Chapter 4
The mechanosensitivity of viscerofugal neurons

INTRODUCTION

Prevertebral sympathetic neurons receive cholinergic inputs from sympathetic preganglionic neurons projecting from the intermediolateral column of the spinal cord (Strack et al., 1988); they also receive some inputs from collateral axons of peptidergic spinal sensory neurons (Peters and Kreulen, 1986, Matthews et al., 1987). Numerous electrophysiological studies in sympathetic neurons of mouse and guinea pig show that they also receive cholinergic synaptic inputs from populations of enteric viscerofugal neurons in the gut wall (Kreulen and Szurszewski, 1979a, Love and Szurszewski, 1987, Anthony and Kreulen, 1990, Stapelfeldt et al., 1993, Miller and Szurszewski, 1997, Skok et al., 1998, Ermilov et al., 2004b). Mechanical distension of the gut evokes increases in the frequency of fast synaptic inputs from enteric viscerofugal neurons (Crowcroft et al., 1971b, Szurszewski and Weems, 1976, Weems and Szurszewski, 1977, 1978, Kreulen and Szurszewski, 1979b, a, Shu et al., 1987, Anthony and Kreulen, 1990, Bywater, 1993, Parkman et al., 1993, Stapelfeldt et al., 1993, Stebbing and Bornstein, 1993, Miller and Szurszewski, 1997, Skok et al., 1998, Miller and Szurszewski, 2002, 2003, Ermilov et al., 2004b). Studies performed on tubular preparations of gut, attached via mesenteric nerve to prevertebral ganglia, suggest that viscerofugal neurons are primarily sensitive to gut volume (circumferential length) rather than intraluminal pressure (tension; Weems and Szurszewski, 1977, Anthony and Kreulen, 1990, Miller and Szurszewski, 1997, 2002, 2003). This has led to the hypothesis that viscerofugal neurons function as volume receptors (Szurszewski et al., 2002). When neurotransmission in the gut (but not in sympathetic ganglia) was blocked using hexamethonium or a low [Ca^{2+}] Krebs solution, the frequency of distension evoked synaptic inputs was reduced, but not

abolished (Bywater, 1993, Parkman et al., 1993, Stebbing and Bornstein, 1993). This suggests that some viscerofugal neurons are directly mechanosensitive. However, the proportion of viscerofugal neurons with this property is unknown. Here, we identified single viscerofugal neurons and showed that all viscerofugal neurons are directly mechanosensitive. We additionally characterized viscerofugal neuron mechanosensitivity in flat sheet preparations to distensions under isotonic and near-isometric conditions, directly showing that change in circumferential length, not tension, affects viscerofugal neuron firing rate. We also show that viscerofugal neurons may respond to circumferential and longitudinal distensions of the gut, without specific sensitivity to stretch in a single direction.

METHODS

Dissection

Adult guinea pigs, 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. 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 and 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 (Zagorodnyuk and Brookes, 2000). Differential extracellular recordings were made between a nerve trunk and the connective tissue strand using 100µm diameter Pt/Ir electrodes. Signals were amplified (ISO80; WPI, Sarasota, FL, USA) and recorded at 20kHz (MacLab16sp, LabChart 7, ADInstruments, Castle Hill, NSW, Australia) and single units were discriminated by amplitude, duration and shape using Spike Histogram and Scope View software (ADInstruments). In some preparations, a 10mm array of hooks

connected the preparation to an isotonic transducer (Harvard Bioscience, model 52-9511, S. Natick, MA, USA) that recorded tissue length and allowed distending loads to be applied in the circumferential axis. In studies of biaxial stretch, an additional transducer was used to record tissue length in the longitudinal axis; connected to the tissue via cotton thread and a 4mm array of hooks (see **figure 4.15**). Alternatively, a 10mm array of hooks was attached to a 'tissue stretcher' (Brookes et al., 1999) – a microprocessor controlled stepper motor with an in-series isometric force transducer (DSC 46-1001-01, Kistler Morse), which imposed controllable changes in length on the preparation while recording elastic force (see **figure 4.03B**). During electrophysiological recordings, preparations were continuously superfused with normal Krebs solution (~1.6ml/min, 35°C).

Localization of viscerofugal neuron cell bodies

As described in detail in **chapter 3**, the nicotinic receptor agonist, DMPP (10^{-3}M in Krebs solution, mixed 1:10 with blue food dye, Rainbow Food Colours, Australia), was applied focally to the tissue through a glass micropipette (10-20µm tip). Drug was delivered using helium gas under pressure (12psi, 15-40ms pulse duration) via a solenoid-operated valve (ejected volume approximately 0.8nl per 30ms pulse at 12psi). The area of tissue covered DMPP ejection was revealed by the dye. Focal mechanical sensitivity was investigated at and around DMPP-responsive sites using calibrated von Frey hairs. **Figure 4.01** shows an example of DMPP ejection at a DMPP-responsive site, including the firing response and micrographs of DMPP ejection onto the tissue.

Drugs

Stock solutions of drugs were made as follows: 10^{-2}M BAY K8644 in dimethyl sulfoxide (Sigma; B112), 10^{-1}M hexamethonium chloride in water (Sigma; H2138), 10^{-1}M 1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) in water (Sigma; D5891), 10^{-2}M N-Vanillylnonanamide (synthetic capsaicin) in ethanol (Sigma; V9130), 10^{-2}M noradrenaline hydrochloride in water (Sigma: A7256), 10^{-2}M serotonin creatinine sulphate complex in water (Sigma; H7752), 10^{-5}M α,β-methyleneadenosine 5′-triphosphate lithium salt in water (Sigma; M6517), 10^{-6}M [βala^{8}]-neurokinin A$^{(4-10)}$ in DMSO (Tocris; 1640) and 10^{-3}M oxotremorine sesquifumarate in water (Sigma; O-9126), 10^{-2}M L-Glutamic acid in water (Sigma; 128430). All drugs were kept refrigerated and diluted to working concentrations in Krebs solution, shortly before use. Vehicle controls were used at the greatest concentrations and quantities used in drug applications. Controls were as follows: 10µl of 10% DMSO in Ca^{2+}-free Krebs solution (1.4 x 10^{-3}M); 30µl of 1% ethanol in Ca^{2+}-free Krebs solution (2 x 10^{-3}M), and 10µl of 1% water in Ca^{2+}-free Krebs solution.

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 (ANOVA, one-way or two-way) using Prism v.5 software (GraphPad Software, Inc., San Diego, CA, USA) and 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". NS denotes a non-significant finding ($P>0.05$).

RESULTS

Mechanical probing studies

The results of DMPP-mapping studies (**chapter 3**) showed that focal compression of the tissue with light von Frey hairs could evoke repeatable bursts of action potentials from a proportion of DMPP-responsive sites (10/24 sites, mean peak firing rate: 20.0±9.0Hz, 10 units, n=6). We aimed to determine the proportion of DMPP-responsive sites that were directly sensitive to mechanical probing, during synaptic blockade. After a desensitising bolus application of capsaicin (1μM), focal ejection of DMPP was used to identify 18 DMPP-responsive sites in flat sheet preparations of guinea pig distal colon (n=6). An example of focal DMPP ejection is shown in **figure 4.01**. The preparations were then perfused with Ca^{2+} free Krebs solution (>60 mins prior to testing, 6mM Mg^{2+}, with 1mM EDTA) and probed with von Frey hairs (1-2mN). Of 18 identified single units, all 18 responded repeatedly to probing with von Frey hairs with bursts of firing (mean increase in firing rate, above basal, 22±11Hz, $p<0.001$, paired t-test). This suggests that all viscerofugal neurons, identified by DMPP-evoked firing, were directly mechanosensitive and their responses to local physical forces are not synaptically driven (**figure 4.02**). In further experiments, DMPP-responsive sites were tested with a series von Frey hairs 0.1mN-5mN. Action potential firing frequency increased in a graded manner with the force applied (0.1-5mN, n=5, **figure 4.02B**).

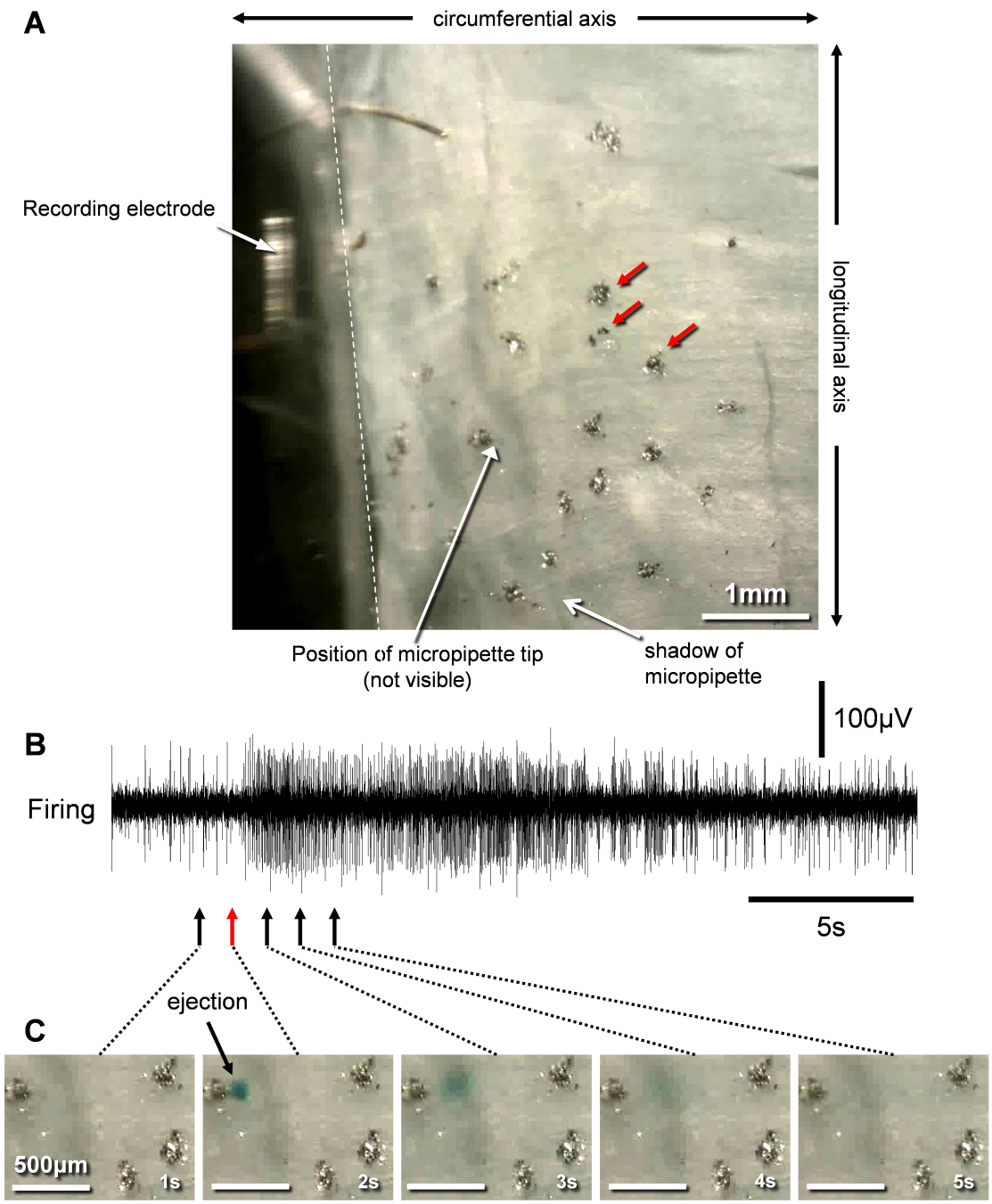

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Figure 4.01

Focal ejection of DMPP. A video frame, taken of a preparation of guinea pig distal colon during extracellular recording is shown in **A**. Multiple carbon markers can be seen on the surface of the circular muscle layer (examples are indicated by red arrows). The micropipette used for ejection is present but barely visible in this micrograph; a faint shadow cast by the pipette is marked and the location of the tip is marked but cannot be seen. Also visible are the recording electrode, which is opposite the glass coverslip (dashed line), which is used as a barrier for isolating the colonic nerve trunk. The firing response to a focal DMPP ejection onto the preparation shown in **A** is shown on the trace in **B**. The point of ejection is indicated as the red arrow; a large burst of firing is promptly evoked by ejection. In **C**, a series of micrographs shows the corresponding ejection of DMPP, which is made visible by a blue food dye. The arrows indicate the corresponding timepoints in the trace shown in **B**. These show that DMPP ejection is first seen in the second frame (marked 2s), as a blue spot of about 100μm. A large burst of firing is recorded in less than 0.5s. After ejection, the blue dye can be seen to diffuse over the following 3 seconds, becoming invisible in the final frame. This DMPP-responsive site was marked on a micrograph, similar to those shown in the figure.

