*Video micrography*

Preparations were imaged at a rate of 25 frames per second, with a microscope eye-piece camera (Dino-Lite AM423C, AnMo Electronics Corporation, Taiwan). Video was synchronized with extracellular and mechanical recordings (Video Capture, ADInstruments). Carbon graphite markers (Aldrich; 28 286-3) were used as references for localization of viscerofugal neurons and as high-contrast markers for digital image correlation (examples can be seen in figure 2). Markers were applied to preparations using a 100mg (1mN) von Frey hair lightly coated in an evaporated sucrose solution ([42](#_ENREF_42)). To calibrate movement of markers, a ruler was visible in the field of view during video micrography.

*Localisation of viscerofugal nerve cell bodies*

The location of viscerofugal neuron cell bodies was inferred using a mapping technique that has previously been validated ([12](#_ENREF_12)). A bolus of capsaicin was added to the recording chamber to reduce the firing of most extrinsic sensory axons (final bath concentration 0.3µM). A second bolus of capsaicin was applied 5 minutes later to confirm desensitization. The nicotinic receptor agonist, 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) was then applied to small sites, from a glass micropipette (10-20μm tip diameter, pressure ejected using Helium at 140kPA for 10-50ms). This produced a cloud of DMPP solution less than 200µm in diameter which was quickly washed away by the superfusing Krebs solution. At a few sites, DMPP promptly and reliably evoked increases in firing frequency recorded in the colonic nerve. These sites have been shown by anterograde dye filling to reliably correspond to the location of viscerofugal neuron cell bodies ([see 12](#_ENREF_12)). Our previous study showed that viscerofugal nerve cell bodies were on average, located 173 ± 156μm from the centre of DMPP-identified sites (24 cells, n = 9; see: 11). In the present study, the locations of DMPP-identified sites were recorded on a photomicrograph of the preparation. An example of firing evoked by focal DMPP application in this study is shown in figure 2. From the DMPP-evoked firing, we were able to discriminate single viscerofugal neuron firing using commercial software (Spike Histogram, ADInstruments, Castle Hill, NSW, Australia).

*Drugs*

Stock solutions of drugs were prepared as follows: 10-1M 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) in water (Sigma; D5891), 10-2M N-Vanillylnonenamide (synthetic capsaicin) in ethanol (Sigma; V9130). Drugs were kept refrigerated and diluted to working concentrations in Krebs solution, shortly before use.

*Statistics*

Statistical analysis was performed by Student’s two-tailed t-test for paired or unpaired data or by repeated measures analysis of variance (ANOVA, one-way or two-way) using IBM SPSS Statistics 20 for Microsoft Windows (release 20.0.0, IBM Corp., 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 lower case “n”. All analyses of viscerofugal neurons firing rate indicate change in firing rate to control for changes in baseline firing throughout experiments.

*Digital Image Correlation*

Digital image correlation (DIC) is a tool for determining the deformation of materials by applied loads, using tracking and image registration techniques ([31](#_ENREF_31)). Cross-correlation is used to track markers in a set of digital images, using pixel intensity values to identify different regions. While DIC is a popular method in engineering for calculating the deformation of materials, it has not been widely used on soft tissues. DIC requires high-contrast points that can be consistently identified and tracked. Gut tissue typically lacks adequate contrast; we therefore applied graphite markers to the tissue surface, as described above.

Several commercial DIC software products exist, such as VIC-2D ([20](#_ENREF_20)), which is optimised and calibrated to track displacement accurately. However, these products are largely developed for materials with high stiffness values, which give rise to small displacements between video frames, and are not compatible with studies of gut tissue because of its high distensibility. Thus, a DIC script ([8](#_ENREF_8)), developed for use with MATLAB software (MathWorks Incorporated, Natick, Massachusetts, USA), was adapted by the authors of this study. This allowed accurate 2-dimensional tracking of the carbon graphite markers over the relatively large displacements that occur during gut distension. After DIC processing, local circumferential and longitudinal strain was calculated, based on marker displacements.