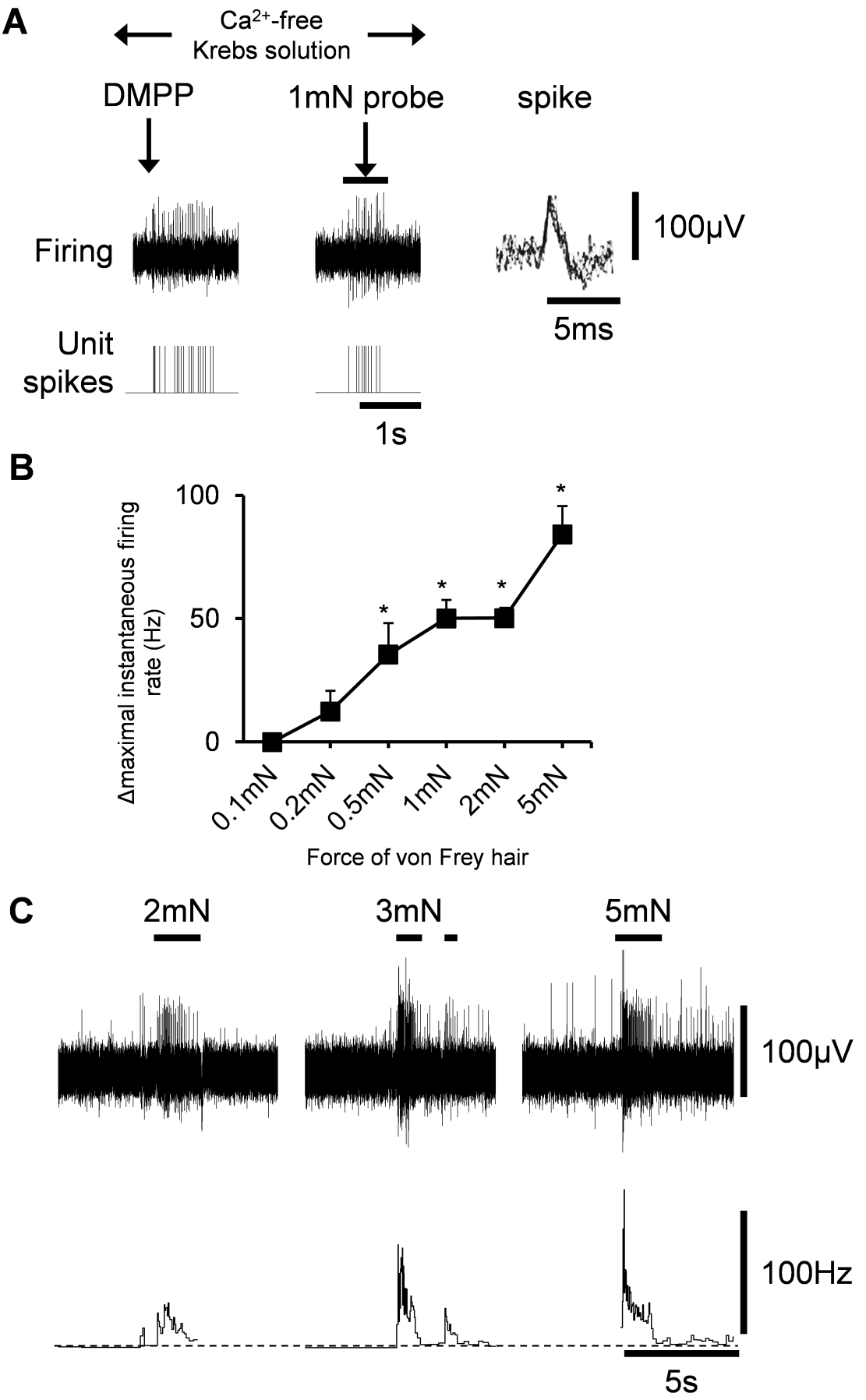

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Figure 4.02

Mechanosensory firing to focal tissue compression. (**A**) Here, a single unit was activated by DMPP ejected onto part of a myenteric ganglion from a micropipette. Focal compression of the same part of the myenteric ganglion using a 1mN von Frey hair evoked a burst of firing from the same unit. Both DMPP and the probe was applied in the presence of Ca^{2+} free Krebs solution. This suggests firing to DMPP and probing resulted from direct activation. (**B**) Increasing force of von Frey hair probing evoked greater maximal instantaneous frequencies from viscerofugal neurons (*$p<0.05$, bonferroni, 1 way ANOVA, repeated measures). (**C**) Examples of mechanically evoked firing by using von Frey hairs of increasing force on the same neuron. It can be seen in the lower traces that greater instantaneous frequencies of firing are evoked with increasing probing force, as also shown in **B**.

Circumferential stretch studies

To assess mechanosensitivity to circumferential stretch, DMPP-sensitive units (14 units, n=13) were tested with distension stimuli after a desensitising exposure to 1μM capsaicin. The average ongoing firing rate of viscerofugal neurons in the absence of applied stimuli (slack preparations) was 4.3 ± 2.9Hz (14 units, n=8). On average, action potentials were of low amplitude (79 ± 29 μV, 14 units), with a duration at half peak amplitude of 0.8 ± 0.4ms (14 units). Preparations were stretched circumferentially by imposed loads of 1-5g, while measuring changes in length with an isotonic transducer (schematic diagram in **figure 4.03A**). Distension evoked modest, load-dependent increases in firing ($p<0.05$, 14 units, n=8, 1 way ANOVA, **figure 4.04**) with a mean increase of 2.8 Hz at the highest (5g) load ($p<0.05$, Bonferroni post hoc, see **figure 4.05**). Hexamethonium (500μM) had no statistically significant effect on the stretch-induced increase in firing rate in these preparations ($F=0.19$, 14 units, n=8, $p>0.05$, 2 way ANOVA, with Bonferroni post hoc correction; examples shown in **figure 4.06**).

Preparations were also stretched by imposing changes in circumferential length, while recording the resultant tension with a force transducer (schematic diagram of the setup shown in **figure 4.03B**, examples of distensions shown in **figure 4.07** and **figure 4.08**). All DMPP-sensitive units (13 units, n=6) responded to distensions of 2mm or more with a significantly increased rate of action potential firing ($p<0.05$, 13 units, n=6, bonferroni post-test, 2 way ANOVA; results summarized in **figure 4.10A**). In the presence of Ca^{2+} free solution (6mM Mg^{2+}, 1mM EDTA, >60 minutes) contractile responses to distension were abolished and resting tension was greatly reduced (**figure 4.10B**). However, spontaneous firing was maintained and

responses to distension were similar to those in normal Krebs solution (examples shown in **figure 4.09**), suggesting that all viscerofugal neurons are directly sensitive to circumferential distension and are not dependent on synaptic activation.

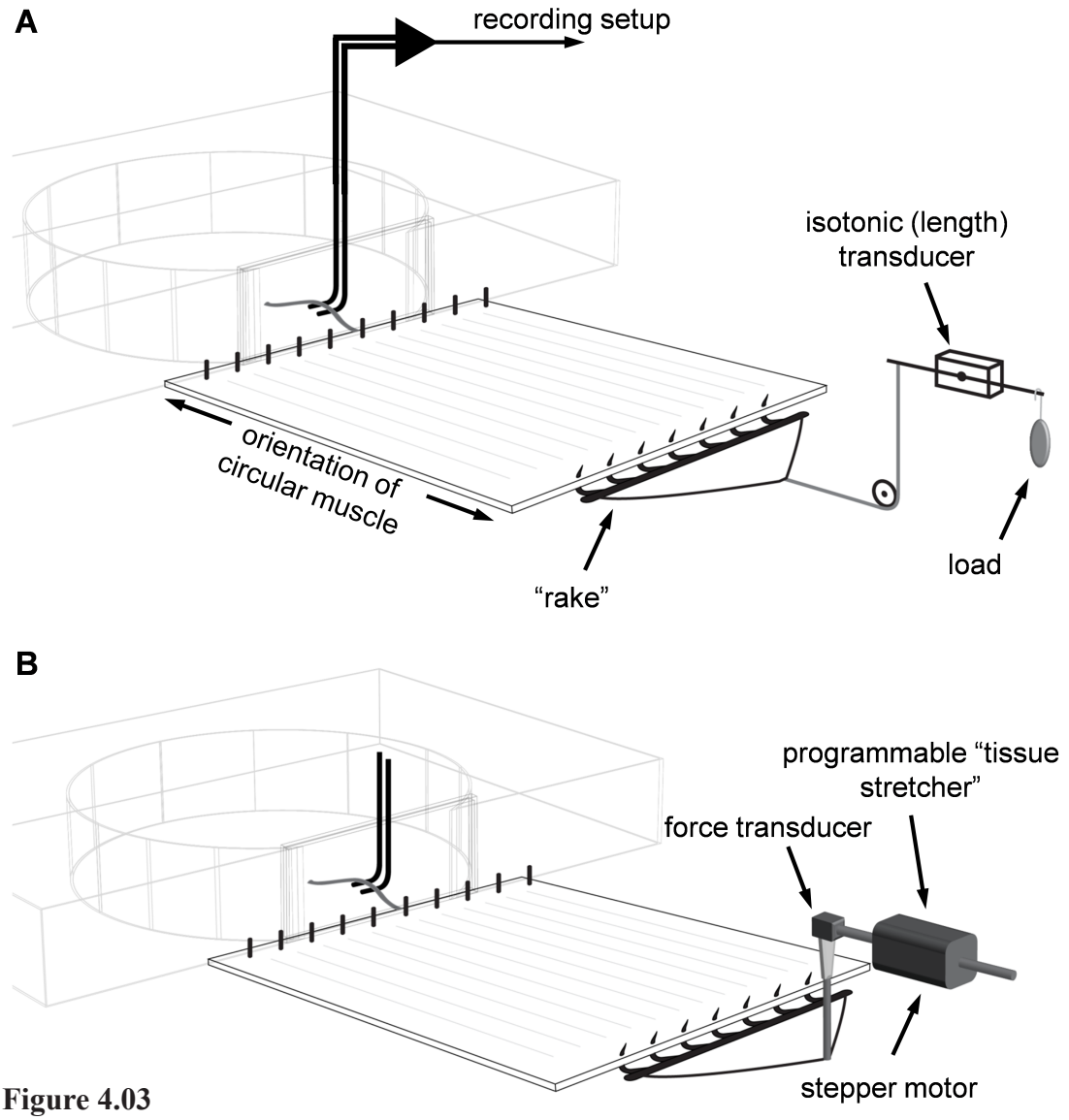

Figure 4.03

Schematic diagrams of the experimental setups used for applying different types of distensions to the circumferential axis of flat sheet preparations of colon. Shown in **A** is a diagram of the setup used for isotonic loading of flat sheet preparations in the circumferential axis during extracellular recordings from colonic nerve trunks. Preparations were pinned at one end near dissected colonic nerve trunks. An isotonic (length) transducer was attached to the preparation via an array of hooks ("rake"), cotton thread and pulley system at the opposite end of the preparation. The transducer measured the circumferential length of the preparation. In addition, varying distending loads could be applied to the preparation via the lever arm of the transducer. In this way, preparations were able to change in length during contractions and relaxations, while under a constant tension (load). A similar setup is shown in **B**, however a programmable tissue stretcher was used to impose circumferential distensions of predetermined length. In this way, the length of a preparation was set, while tension was variable and measured by an in-series force transducer attached to a rigid vertical bar that held the array of hooks. A stepper motor under computer control imposed changes in length to the preparation.

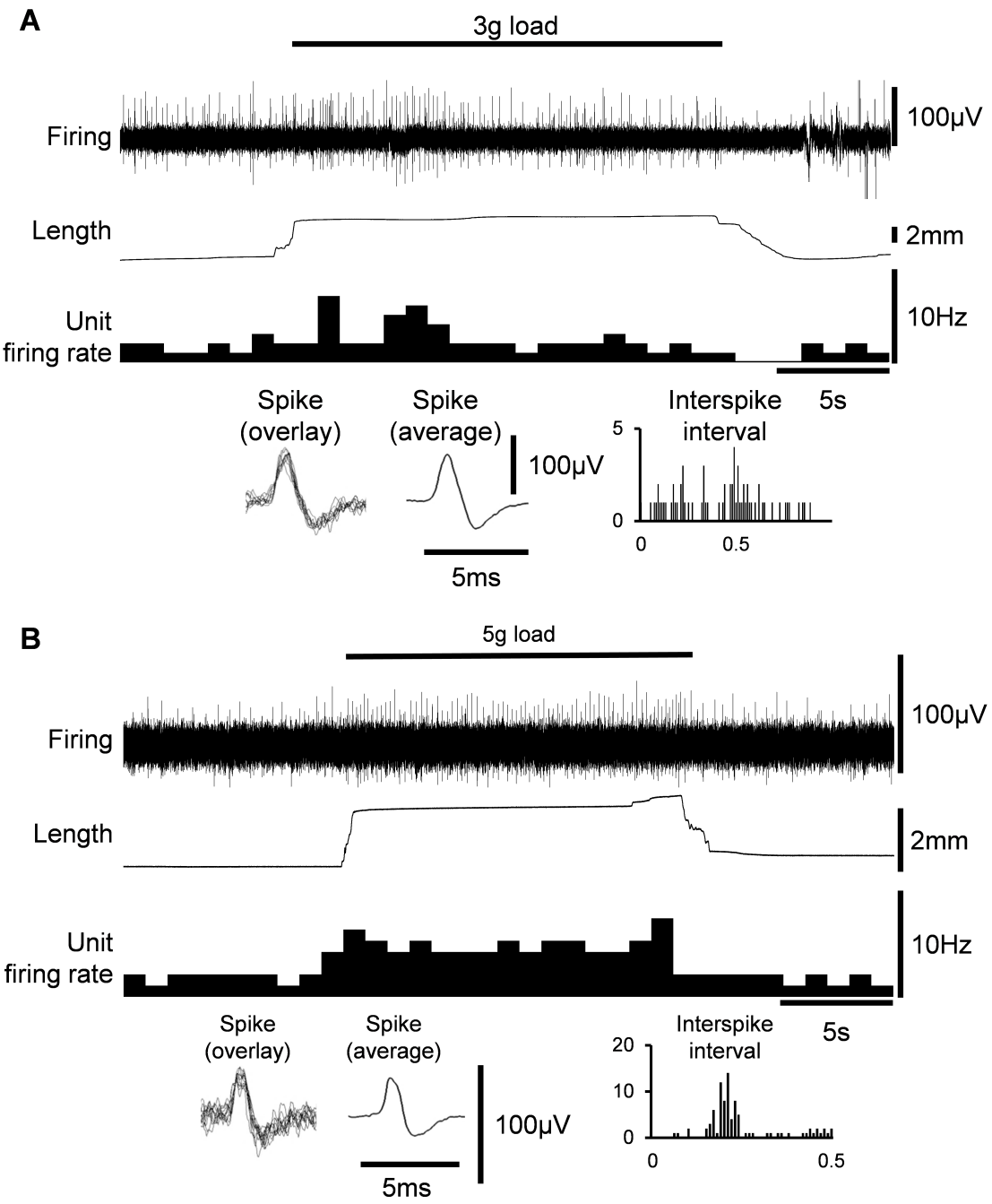

Figure 4.04

Firing evoked by circumferential isotonic distensions. **A** and **B** show examples of circumferential distensions that were produced by applying constant loads of 3g and 5g, respectively. Single units were discriminated by spike amplitude, duration and shape; average and overlayed individual spikes are shown under the graph of unit firing rate. Also, shown are their interspike interval histograms for the periods corresponding to the traces shown. These indicate firing rates consistent with a single neuron. The increases in firing rates produced by distensions up to 5g were modest. The highest load (5g) is shown in **B**, which approximately doubled the spontaneous firing rate (2Hz) to about 5Hz.

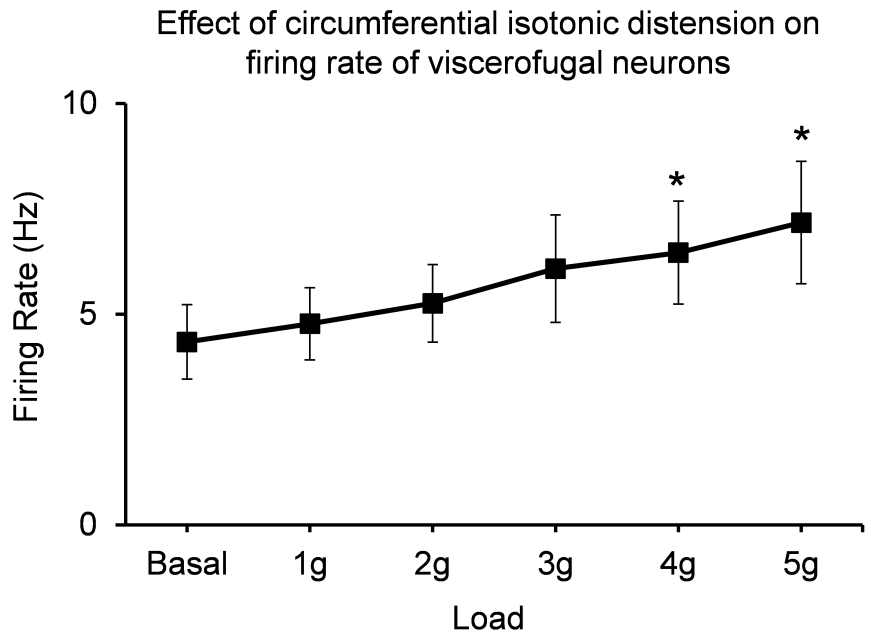

Figure 4.05

Effect of circumferential isotonic distensions on the firing rate of viscerofugal neurons. It can be seen that circumferential isotonic distensions evoked significant graded increases in firing rate (*$p<0.05$, Bonferroni post hoc analysis). The increases in firing rate were modest, with a mean increase of 2.8Hz above basal firing rate at the highest load of 5g.

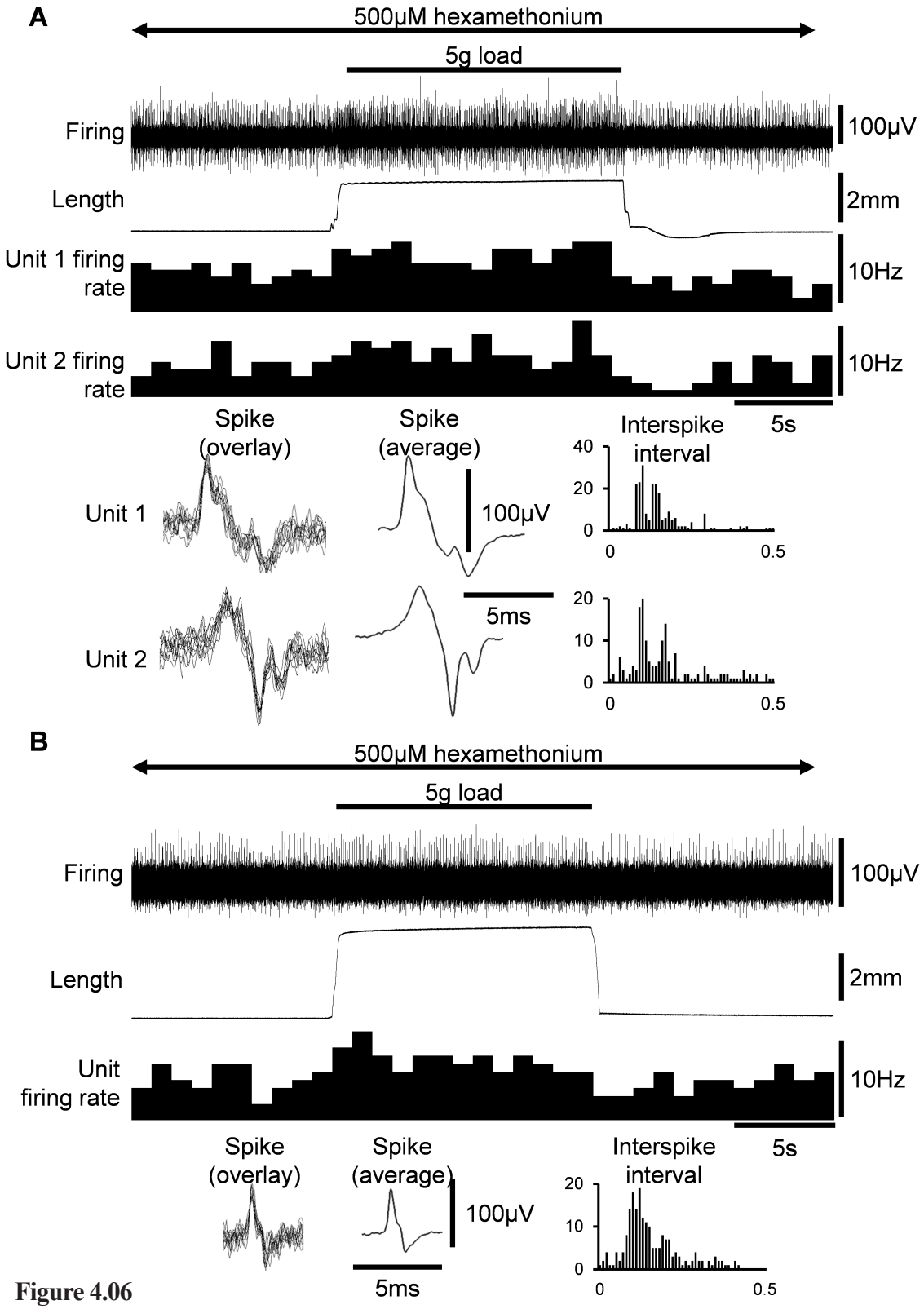

Figure 4.06

Firing evoked by circumferential isotonic distensions in hexamethonium. **A** and **B** show examples of 5g isotonic distensions in two different preparations during perfusion of the organ bath with Krebs solution containing the nicotinic receptor blocker hexamethonium (500µM). There were no differences in firing rates evoked by distensions up to 5g in hexamethonium when compared with control distensions without hexamethonium ($F=0.19$, 14 units, $n=8$, $p>0.05$, 2 way ANOVA, with Bonferroni post hoc correction). Both **A** and **B** show typical modest elevations in the firing rate of the discriminated single units while under load.

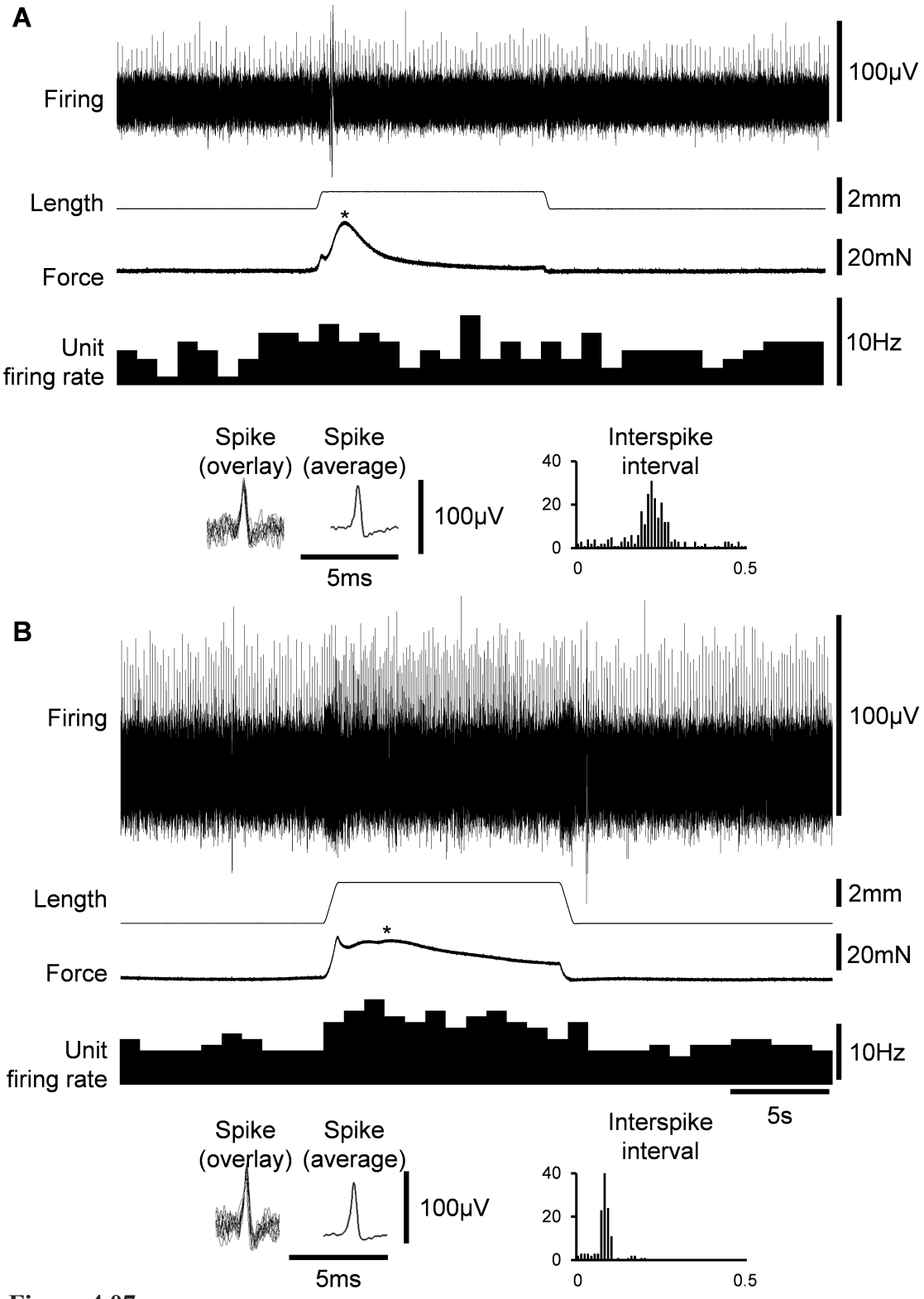

Figure 4.07

Effect of imposed circumferential displacement on firing. In these examples, preparations were lengthened by 1mm (**A**) and 3mm (**B**), respectively. Note the initial passive increase in tension evoked by tissue lengthening, followed by longer increases in tension resulting from active contractions of circular muscle (peaks are marked with asterisks). Little change in firing is apparent with 1mm lengthening (**A**); a greater increase in firing rate is observed in the 3mm distension (**B**).

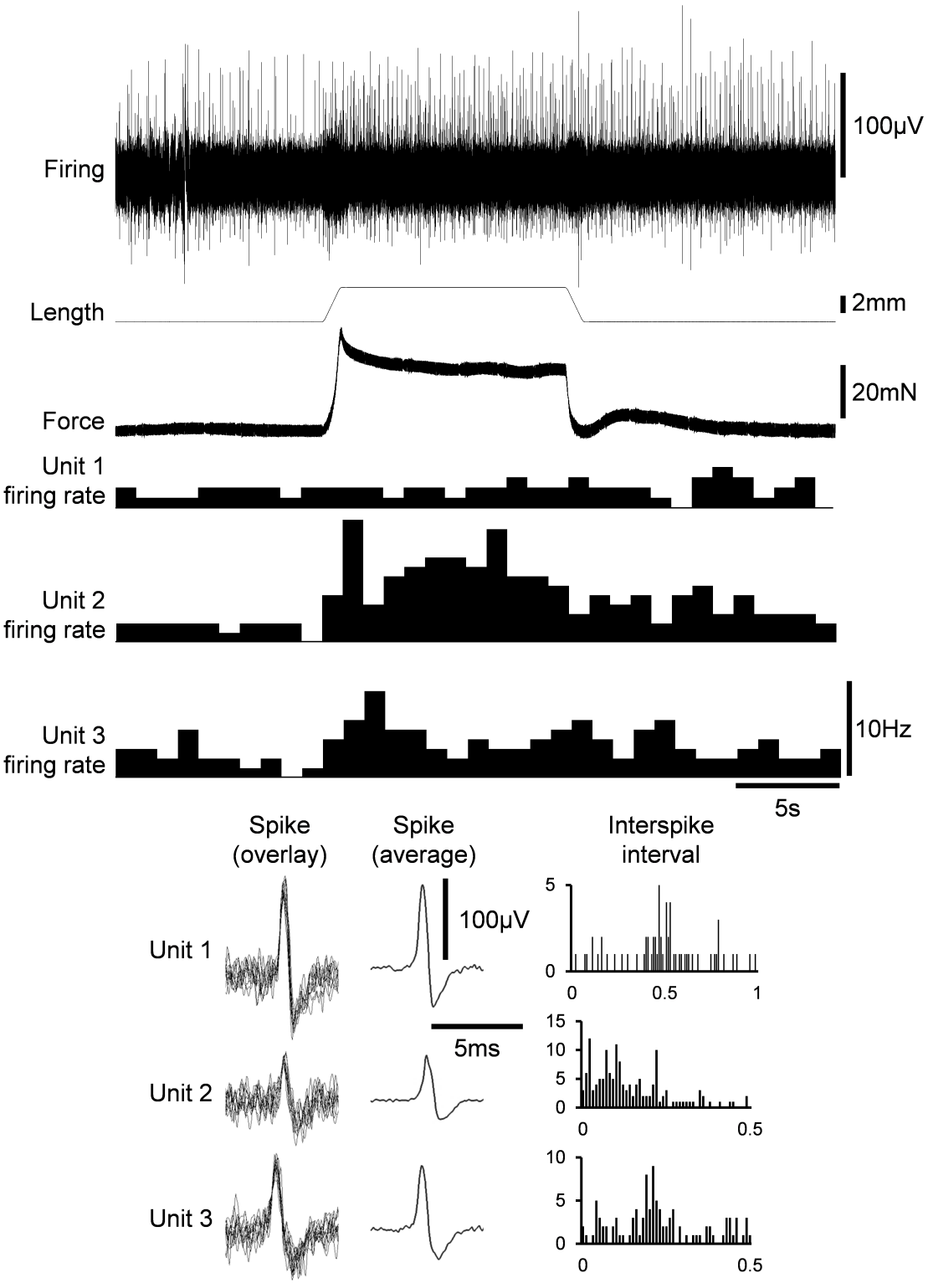

Figure 4.08

Effect of imposed circumferential displacement on firing. This example shows the effect of a 4mm lengthening of the preparation on the firing rate of 3 single units. Small increases in firing rate occurred in units 1 and 2; unit 3 showed little change in firing rate.