*Combination strains*

Mechanical activation of viscerofugal neurons could be mediated by strain occurring in multiple axes (circumferentially, longitudinally and transversely). Thus, we compared several different measures of strain with mechanically-evoked viscerofugal neuron firing behaviour. These included 3 measurements of strain in a single plane (the circumferential and longitudinal axes of the gut). They were: first analogue of strain, differential strain, and maximum shear strain. Additionally, measurements of strain that included a calculated z-axis component were assessed (see below). They were: hydrostatic strain, and equivalent strain. The equations used for these are listed in table 1. The first analogue of strain is a calculation of the total combined amount of elongation (or compression) in both the circumferential and longitudinal axes of the gut. Differential strain calculates asymmetry of stretch between two axes. This measure may be expected to correlate closely with firing of neurons sensitive to uniaxial distension. Maximum shear strain calculates relative lateral displacement of the circumferential and longitudinal axes. Like normal strain, shear movement may distort myenteric ganglia. Hydrostatic strain (also known as dilation) and equivalent strain calculate the total volume strain (average strain) and total distortion in the tissue due to shear, respectively. Note that both hydrostatic and equivalent strain include a component of strain in the *z* direction (transverse strain, perpendicular to both the circumferential and longitudinal muscle). This cannot be calculated using 2-dimensional DIC methods; we therefore predicted this z-strain using the linear elasticity relationship:

$σ\_{x}=2Gε\_{xx}+\left(k-^{2}/\_{3}G\right)\left(ε\_{xx}+ε\_{yy}+ε\_{zz}\right)$,

$σ\_{y}=2Gε\_{yy}+\left(k-^{2}/\_{3}G\right)\left(ε\_{xx}+ε\_{yy}+ε\_{zz}\right)$,

$σ\_{z}=2Gε\_{zz}+\left(k-^{2}/\_{3}G\right)\left(ε\_{xx}+ε\_{yy}+ε\_{zz}\right)$,

where $σ\_{x}$, $σ\_{y}$, and $σ\_{z}$ are the stresses in circumferential, longitudinal, and *z* direction respectively, $ε\_{xx}$, $ε\_{yy}$, and $ε\_{zz}$ are the strains in circumferential, longitudinal, and *z* direction respectively,$ G$ is the shear modulus, and $k$ is the bulk modulus. Since the experiments were carried out in plane stress, the stress in the *z* direction is zero. The shear modulus and bulk modulus are calculated using the global stresses and strains only.

RESULTS

*Effect of stretch on viscerofugal neuron firing*

A full set of recordings, in which the whole experimental protocol was completed, was made from 6 single viscerofugal neurons (n = 6). Capsaicin activates 85% of medium-to-high threshold spinal afferent neurons in guinea pig colon ([37](#_ENREF_37)). In all preparations, a bolus of capsaicin was applied directly onto the tissue (final bath concentration 0.3μM). As found previously ([12](#_ENREF_12)), capsaicin evoked bursts of action potentials for up to 90s, followed by quiescence of the activated units, presumably due to desensitization ([12](#_ENREF_12)). Subsequent applications of capsaicin failed to evoke any change in action potential discharge (n = 6). This treatment was used to reduce, as much as possible, the number of distension-sensitive spinal afferent axons that may have contaminated recordings of viscerofugal neuron firing. Following this, synaptic blockade and muscle paralysis were induced by switching the superfusing solution to Ca2+-free Krebs solution with raised [Mg2+]. Viscerofugal neurons were then identified by focally applying DMPP pressure ejected onto small areas of preparation (6 cells, n = 6). Previous studies have shown that viscerofugal neurones respond primarily to increased length of the gut wall, rather than wall tension ([12](#_ENREF_12)). Use of Ca2+-free solution prevented both spontaneous and reflex-induced motor activity that would have complicated analysis. An example of DMPP-evoked firing is shown in figure 2. The basal firing rate of viscerofugal neurons in unstretched preparations was 1.8 ± 1.1Hz (6 cells, n = 6). Preparations were stretched by loads up to 9g applied in the circumferential axis, longitudinal axis or in both axes, while recording identified single viscerofugal neuron firing. Either circumferential or longitudinal distensions evoked graded increases in firing rate of viscerofugal neurons (uniaxial distensions; p < 0.05, 2-way repeated measures ANOVA, n = 6; figures 3 and 4). This suggests that viscerofugal neurons are directly mechanosensitive to distension in either the circumferential or longitudinal axes. There was a tendency for circumferential distensions to evoke higher rates of firing than longitudinal distensions, but the difference was not significant with a sample size of n = 6 (see figure 3, p = 0.16, 2-way repeated measures ANOVA).