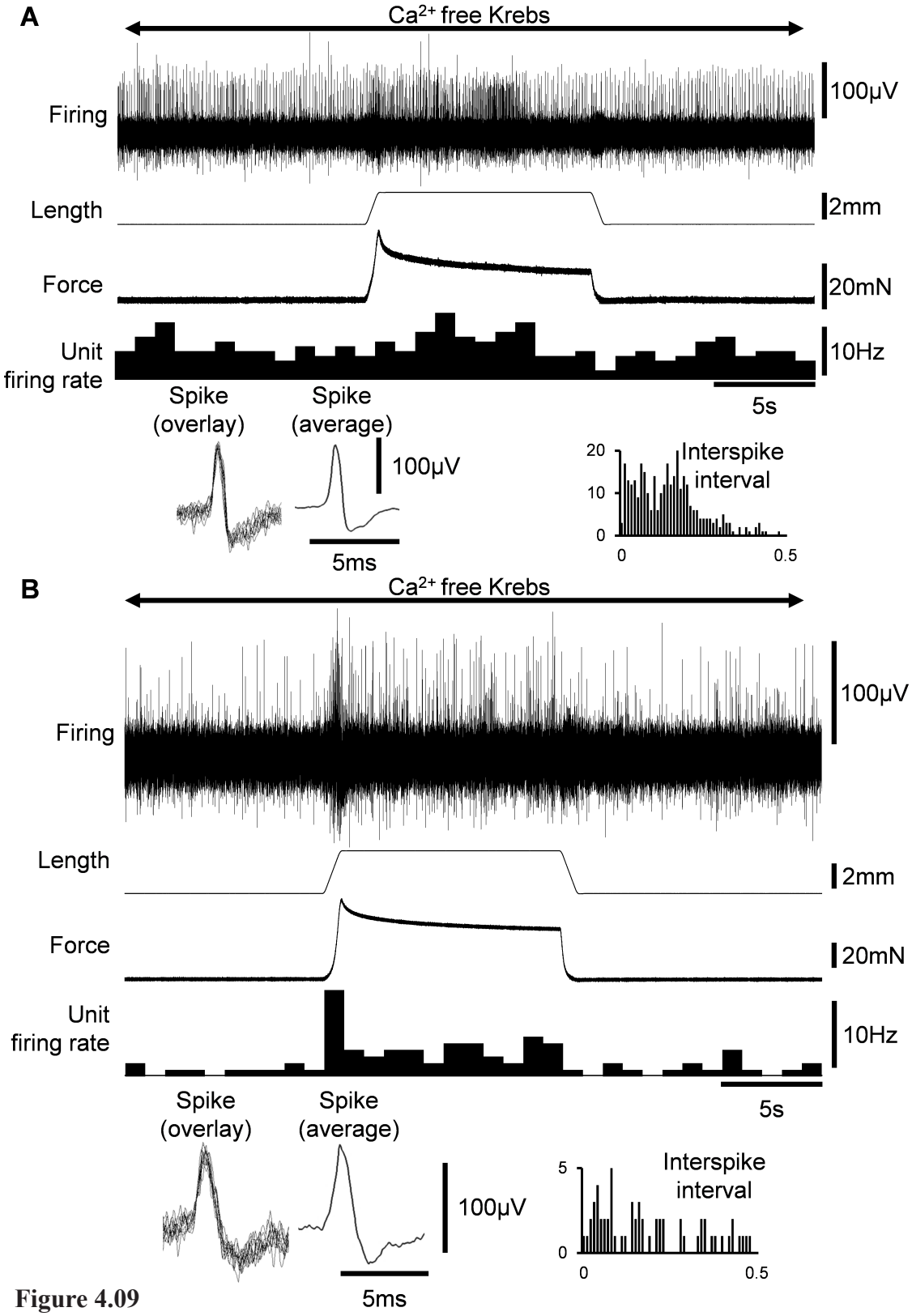

Figure 4.09

The effect of imposed circumferential displacement on firing in Ca^{2+} free Krebs solution. A Ca^{2+}-free Krebs solution was used to block synaptic transmission and paralyse smooth muscle. Note the absence of active increases in tension evoked by distension in **A** and **B**. Tension peaked at the onset of stretch and declined exponentially to a level above resting tension. **A** and **B** show examples of 3 and 4mm distensions, respectively. Both examples show modestly increased firing rate during distension. This suggests that viscerofugal neurons are directly mechanosensitive to distension.

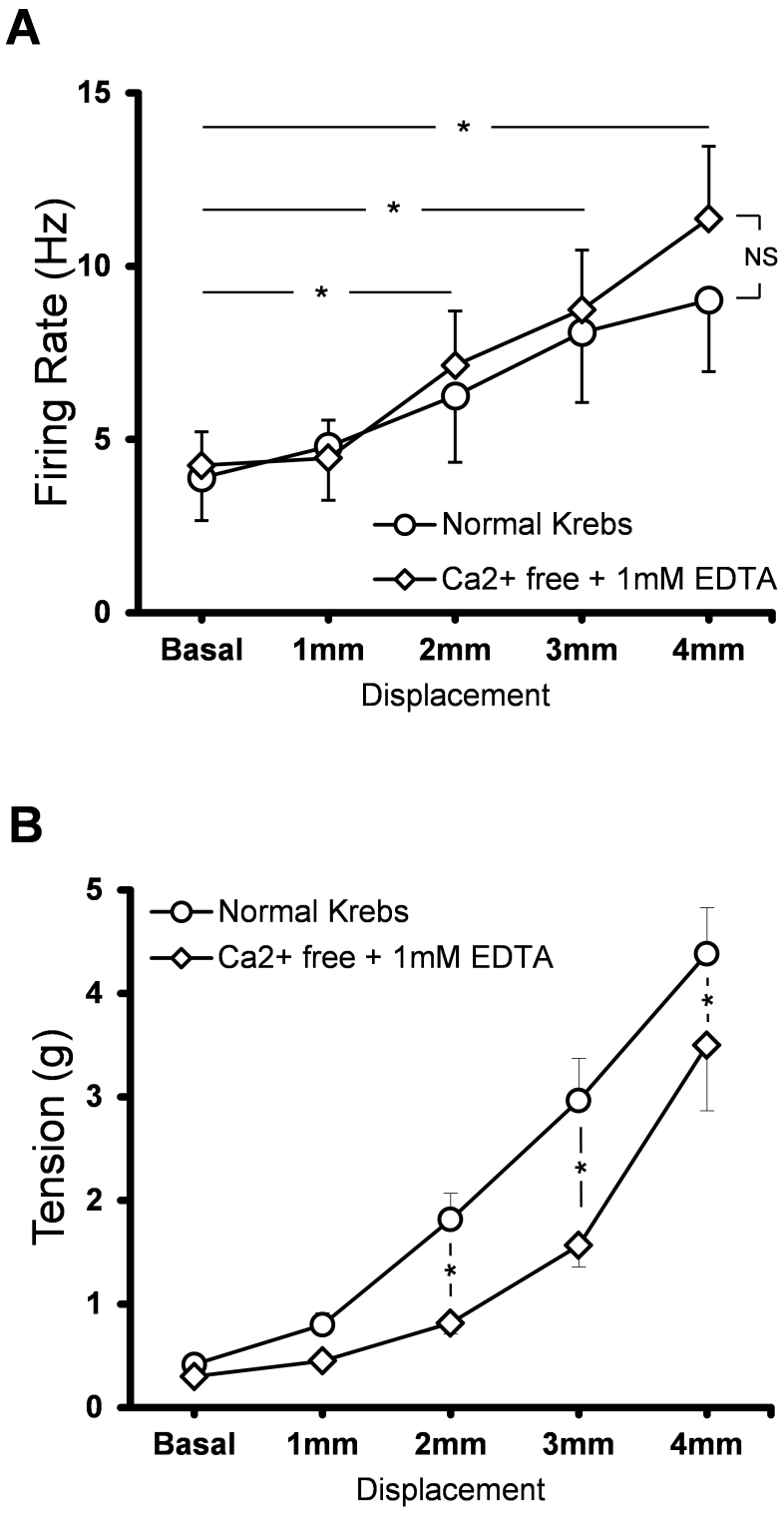

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Figure 4.10

Effect of imposed circumferential displacement on firing rate and tension in normal Krebs solution and in Ca^{2+} free Krebs solution. (**A**) Imposed circumferential displacements of up to 4mm evoked significantly increased firing rate in viscerofugal neurons in distensions 2mm and greater ($p<0.05$, 13 units, n=6, bonferroni post-test, 2 way ANOVA). Firing responses to distensions carried out in Ca^{2+}-free Krebs solution were not significantly different to normal Krebs solution, suggesting that viscerofugal neurons were directly mechanosensitive. Shown in **B** is the effect of imposed circumferential displacement on the tension in the circumferential axis. In both conditions, increasing amounts of displacement produced greater tensions. However, the tension of preparations in Ca^{2+}-free Krebs solution was significantly less than in normal Krebs solution, for displacements of 2mm and greater ($p<0.001$, 2-way ANOVA). This is probably the result of the inability of circular muscle to generate force in the Ca^{2+}-free Krebs solution. Note that despite the significantly decreased tension produced by circumferential displacement in Ca^{2+}-free Krebs solution, firing under these conditions was not significantly different from control. This is compatible with sensitivity to the changes in the length of the tissue, rather than the tension.

Studies of isometric and isotonic contractions

During distension by imposed load, the circumferential length of the preparation necessarily changes in parallel. Likewise, when preparations were stretched by imposing circumferential length changes in the tissue, the intramural tension increased in parallel. To test whether viscerofugal neurons were specifically sensitive to changes in tension, we held preparations at a constant length and increased tension pharmacologically by applying the L-type calcium channel agonist BAY K8644 (1μM). This series of experiments was performed using the setup shown in **figure 4.03B**. BAY K8644 (1μM) increased circular muscle contractility under near-isometric conditions and augmented mean tension to approximately 250% of the basal tension (25±10 mN basal, compared to 60 ± 25 mN in 1μM BAY K8644, $p<0.001$ paired t-test, n=6). Firing of viscerofugal neurons (7 units, n=6) did not change significantly despite the significant increase in tension (2.0±2.3Hz control; 2.0±2.0Hz in BAY K8644, p=0.7 paired t-test, 7 units, n=6, **figure 4.11**).

To assess the specific effect of circumferential length on viscerofugal neuron firing, preparations were held under conditions of constant load, while contractions of the circular muscle were stimulated by BAY K8644. Preparations (n=5) were attached circumferentially to an isotonic transducer and length recorded under fixed loads (1-5 g). After a control period, BAY K8644 (10μM) was added to the recording chamber, evoking summating phasic contractions which shortened the preparation significantly (by 36±7% of total circumferential length). Under these conditions, viscerofugal neuron firing decreased during periods of shortening and increased when the circumference lengthened (**figure 4.12**). When tissue length was recorded at each viscerofugal neuron action potential, firing was significantly concentrated at longer

average tissue lengths than randomly generated time points in the same traces ($p<0.001$, n=5, paired t-tests, loads 2-5g, **figure 4.13A**). The mean firing rate was assessed at different lengths of the preparation during BAY K8644-evoked contractile activity. Mean firing rate showed graded increases with tissue length, for any given load ($p<0.001$, 9 units, n=5, Bonferroni post-test, 2 way ANOVA, **figure 4.13B**). The relationship between tissue length and mean firing rate appeared linear ($R^2=0.82$, slope=6.5, $p<0.001$, **figure 4.13C**). Although heavier loads typically result in greater tissue lengths, load *per se* did not significantly affect firing rate (NS, 9 units, n=5, 2 way ANOVA, see **figure 4.13B**).

In pilot experiments, the β adrenergic receptor agonist isoprenaline (1μM) was used to evoke smooth muscle relaxation (Scheid et al., 1979). Changes in muscle length were less reliably evoked by isoprenaline, compared to BAY K8644. However, increases in length evoked by isoprenaline, were also associated with increased firing rate. Examples of increased firing to isotonic lengthening of preparations are shown in **figure 4.14**. Isoprenaline (1μM) and BAY K8644 (10μM) had no significant effect on firing rate when either drug was added to preparations in the presence of a Ca^{2+} free Krebs solution (high Mg^{2+}, 1mM EDTA) to paralyse smooth muscle and block synaptic transmission, (4 units, n=3 $p<0.05$, paired t-test). This suggests that these drugs did not alter viscerofugal neuron firing rate by a direct action on the nerve cell itself.

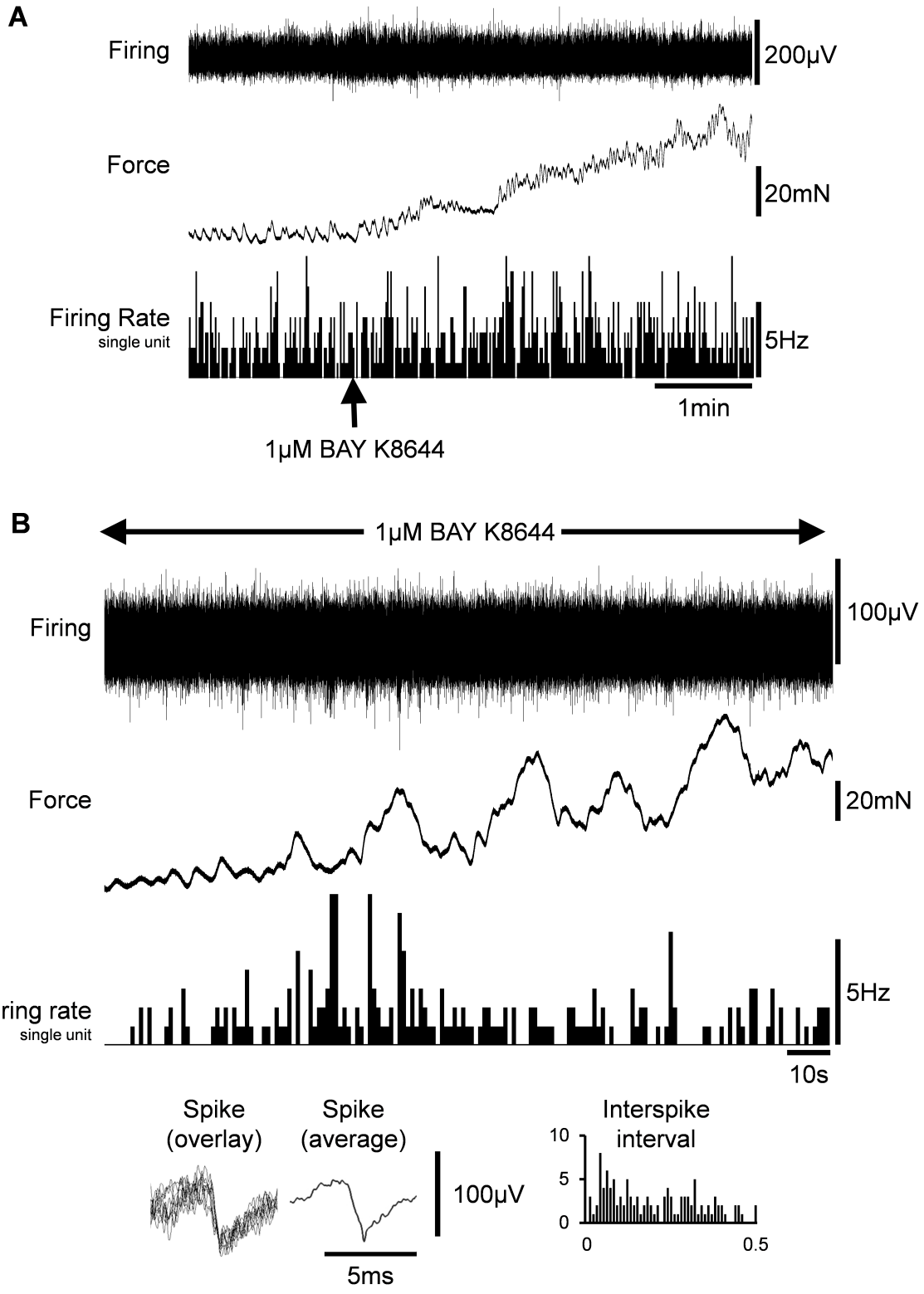

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Figure 4.11

Isometric circular muscle contractions: modulation of circular muscle tension while held at a constant length. Shown in **A** and **B** are examples of two different preparations in which smooth muscle contractions were enhanced by adding the L-type Calcium channel agonist BAY K8644 (1μM) to the organ bath. Preparations were held at a fixed length. After 3-4 minutes for adaptation, BAY K8644 was added. In **A**, the drug is added at the point indicated by the arrow, in **B** the drug was present throughout the period shown by the trace. In both examples, BAY K8644 evoked large increases in circular muscle tension; up to about 5g (~50mN) and 8g (~80mN) in **A** and **B**, respectively. However, the large increases in tension were not accompanied by a change in firing rate. On average, tension typically increased to 2-3 times the resting level, but no increase in viscerofugal neuron firing was observed. This suggests that viscerofugal neurons are not mechanically activated by tension.

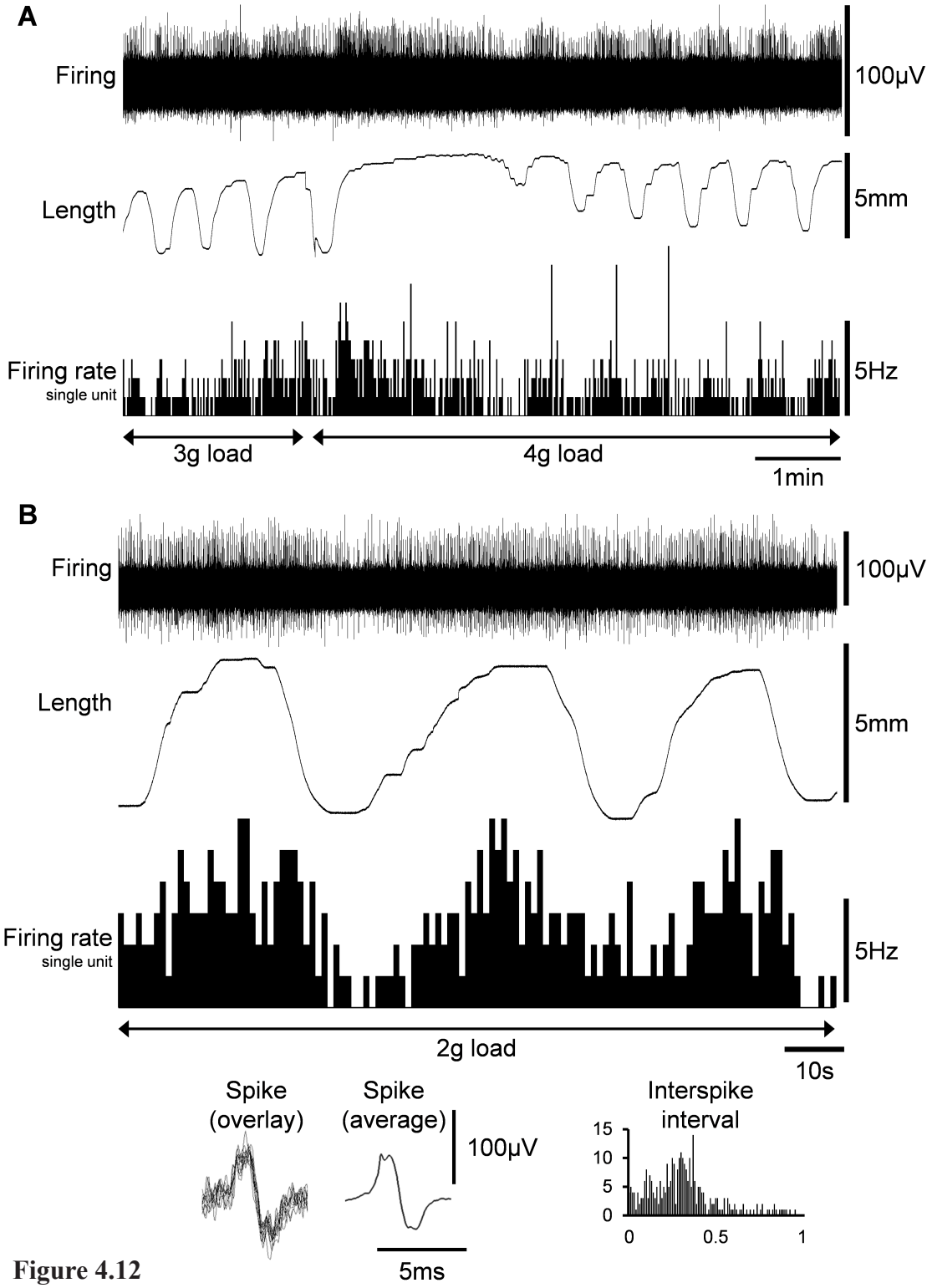

Figure 4.12

Isotonic circular muscle contractions: modulating the length of preparations held under constant tension. Constant distending loads (1-5g) were applied circumferentially. BAY K8644 (10µM) applied to the organ bath, increased phasic contractions. Examples of phasic contractions (downward deflections correspond to decreased length) of the circular muscle are shown in **A** and **B**. In these examples the contractions of the circular muscle caused 3-4mm changes in the length of the preparations. Firing rate was increased at greater lengths, and markedly inhibited around the peak of contractions, when the circular muscle was at its shortest. These results suggest that viscerofugal neurons are mechanically sensitive to circumferential length.

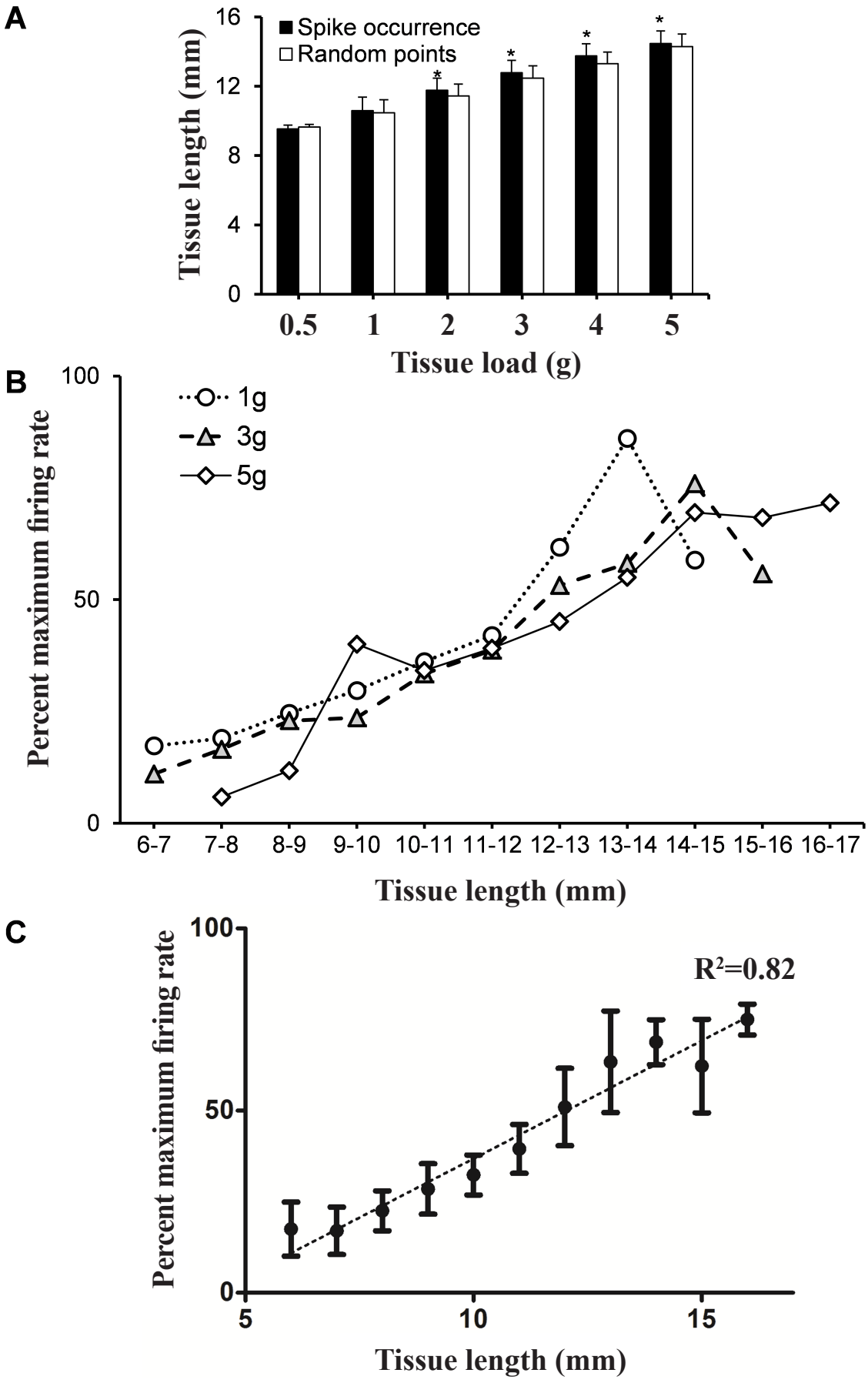

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Figure 4.13

The effect of circumferential tissue length on viscerofugal neuron firing rate. (**A**) This figure shows that action potentials of viscerofugal neurons occurred at significantly greater tissue lengths than expected by chance. At each tissue load (0.5-5g), the length at which each viscerofugal action potential occurred was averaged. These values were compared to random timepoints in the same traces, under the same load. Viscerofugal neurons fired at greater tissue lengths than would be expected if they occurred at random, under loads of 2g and higher (*$p<0.001$, paired t-tests). (**B**) In this figure, viscerofugal neuron firing rate during contractile activity was plotted against the circumferential tissue length, while under different maintained loads (1, 3, 5g). Firing rate increased significantly with length ($p<0.001$, 9 units, n=5, Bonferroni post-test, 2 way ANOVA). Load did not significantly affect the firing rate (NS, 9 units, n=5, 2 way ANOVA). The lack of a significant effect of load on firing rate is seen in this graph by comparing the change in firing rate between the different loads (1, 3, 5g). This suggests that viscerofugal neurons were sensitive to circumferential length, not tension. (**C**) This graph shows the effect of circumferential tissue length on viscerofugal neuron firing rate, regardless of load. The relationship between length and firing rate was linear ($R^2=0.82$, slope=6.5, $p<0.001$).