Mechanosensory firing of viscerofugal neurons was also evoked when the circumferential and the longitudinal axes were stretched together (biaxial distensions; figure 4). In some cases, additive effects on firing rate were observed upon the addition of a second, orthogonal load although this was not seen in all preparations (see figure 4**A**). The overall effects of loads applied in both axes are shown in figures 4**B**-**D**. For biaxial distensions, the effect of increasing circumferential loads on firing rate was significant (p < 0.05, 2-way ANOVA, n = 6), but adding longitudinal loads had a smaller effect which was not significant with a sample of n=6 (p = 0.08, 2-way ANOVA). Overall, the greatest increases in firing rate occurred where combined strain was greatest. Thus, the peak average mechanosensitive firing rate occurred under the peak loading conditions (i.e. 9g circumferentially together with 9g longitudinally; see figures 4**B**-**D**).

*Effect of load on global tissue strain*

Load-evoked tissue strain across the whole preparation (global strain) was calculated for all uniaxial and biaxial distensions. The effect of biaxial loading on global tissue strain is shown in figures 5**A**-5**D**, and figures 7**A**-7**E**. In both axes, loads evoked significant increases in strain in the same direction as the applied load (as would be expected), while significantly decreasing strain in the orthogonal axis (due to the "Poisson Effect", p < 0.001, 2-way repeated measures ANOVA, n = 6, examples can be seen in figures 3**A**-**B**, and figure 4**A**). Application of circumferential loads evoked significantly greater tissue strains than identical longitudinal loads (circumferential loads: F = 61.05, longitudinal loads: F = 23.95; see figure 3**D**, and figures 5-7 for comparisons). This indicates that the gut was more compliant circumferentially than longitudinally, suggesting that differences in firing evoked by circumferential and longitudinal loads may be explicable by different strains evoked by those loads. Indeed, when viscerofugal neuron firing was plotted against strain evoked during uniaxial distensions, differences in firing rates between the two major axes were abolished (seefigure3**E**). Thus, the higher magnitude strains evoked by circumferential loads in biaxial distensions (compared to identical longitudinal loads) probably underlies the greater effect of circumferential loading on viscerofugal neuron firing (figure 3**C**).

*Effect of load on local tissue strain*

DIC revealed heterogeneity of strain within the tissue during distensions. Local strain was typically greater toward the centre of preparations compared to the fixed edges (figure 6). The circumferential and longitudinal load-evoked strains were calculated at the site of the viscerofugal nerve cell body (identified by DMPP-evoked firing) and are referred to as "local strain". These measurements revealed that the overall local strain approximately paralleled global strain. However, local strains were more variable and significantly smaller than global strains (p < 0.001, paired t-tests; figure 7), probably because viscerofugal nerve cell bodies are preferentially located nearer to the mesenteric border of the gut than to the antimesenteric border ([27](#_ENREF_27), [28](#_ENREF_28)).

*Relationship between stretch-evoked firing and tissue strain*

The highest firing rates of viscerofugal neurons were evoked by the largest combined loads. This suggests that strains in both the circumferential and longitudinal directions can drive mechanosensitive firing. For this reason viscerofugal neuron firing was quantified during both single axis strains as well as multi-axis, combination strains (table 1). There were relatively weak relationships between viscerofugal neuron firing rate and single axis strains (figure 8). In comparison, combination strains were more strongly related to viscerofugal neuron firing. The linear correlation coefficients observed between combination strains (see methods) and firing rates are shown in table 2. Close linear relationships between viscerofugal neuron firing rate and strain was observed with local first analogue of strain, maximum shear strain, and equivalent strain (R = ~0.6, p < 0.005; see table 2). These are combination strains that calculate tissue shearing and changing area. In each of these cases, stronger relationships were seen between firing rate and local strain, compared to global strains (figure 8). The observed pattern of tissue strains evoked during biaxial distensions are shown in figure 7. This method of visualisation shows that the local pattern of strain for first analogue of strain and equivalent strain are most closely associated with viscerofugal firing rate.