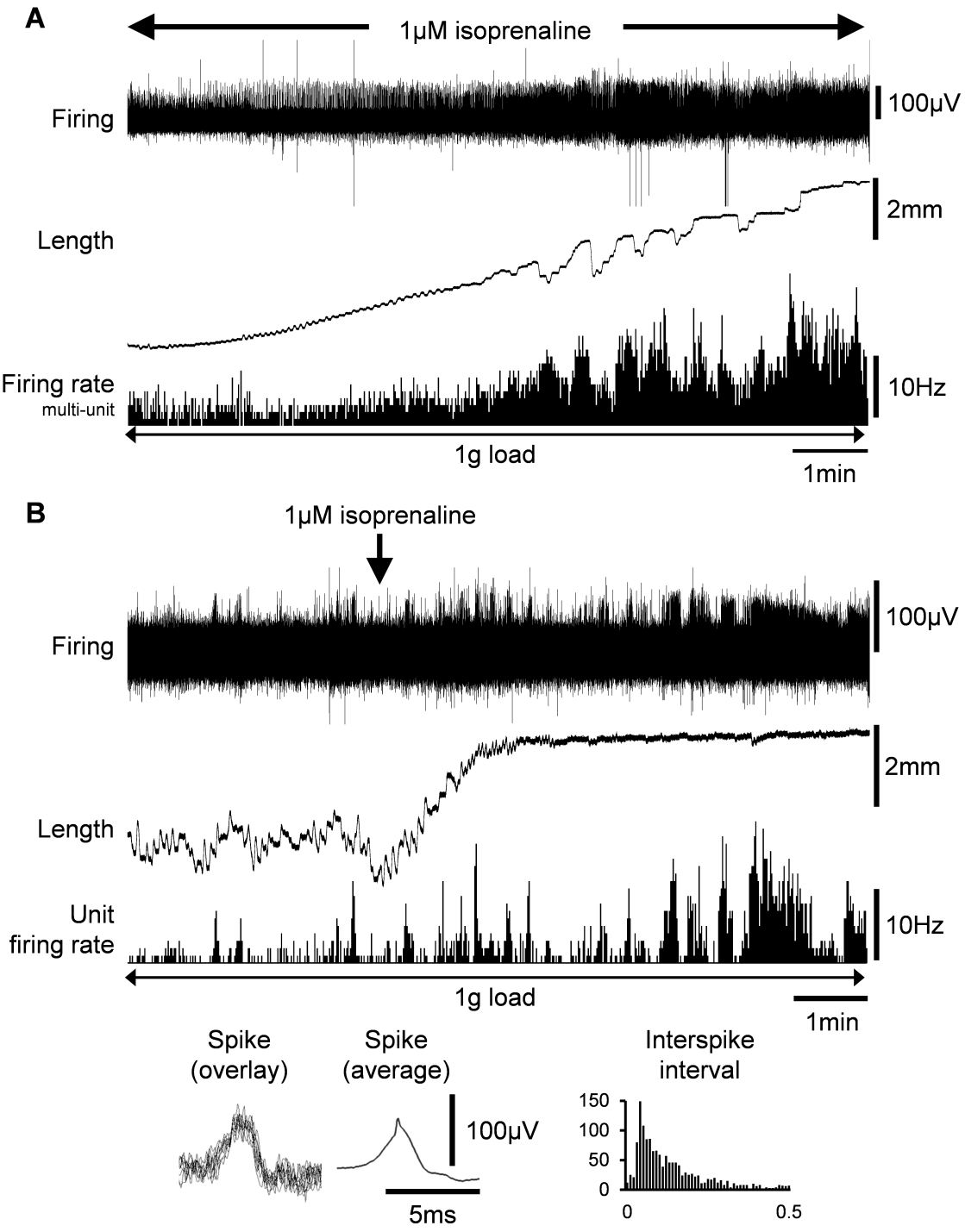

Figure 4.14

Pharmacologically increasing the length of the circular muscle under constant tension evoked increased firing rates. Shown in **A** and **B** are examples of isotonic relaxations that were evoked by the non-selective β adrenergic receptor agonist isoprenaline (1μM). Increased firing rates can be seen in both examples as the circular muscle lengthened. A 1g counterweight was applied in both examples. Later application of isoprenaline in a Ca^{2+} Krebs solution did not significantly affect firing rate, suggesting that the effect of isoprenaline was not a direct pharmacological action on viscerofugal neurons.

Studies of circumferential and longitudinal isotonic distensions

In a previous series of experiments (**chapter 2**), distension by loads up to 4g did not significantly affect viscerofugal neuron firing rate when applied in the longitudinal axis. We were interested in whether greater distending loads applied to the longitudinal axis could evoke mechanosensory firing. In addition, it is possible that the receptive field of viscerofugal neurons may sometimes lie outside the area of the preparation directly affected by the load. This could arise due to the arrangements of pins required to immobilise colonic nerve trunks during electrophysiological recordings. By using focal ejection of DMPP (**figure 4.01**), we could approximate the location of viscerofugal neuron cell bodies in the tissue. This would allow us to see whether either circumferential or longitudinal distensions actually affected the receptive field of viscerofugal neurons. Thus, we set up preparations with isotonic transducers attached circumferentially and longitudinally (schematic diagram in **figure 4.15**). The locations of viscerofugal nerve cell bodies were identified by focal DMPP application after a desensitizing bolus of capsaicin (6 units, basal firing rate 1.8 ± 1.1Hz, n=6). Each unit included in this series of experiments responded to DMPP at a single site on the tissue which was aligned with both arrays of hooks used to apply distensions. Preparations lacking appropriately located DMPP-responsive sites were rejected. To assess direct mechanosensory responses, all experiments were carried out in Ca^{2+} free Krebs solution (high Mg^{2+}, 1mM EDTA), as in the circumferential stretch and focal mechanical probing studies above.

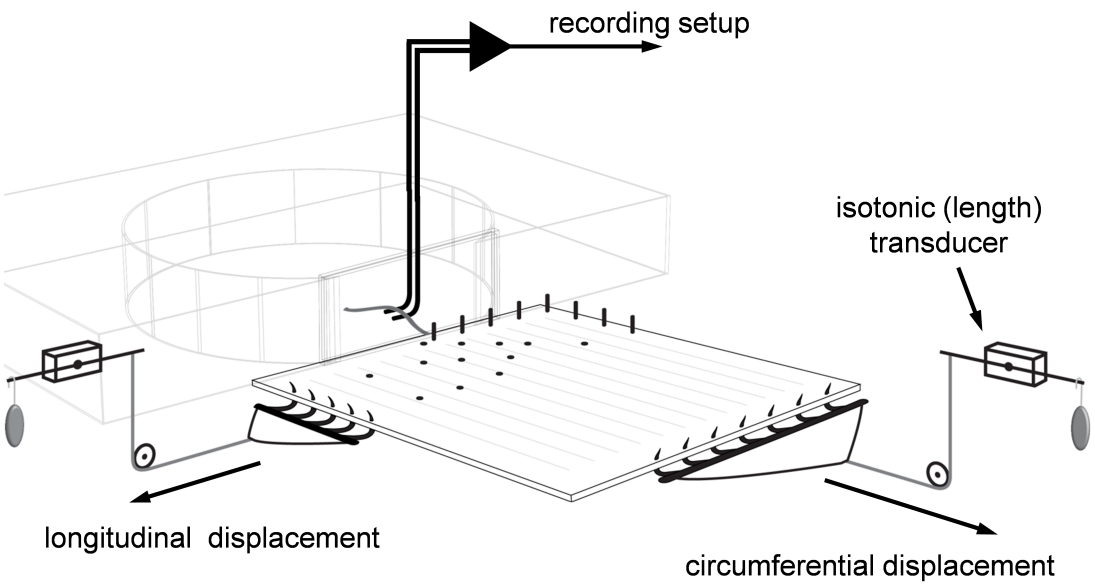

Figure 4.15

Experimental setup used for performing biaxial distensions of the guinea pig colon. The circumferential and longitudinal axes of each preparation were connected to an isotonic transducer via cotton threads and an array of hooks. The transducers measured changes in length of the preparations in their corresponding axes. Varying distending loads could be applied to the contralateral arms of the transducers. Loads of 1, 3, 6 and 9g were applied to each axis alone or in combinations. The preparations were pinned in line with the array of hooks on the opposing side. Multiple graphite markers were applied to the surface of the circular muscle. These markers served as reference points as described in **chapter 3.** Focal ejection of DMPP from a micropipette was used to localize viscerofugal neuron cell bodies as they were previously demonstrated statistically to correspond to viscerofugal neuron cell bodies. DMPP-responsive sites were marked on micrographs of the preparation.

Uniaxial distensions

Single distending loads of 1, 3, 6, or 9g were applied to preparations in either the circumferential or longitudinal axis. Loading evoked a graded lengthening of the preparation in the axis of the applied load, while usually shortening the preparation in the orthogonal axis (the "Poisson effect"). As reported above in studies of circumferential distension alone, firing was evoked by distension applied circumferentially (**figure 4.16A**). Firing was also evoked by longitudinal distensions (**figure 4.16B**). The increases in firing rates evoked by both circumferential and longitudinal distensions were statistically significant ($p<0.05$, 2-way repeated measures ANOVA, n=6, **figure 4.17A**). Circumferential distensions tended to evoke higher rates of firing than longitudinal distensions but the difference was not significant with a sample of n=6 ($p>0.05$, 2-way repeated measures ANOVA).

To determine whether the distending effects of loads were the same in both axes, we quantified tissue strains, calculating the displacement of the tissue as a proportion of its resting length, thus normalizing data across preparations. Strain was calculated thus:

$$\varepsilon_{circ} = \frac{\Delta l_{circ}}{l_{circ}}, \text{ and } \varepsilon_{long} = \frac{\Delta l_{long}}{l_{long}}$$

Here, ε_{circ} and ε_{long} are the circumferential and longitudinal strains respectively; Δl_{circ} and Δl_{long} are the displacements evoked by loading; and l_{circ} and l_{long} are the resting lengths of the preparations.

Predictably, increasing loads evoked significant increases in strain in the same axis of the applied load, and significantly decreased strain in the perpendicular axis ($p<0.001$, 2-way repeated measures ANOVA, n=6). The gut was significantly more compliant in the circumferential axis than in the longitudinal axis. Applied loads evoked significantly greater strain in the circumferential axis, compared to the same loads in the longitudinal axis ($p<0.05$, 2-way repeated measures ANOVA, n=6, **figure 4.17B**). Thus, the greater strains evoked by circumferential loading may partly explain why slightly higher firing rates were observed during circumferential loading. When firing rate was plotted against strain, the differences between the circumferential and longitudinal axes were abolished (see **figures 4.17C-E**).

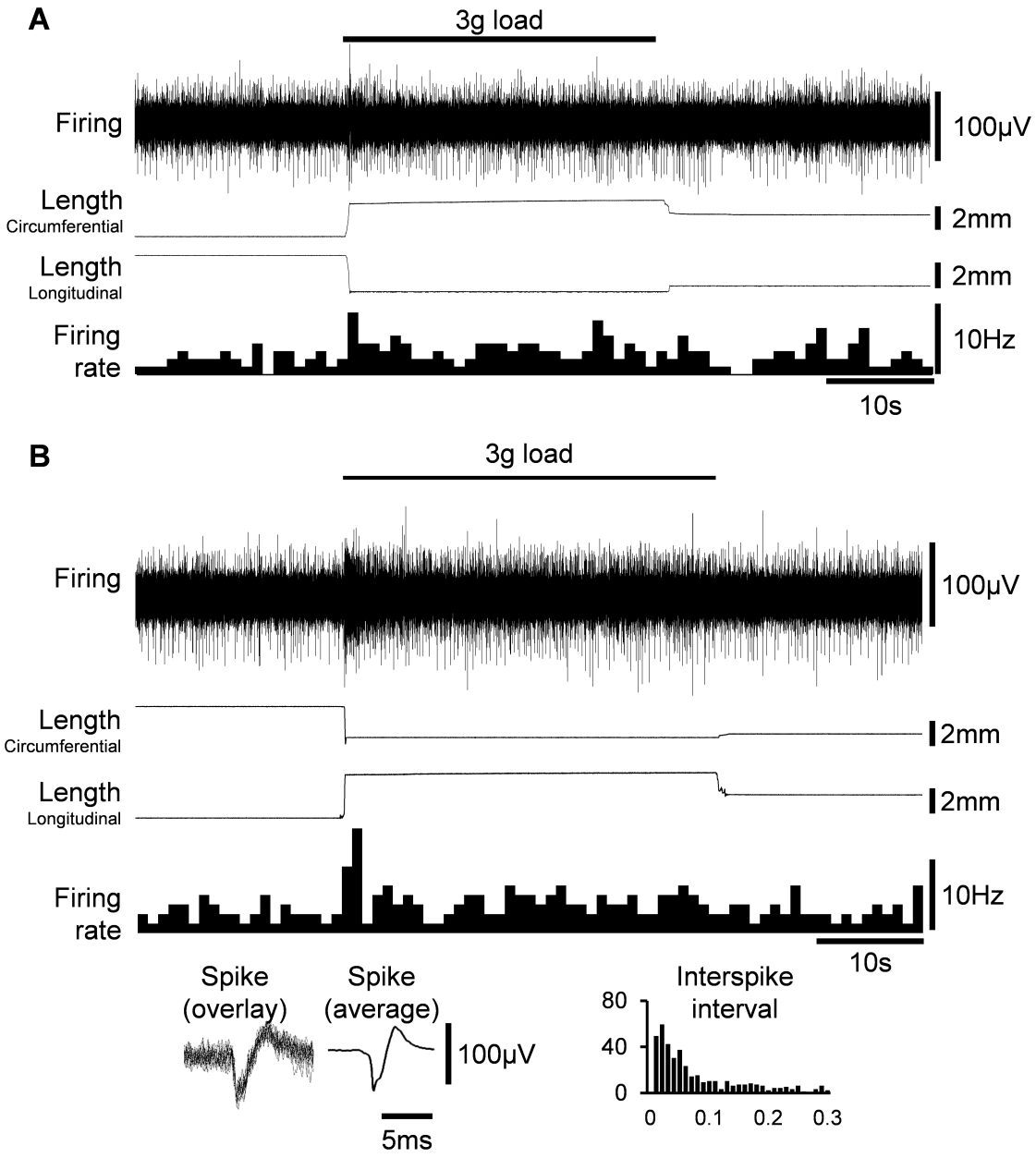

Figure 4.16

Examples of isotonic distensions applied in the circumferential and longitudinal directions. A circumferential distension is shown in **A**. Note the circumferential axis is lengthened by the load while the longitudinal axis is shortened. The distension evoked a modest increase in the firing rate throughout the distension. Shown in **B** is an example of a 3g longitudinal distension. Note the lengthening of the longitudinal axis and concomitant shortening of the circumferential axis (the "Poisson" effect). This distension evoked brief burst of firing evoked at the onset of the longitudinal distension.

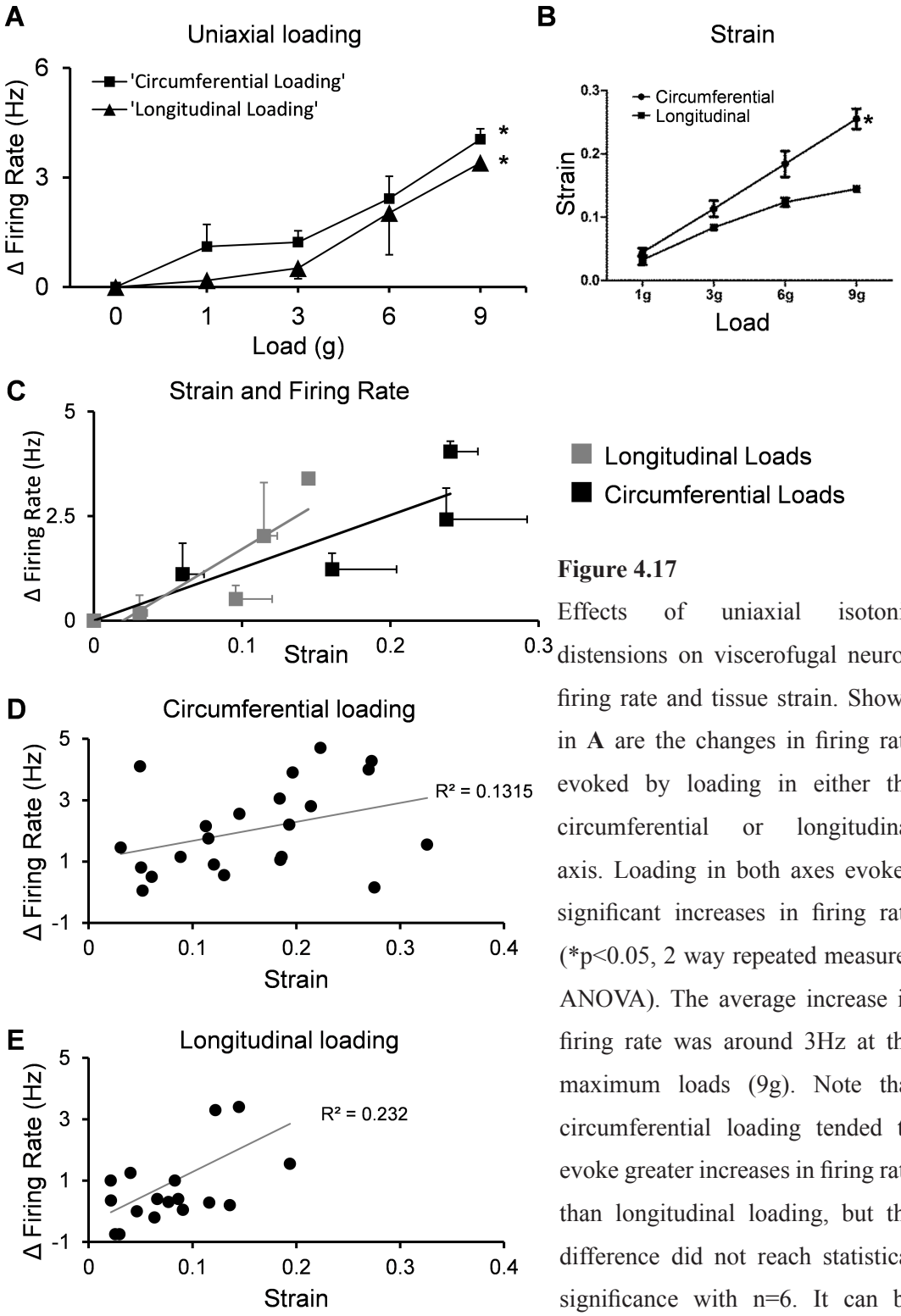

Figure 4.17

Effects of uniaxial isotonic distensions on viscerofugal neuron firing rate and tissue strain. Shown in **A** are the changes in firing rate evoked by loading in either the circumferential or longitudinal axis. Loading in both axes evoked significant increases in firing rate (*$p<0.05$, 2 way repeated measures ANOVA). The average increase in firing rate was around 3Hz at the maximum loads (9g). Note that circumferential loading tended to evoke greater increases in firing rate than longitudinal loading, but the difference did not reach statistical significance with n=6. It can be seen in **B** that loads applied in the circumferential axis evoked greater tissue strains than the same loads applied longitudinally (*$p<0.05$, 2 way repeated measured ANOVA). This may underlie the small differences observed in **A**. When the average tissue strains were plotted against firing rates there was no difference in the relationship whether strains were evoked by circumferential or longitudinal loads, as shown in **C**. All individual cases of load evoked tissue strain are plotted against the change in firing rate for circumferential and longitudinal loading in **D** and **E**, respectively. As in **C**, these show a general increase in firing rate with increasing tissue strain. Note the smaller strains evoked by longitudinal loads in **C** and **E**.

Biaxial isotonic distensions

In addition to distensions in a single axis, preparations were also distended biaxially. That is, combinations of loads were applied, at the same time, to the circumferential and longitudinal axes (1, 3, 6, and 9g loads; examples shown in **figure 4.18**). Biaxial loads significantly increased total tissue strain ($p<0.001$, 2 way ANOVA; **figure 4.19** and **figure 4.20**). However, as in uniaxial distensions, the circumferential loads evoked significantly greater tissue strains than longitudinal loads (circumferential loads: $F=61.05$, longitudinal loads: $F=23.95$; **figure 4.19** and **figure 4.20**). Mechanosensitive firing was evoked by biaxial loading (**figure 4.18** and **figure 4.21**). Probably due to the higher strains evoked by circumferential loads in biaxial distensions, circumferential loads appeared more effective than longitudinal loads in increasing firing rate (**figure 4.21**). Overall, the effect of increasing circumferential loads on firing rate was significant ($p<0.05$, 2-way ANOVA, $n=6$), but the effect of longitudinal loads was not significant ($p=0.08$, 2 way-ANOVA, $n=6$). The greatest increases in firing rate occurred where combined strain was greatest; the peak average firing rate occurred with the greatest total combined loads (9g x 9g; see **figure 4.20** and **figure 4.21**). Regression analysis revealed the strongest linear relationship was between the combined tissue strain of both axes (circumferential + longitudinal) and the change in firing rate (slope 12.7 ± 4.0, adjusted $R^2=0.276$, $F=9.8$, $p<0.01$; **figure 4.22**). By comparison, weaker relationships were seen between firing rate and strain in either single axis (circumferential: slope 3.9 ± 3.0, adjusted $R^2=0.073$, $F=1.7$, $p=0.2$; longitudinal: slope 4.6 ± 4.0 adjusted $R^2=0.057$, $F=1.3$, $p=0.3$; **figure 4.22**). This suggests that viscerofugal neurons are sensitive to the total tissue strain, regardless of direction.

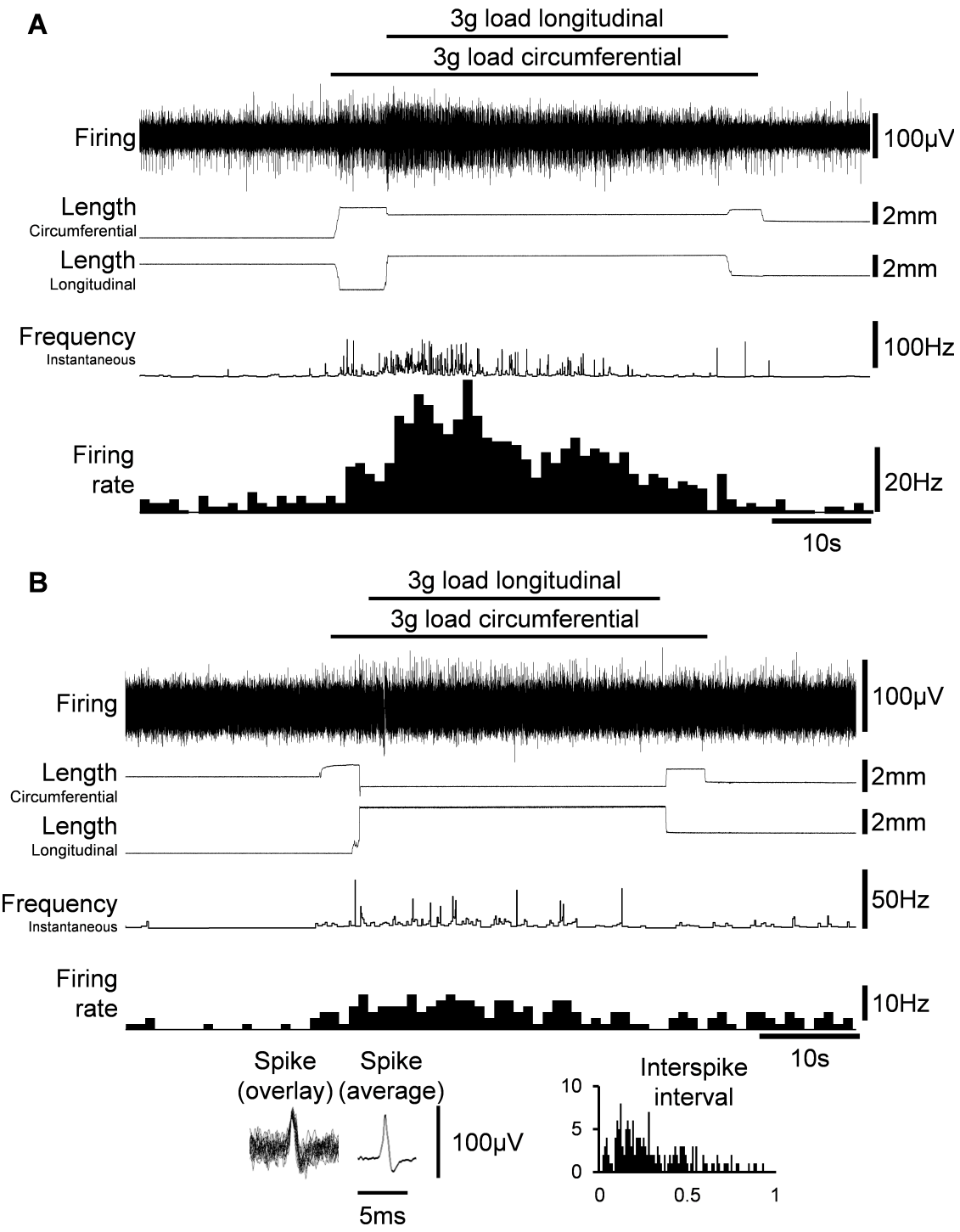

Figure 4.18 see next page for figure legend

Figure 4.18

Examples of biaxial isotonic distensions. These examples show combined 3g circumferential and longitudinal distensions in two different preparations (**A**) In this example, a 3g load is first applied to the circumferential axis, followed by the addition of 3g to the longitudinal axis. Note the addition of a longitudinal load reduces tissue length in the circumferential axis. The firing rate clearly increases upon the addition of both loads and appears to adapt slowly during the maintained distension. A separate preparation is shown in **B**. Here, a more modest increase in firing rate is observed under the same loading conditions. However, increases in firing rate can again be seen at the addition of loads in both axes; circumferential loading increases firing rate modestly, and there is a brief burst of firing on the addition of the longitudinal load, seen in the trace of instantaneous frequency. There is also a slow adaptation of firing rate over the course of the distensions, which lasted approximately 30s. Shown below is the shape of the discriminated unit as an overlay of several spikes, and shown as an average of all spikes in the trace (189 spikes). The interspike interval is also shown here, showing spike intervals consistent with a single unit firing.

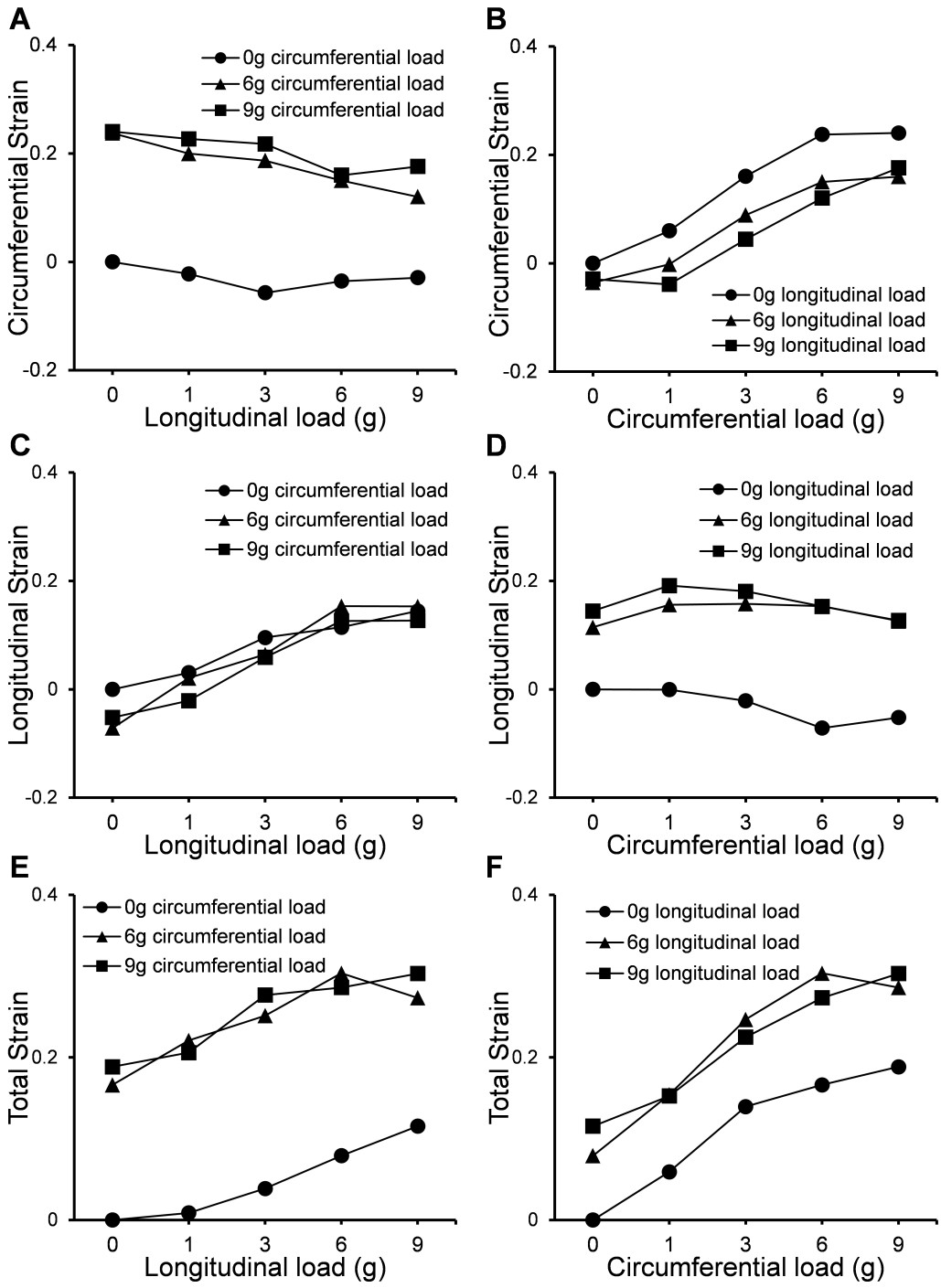

Figure 4.19

The effect of biaxial loading on tissue strain. **A**, **C** and **E** summarizes the effect of adding longitudinal loads on strain in the circumferential axis (**A**), the longitudinal axis (**C**), and the combined strain of both axes (**E**). Note how longitudinal loads increasingly decrease circumferential strain (the "Poisson effect"), and increases longitudinal and total strain. These are shown under varying circumferential loading conditions (0, 6 and 9g). The converse is shown in **B**, **D** and **F**. Circumferential loading increased circumferential and total strain. Note that the Poisson effect was less pronounced for circumferential loading (**D**). It can be seen by comparing **E** and **F** that the addition of circumferential loads (**F**) evoked greater increases in total strain than longitudinal loads (**E**).