DISCUSSION

The present study combines a well-established engineering method, DIC, with electrophysiological recordings from the axons of viscerofugal neurons, a well-characterised class of mechanosensitive enteric neurons. A DIC script was modified by the authors to analyse the large deformations observed in intestinal tissue and calculate local strain within the receptive field of mechanosensitive neurons. Enteric viscerofugal neurons were chosen for this study because they have a single, punctate transduction site (their cell body) which can be precisely localised using focal application of a nicotinic agonist (DMPP), as characterized previously ([12](#_ENREF_12)). They are also directly mechanosensitive ([12](#_ENREF_12)), sensitive to strain, and have a single punctate receptive field which corresponds to their nerve cell body ([12](#_ENREF_12), [13](#_ENREF_13)). This study provides proof-of-principle that other mechanosensory neurons, such as extrinsic primary afferent neurons, with more complex mechanotransduction sites in the gut wall, may be analysed using the DIC method ([21](#_ENREF_21), [37](#_ENREF_37), [42](#_ENREF_42)).

*Heterogeneity of strain*

Video recordings have been used previously to observe micro-contractile events in the receptive field of spinal sensory neurons to the guinea pig rectum in vitro ([22](#_ENREF_22)). Local mechanical events in colorectal preparations differed from global measurements made from the whole isolated preparation, and correlated more strongly with mechanoreceptor firing, similar to the present findings. In the present study, preparations were pharmacologically paralysed with the low [Ca2+]/raised [Mg2+] solution to prevent stretch-induced reflex contractile activity that would have complicated analysis. This is valid because we have previously shown that viscerofugal firing is closely associated with increases in gut diameter and not with increases in wall tension. Indeed, active contractions that increased tension and shortened the gut wall caused decreases in viscerofugal firing ([12](#_ENREF_12)).

In the present study, distension evoked lower strains near the fixed borders of preparations, with higher strain values in the centre. Immobilisation of the tissue is the most likely explanation for the lower strains observed near fixed edges. However, the possibility of structural differences around the circumference of the intestine cannot be ruled out. Different densities of ganglia have been observed around the circumference of guinea pig, mouse and rat colon ([35](#_ENREF_35)).

Flat-sheet preparations used for neurophysiological recordings of gastrointestinal afferent neurons are typically pinned at the mesenteric border where mesenteric/colonic/rectal nerves enter the preparation. Viscerofugal neuron cell bodies ([27](#_ENREF_27), [28](#_ENREF_28)), and the transduction sites of many splanchnic afferent neurons ([2](#_ENREF_2)), are concentrated near the intestinal mesenteric border; this was where lower local strains were observed in the present study. Indeed, the local strains evoked at viscerofugal nerve cell bodies were, on average, lower than global strain values. This suggests that the activation thresholds for individual afferent neurons are influenced by the location of their receptive endings in the preparation. Thus thresholds for afferents with receptive fields close to pinned edges of preparations may be overestimated when global strain measurements are used.

*Activation of viscerofugal neurons by longitudinal distension*

In the present study, loads applied in the circumferential axis evoked, on average, more viscerofugal neuron firing than the same loads applied in the longitudinal axis. Loads of less than 4g in the longitudinal axis did not increase viscerofugal neuron firing rate ([13](#_ENREF_13)), but the same small loads applied in the circumferential axis reliably evoked faster firing. The differences in the load/firing curves for circumferential and longitudinal axes were not significant; it is possible that this reflected the sample size (n=6).

The anisotropic responses to longitudinal and circumferential loads are explicable by the higher compliance of the gut in the circumferential axis (see figure 3**D**). Miller & Szurszewski ([29](#_ENREF_29)) recorded synaptic inputs in prevertebral ganglion cells in the mouse as a measure of viscerofugal activation. When tubular and flat sheet preparations were stretched circumferentially by up to 15% of resting length (a strain of 0.15), there was an increase in the frequency of synaptic events. However, when the same preparations were stretched by 20% of resting length longitudinally (a strain of 0.2) no increase in synaptic inputs was observed. The strains applied by Miller & Szurszewski ([29](#_ENREF_29)) are similar to the resultant strains in the present study. One important difference is that in the present study, preparations were distended under synaptic blockade with a Ca2+-free Krebs solution. This was used to ensure that all responses were mediated by direct mechanosensitivity, rather than by indirect synaptic activation of viscerofugal neurons by enteric reflex pathways ([29](#_ENREF_29)). It is possible that in the previous study ([29](#_ENREF_29)), enteric neural circuits in the colon were strongly activated by circumferential distension, leading to activation of viscerofugal neurons via nicotinic synaptic inputs ([4](#_ENREF_4)). It has been shown that longitudinal distension may extensively inhibit enteric neurons by release of nitric oxide ([6](#_ENREF_6), [7](#_ENREF_7)). Viscerofugal neurons may have been inhibited by elongation of the preparation in the previous study (25); this would have reduced their apparent mechanosensitivity in the longitudinal axis. This consideration does not apply to the present study, because the low [Ca2+]/high [Mg2+] solution would have blocked inhibitory synaptic transmission.

*Multi-dimensional mechanosensitivity*

Viscerofugal enteric neurons are mechanosensitive interneurons that are activated primarily by increases in wall strain rather than by wall tension ([1](#_ENREF_1), [12](#_ENREF_12)). All viscerofugal neurons appear to be mechanosensitive ([12](#_ENREF_12)) and mechanotransduction probably occurs at the nerve cell body ([12](#_ENREF_12), [13](#_ENREF_13)). Viscerofugal neurons were mechanically activated by both circumferential and longitudinal distensions in the present study. In addition, they can be activated by focal compression with von Frey hairs ([12](#_ENREF_12), [13](#_ENREF_13)). Thus, it is likely they can be activated by mechanical distortion of their cell bodies in all 3 axes. Consistent with this, viscerofugal neurons did not appear to preferentially detect strain in any of these axes, including the transverse axis (z-axis; see table 2). This is comparable to afferents in the feline knee joint capsule which similarly lack directional sensitivity ([14](#_ENREF_14)). Consistent with our observations, mechanosensory firing of viscerofugal neurons was most closely related to local multiaxial strains: maximum shear strain, equivalent strain, and first analogue of strain (table 2). Maximum shear strain and equivalent strain indicates angular distortion (shearing strain) in two and three dimensions, respectively. First analogue of strain calculates combined displacement of the circumferential and longitudinal axes. In the gut, these strains may cause distortion of myenteric ganglia by skewing their axes (shearing; maximum shear strain or equivalent strain) or by increasing their area (first analogue of strain). Both effects are likely to be relevant during normal gut movements. Ganglionic shearing occurs during normal contractile activity of the ileum ([26](#_ENREF_26)). The area of myenteric ganglia and their nerve cell bodies may change by as much as 50% in ileum and rectum, depending on contractile status ([10](#_ENREF_10)). Hydrostatic and differential strain was not significantly associated with viscerofugal neuron firing. Hydrostatic strain is a measure of volumetric distortion; this occurs with osmotic distension which was not used in the present study. Differential strain measures stretch asymmetry; this would be expected to correlate well with neurons sensitive to uniaxial distension. This is consistent with observation in the present study that both circumferential and longitudinal distensions evoked firing in viscerofugal neurons.

Sensitivity to mechanical distortion in all directions is consistent with the morphology of viscerofugal neurons. They are mostly simple ovoid cell bodies with Dogiel type I morphology in the guinea pig colon ([i.e. uniaxonal neurons with small- or medium-sized nerve cell bodies, and either short lamellar dendrites or no dendrites; 3](#_ENREF_3)). A small population of viscerofugal neurons with Dogiel type II morphology exists, but they have not been functionally distinguished ([9](#_ENREF_9), [11](#_ENREF_11)) and were not encountered in this study ([11-13](#_ENREF_11)). Dogiel type II neurons have filamentous processes orientated circumferentially, however, at least in the small intestine, Dogiel type II “AH” neurons respond equally to stretch in the circumferential and longitudinal directions ([18](#_ENREF_18)). Interestingly, Dogiel type II neurons are sensitive to wall tension, unlike viscerofugal neurons ([19](#_ENREF_19)). Other mechanosensitive neurons with Dogiel type I morphology have been identified in myenteric ganglia. These include the length-sensitive S-neurons, identified by Spencer and colleagues ([38](#_ENREF_38)), and rapidly-adapting mechanosensitive enteric neurons (RAMEN), identified by Mazzuoli and Schemann ([25](#_ENREF_25), [26](#_ENREF_26)). The latter comprise motor neurons and interneurons that discharge phasically to myenteric ganglion deformation. We speculate these classes of enteric neurons may also be sensitive to tissue distortion in multiple axes, similar to viscerofugal neurons.

Many enteric interneurons and motor neurons show direct mechanosensitivity ([26](#_ENREF_26)). This suggests that neurons driving intestinal motor patterns are modulated by the mechanical consequences of motor patterns that they contribute to ([26](#_ENREF_26)). Viscerofugal neurons are also exposed to mechanical effects of gut motility and may therefore transmit this information to sympathetic prevertebral ganglia ([39](#_ENREF_39)). The results of the present study suggest that viscerofugal neurons are activated by distortion of their cell bodies associated with increased strain in either the circumferential or longitudinal axis of the gut. We speculate that this very specific type of mechanosensitivity may allow viscerofugal neurons to encode information about the volume of the region of intestine that they innervate. Viscerofugal neurons also receive synaptic inputs from enteric motor circuits ([11-13](#_ENREF_11)). Presumably, synaptic input combines with mechanosensory responses to drive viscerofugal inputs to sympathetic reflex arcs that feed back to inhibit motility ([15-17](#_ENREF_15)). Previous studies have indicated that viscerofugal neurons are not responsive to increases in intramural tension ([12](#_ENREF_12), [29](#_ENREF_29)). Thus, DIC was an ideal method to study their mechanosensitivity to local strains. It has been reported that circumferential, but not longitudinal strain activates sympathetic prevertebral neurons ([via viscerofugal neurons; 29](#_ENREF_29)). Using DIC, the present study suggests that under more controlled conditions, longitudinal strain does activate viscerofugal neurons. This may reflect an absence of enteric inhibitory neurotransmission activated during gut elongation ([7](#_ENREF_7)).

In conclusion, this study has shown that DIC can be used to measure local strain in the receptive fields of enteric viscerofugal neurons in flat sheet preparations of colon. Local strains calculated in this way were better predictors of firing than global strains. Strain values calculated at viscerofugal nerve cell bodies were smaller than strain averaged across the entire preparation and more variable. Viscerofugal neurons lacked preferential direction-sensitivity in their mechanically-driven responses: they can be directly activated by both circumferential and longitudinal gut distensions. Their firing correlates most closely with combinations of strain in multiple axes that indicate shearing or changing area. DIC may prove a useful method to characterise strains in the receptive fields of sensory neurons innervating other soft tissues. Whether it can be applied to sensory neurons with larger receptive fields, in more complex tubular preparations with ongoing contractility and in sensory neurons that are responsive to wall stress (in addition to strain) remains to be determined.

ACKNOWLEDGEMENTS

We would like to thank the staff of Flinders Biomedical Engineering for the development of organ baths, heat baths, and pulley systems used in this study.

GRANTS

This work was supported, in part by NHMRC project grant #1048195; TR was supported by a University Research Fellowship from The Royal Society; GP was funded by Engineering and Physical Sciences Research Council (EPSRC) grant EP/G03690X/1. TJH was supported by a Flinders University Research Scholarship.

DISCLOSURES

The authors have no conflicts of interest that have any bearing on the material presented in this paper

AUTHOR CONTRIBUTION

TJH made the recordings of viscerofugal neurons. TJH, SJHB and MT planned the study, designed the experiments and assisted with data analysis and interpretation. GP and TR carried out the DIC analysis and interpretation. All authors contributed to the preparation of the manuscript.

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TABLES

**Table 1** Combination strains calculated in the present study

|  |  |
| --- | --- |
| **Strain** | **Equation** |
| Hydrostatic Strain | $$γ=(ε\_{x}+ε\_{y}+ε\_{z})/3$$ |
| Differential Strain | $$γ=ε\_{x}-ε\_{y}$$ |
| Equivalent Strain | $$γ=\sqrt{0.5\left(\left(ε\_{x}-ε\_{y}\right)^{2}+\left(ε\_{y}-ε\_{z}\right)^{2}+\left(ε\_{x}-ε\_{z}\right)^{2}\right)}$$ |
| 1st Analogue of Strain | $$γ=ε\_{x}+ε\_{y}$$ |
| Maximum Shear Strain | $$γ=\frac{1}{2}\left|ε\_{x}+ε\_{y}\right|$$ |

**Table 2** Pearson’s linear correlation between tissue strain and viscerofugal neuron firing

|  |  |  |
| --- | --- | --- |
| Strain | Correlation coefficient | P |
| Global | Local | Global | Local |
| Circumferential | 0.264 | 0.462 | 0.223 | 0.026 |
| Longitudinal | 0.218 | -0.096 | 0.317 | 0.662 |
| Z | 0.430 | -0.008 | 0.041 | 0.970 |
| 1st Analogue of strain | 0.498 | 0.596 | 0.016 | 0.003 |
| Hydrostatic | 0.557 | 0.296 | 0.006 | 0.171 |
| Differential | 0.035 | 0.361 | 0.874 | 0.091 |
| Equivalent | 0.095 | 0.622 | 0.667 | 0.002 |
| Maximum Shear | 0.498 | 0.601 | 0.016 | 0.002 |

**FIGURE LEGENDS**

Figure 1: *Schematic diagram of the recording setup.* Conventional extracellular recordings were made from colonic nerve trunks close to the gut wall isolated in a small, paraffin oil-filled chamber using platinum/iridium electrodes. Load-evoked distensions were applied by adding weights to the opposing arm of isotonic transducers. An array of hooks and pulley system connected transducers to the circumferential and longitudinal axes of a preparation. Black graphite markers were applied to the surface of the preparations and used to track the local circumferential and longitudinal strains within the preparation; isotonic transducers recorded gross strains.

Figure 2: *Localisation of a viscerofugal nerve cell body by focal application of DMPP.* **A** A micrograph of a flat-sheet preparation of guinea pig distal colon with carbon/graphite markers (asterisks). The tip of a glass micropipette containing DMPP was positioned close to the preparation (arrow) and DMPP was pressure ejected focally onto the preparation **B**. The time of ejection is indicated by the red arrow. A large burst of action potentials was evoked by the DMPP. The cloud of ejected DMPP is visible by the blue marker, and is about 100μm diameter, allowing reliable localisation of the viscerofugal nerve cell body see ([12](#_ENREF_12)).

Figure 3: *Effect of loads on mechanosensitive viscerofugal neuron firing.* **A** A 3g load applied in the circumferential axis causes circumferential elongation and simultaneous longitudinal shortening (the latter due to the Poisson effect). There is small initial burst of firing and modest level of maintained firing for the duration of the load. **B** A 3g load applied in the longitudinal axis of the same preparation, caused longitudinal elongation and circumferential shortening (again, due to the Poisson effect). It evoked a similar pattern of firing. **C** Combined data (n = 6) showing that loads applied in longitudinal and circular axes both increase viscerofugal neuron firing (p < 0.05, 2-way repeated measures ANOVA, n = 6); note the tendency for slightly larger effects for circumferential loads (not significant). **D** Combined data (n = 6) plotting strain against load, showing that loads cause larger strains in the circular axis, compared to longitudinal (p < 0.05, 2-way repeated measures ANOVA, n = 6), reflecting greater circular compliance. This may contribute to the higher firing in **C. E** Strain plotted against firing rate for individual uniaxial distensions. Note that higher strains were evoked by circumferential loading. This graph shows that the relationship between strain and viscerofugal neurons firing is similar in the circumferential and longitudinal axes, despite the differences in compliance.

Figure 4: *Effect of biaxial distensions on mechanosensitive viscerofugal neuron firing.* **A** A 3g load was first applied to the circumferential axis, followed by the a 3g applied to the longitudinal axis about 4 seconds later. Note the addition of a longitudinal load reduces tissue length in the circumferential axis (Poisson effect). Viscerofugal neuron firing increases upon the addition of each load, but adapts slowly when the distensions are maintained. **B** Average effect of biaxial distensions on viscerofugal neuron firingfor different loads applied in the longitudinal axis, in the presence of existing load in the circular axis (n = 6). Note that firing is higher for every longitudinal load when there is a 9g load applied in the circular axis (filled squares). **C** Here, the effect of increasing circumferential loads in the presence of existing longitudinal loads (0g, 6g, 9g) are shown. Note that all circumferential loads evoked more firing when there is a pre-existing longitudinal load of 9g (filled squares) compared to lower longitudinal loads. **D** Combined data for loads in both axes, where firing rate is represented by greyscale; darker shades indicate higher firing (see scale). Both circumferential and longtudinal distensions increased viscerofugal neuron firing, but the maximal firing was when loads in both axes were maximal.

Figure 5: *Interactions of biaxial loads on tissue strain.* **A** increasing longitudinal loads caused decreased circumferential strain (Poisson effect) irrespective of circular load. (**B**) Increased circumferential load, as would be expected, increased circumferential strain, but in a manner that was influenced by existing longitudinal load. **C** Increasing longitudinal load increased longitudinal strain (as would be expected), irrespective of circumferential loads. D. Increasing circumferential loads had small effect but tended to cause decreasing strain in the longitudinal axis (Poisson effect). Note that Figure 5 does not include data on afferent firing.

Figure 6: *Hetergeneity of load-evoked strain revealed by digital image correlation* **A** Localised circumferential strain shown in the region surrounding a viscerofugal nerve cell body (white arrow). **B** Localised longitudinal strain in the same region during the same 3g x 3g stretch. Note that local strain evoked by applied load varies within the tissue, even when smooth muscle was paralysed. Markers were displaced by load by variable distances, according to their location in the tissue, with 0 µm at the fixed edge, up to 70μm at the medial edge of the receptive field. Furthermore, this effect was more marked in the circumferential axis than in the longitudinal axis. Dashed lines indicate the mesenteric border of the preparation.

Figure 7: *Effect of biaxial loads on tissue strain* Positive strain is shown as greyscale with darker shades representing higher strain. Negative strain (ie shortening) is shown in red; white is zero strain. Applied loads in the circumferential and longitudinal axes are expressed on the X and Y axes, respectively. Global strains are shown in the left column and local strains (close to viscerofugal neuron cell bodies) are shown in the right column for comparison. **A**. Colours represent circumferential strain. Circumferential loads increase circumferential strain, irrespective of longitudinal load (squares are increasingly darker moving from left to right). However, longitudinal loads cause negative circumferential strain (in red) due to the Poisson effect. The overall distribution of strains is similar for global and local sites, but with more variability locally **B**. Colours represent longitudinal strain. Increasing longitudinal load increased longitudinal strain, whereas circumferential loading decreased longitudinal strain (shown in red; the Poisson effect). By comparing circumferential and longitudinal strain in **A** and **B** (same strain scale is used in each figure) similar patterns of strain occurred locally but these were lower than global strains and were more variable, with less smoothly graded changes. **C**-**E** show combination strains (table 1): 1st analogue of strain, equivalent strain, and hydrostatic strain evoked by biaxial loads. All figures show the average effect of loading on tissue strain (n = 6). Note that maximum shear strain (not shown), produces a similar relative strain pattern to the first analogue of strain.

Figure 8: *Relationship between tissue strain and viscerofugal neuron firing rate.* Average firing rate (Hz averaged over 10s at the onset of load application, n = 6) plotted against various strains, with regression line and P values calculated from Pearson’s R value. Weak positive correlations were seen between average firing and both global and local circumferential strains (**A**, **B**), but not for global and local longitudinal strains (**C**, **D**). By comparison, taking both circumferential and longitudinal strains into account as first analogue of strain (global and local) showed closer correlations with firing (**E**, **F**).