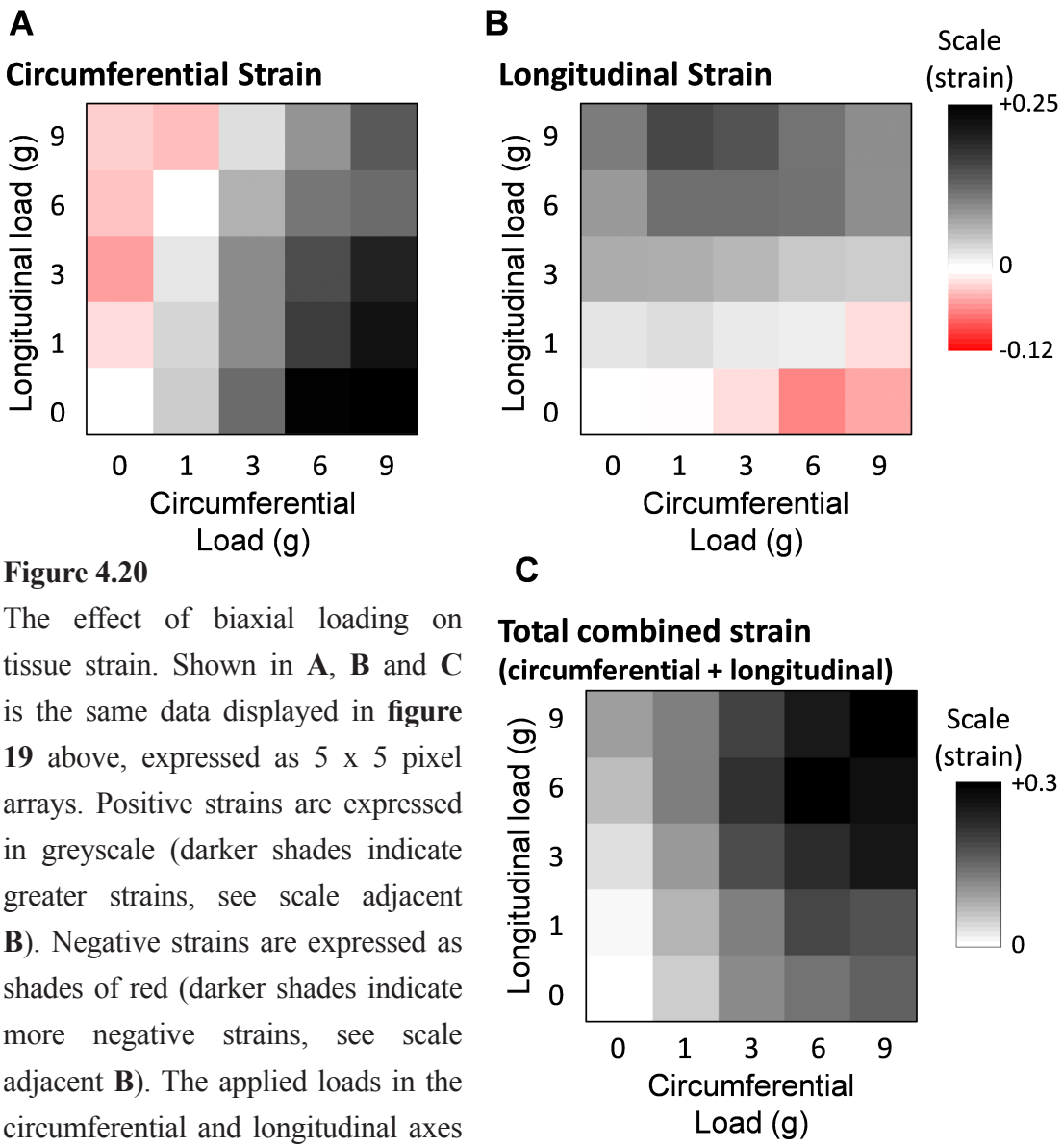

Figure 4.20

The effect of biaxial loading on tissue strain. Shown in **A**, **B** and **C** is the same data displayed in **figure 19** above, expressed as 5 x 5 pixel arrays. Positive strains are expressed in greyscale (darker shades indicate greater strains, see scale adjacent **B**). Negative strains are expressed as shades of red (darker shades indicate more negative strains, see scale adjacent **B**). The applied loads in the circumferential and longitudinal axes are expressed on the X and Y axes, respectively. Circumferential strains are shown in **A**. Increasing circumferential loads can be seen to increase circumferential strain, regardless of the longitudinal load (squares are increasingly darker moving from left to right). Note how increasing longitudinal loads reduce circumferential strain (squares become lighter, and even negative (red) moving from the lower squares to the top). Longitudinal strains are shown in **B**. Note the increasing longitudinal strain as load increases from the bottom to the top of the array. Circumferential loading tended to decrease longitudinal strain, moving from left to right. By comparing circumferential and longitudinal strain in **A** and **B** (which use the same scale, adjacent **B**), it can be seen that the greatest strains were produced in the circumferential axis, by the greatest circumferential loads. Shown in **C** is the total tissue strain by the addition of strains in the circumferential and longitudinal axis. The more prominent contribution of circumferential loading to total strain, compared to longitudinal loading, is clearly evident in this figure. Note the sharper transition to darker value moving left to right than from the bottom to the top of the array. It may also be seen that the greatest total strains were evoked by the greatest combined loads (9g x 9g).

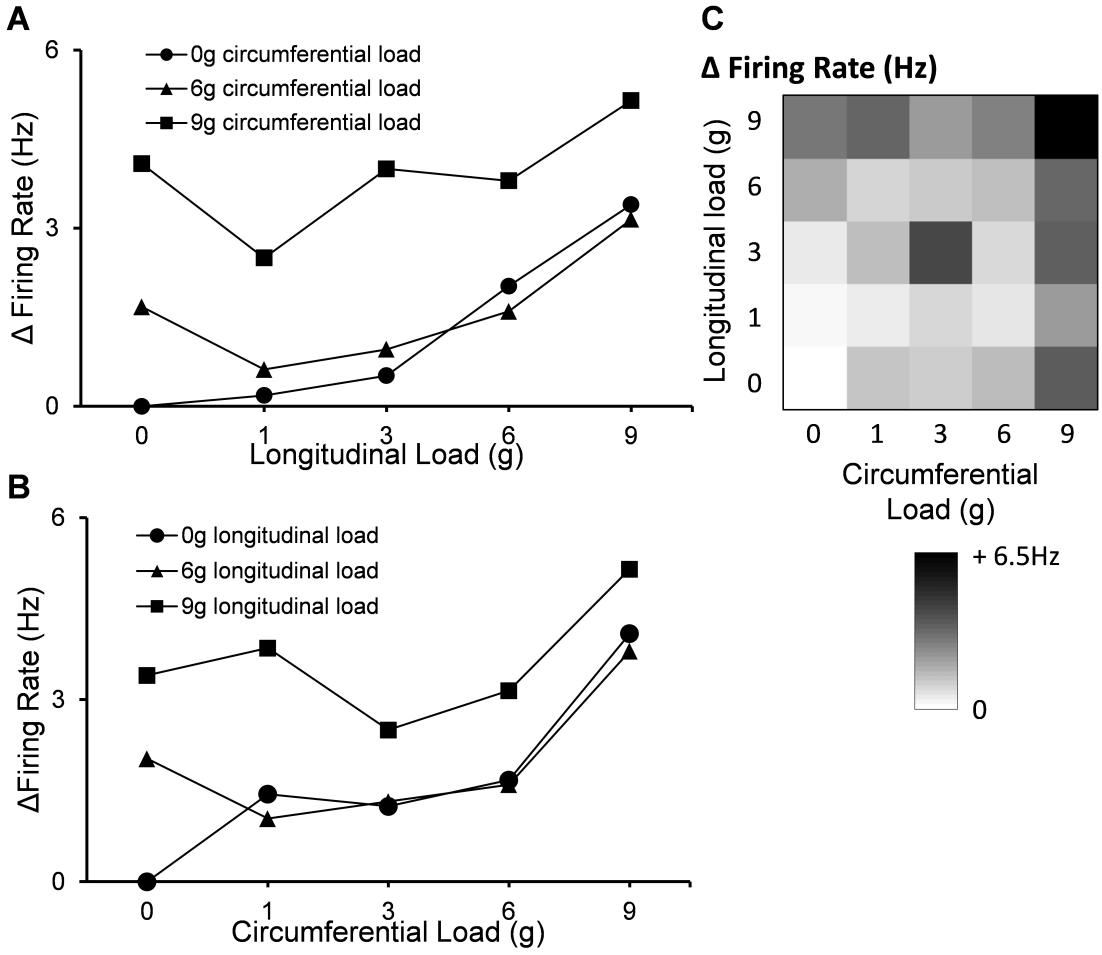

Figure 4.21

The effect of biaxial loading on firing rate. The effect of longitudinal and circumferential loads on firing rate is shown in **A** and **B**, respectively. Note the general trend toward increased firing as the load in either axis increased. The peak average increase in firing rate in both was evoked with the maximum combined loads (9 x 9g). Shown in **C** is the effect of biaxial loading on firing rate expressed in a 5 x 5 pixel array. As in the graphs above, this indicates a general trend toward increased firing as loading increased. The peak average increase in firing was evoked under 9g x 9g loading conditions. This coincided with the maximum tissue strain (see **figure 4.20**).

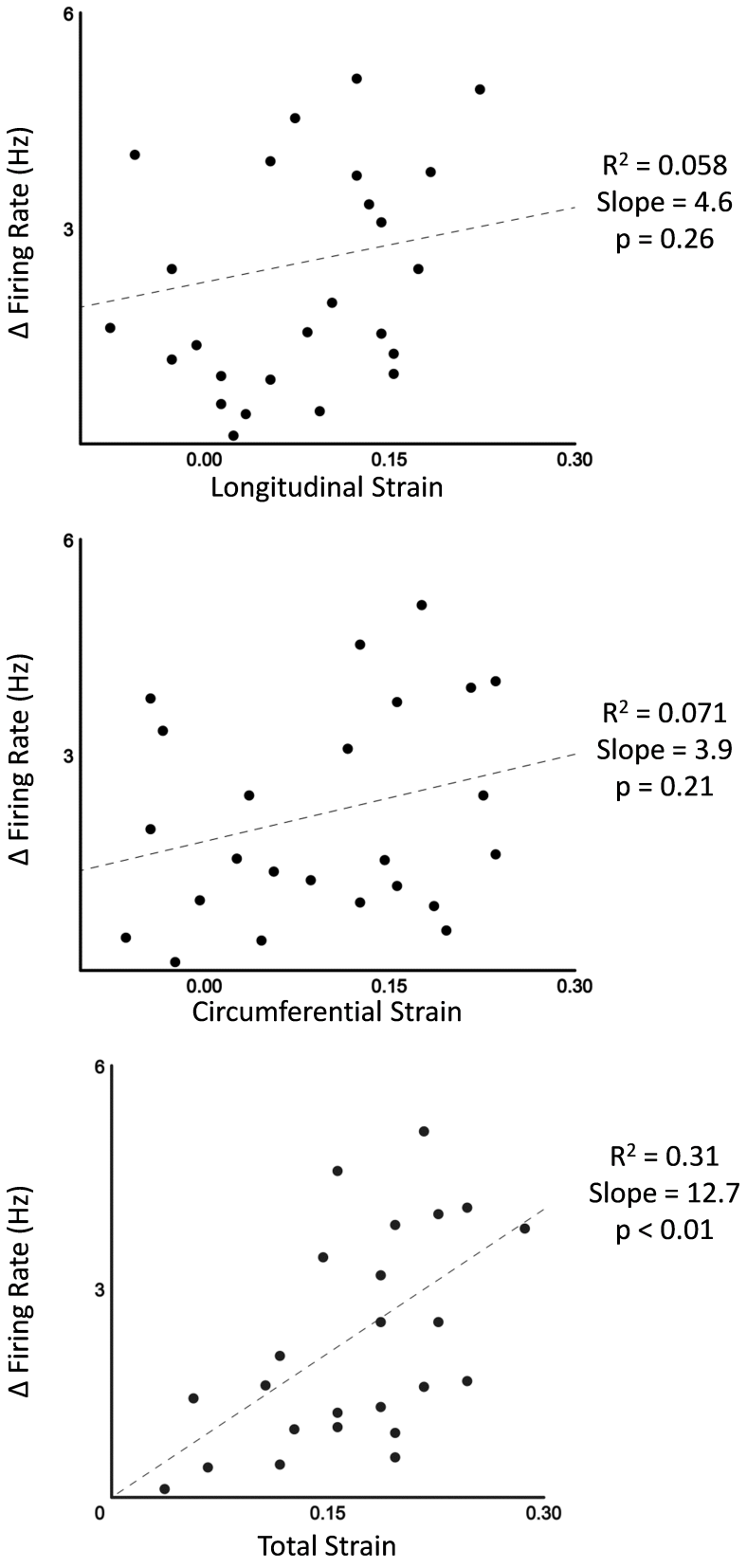

Figure 4.22

Relationship between tissue strain and firing rate. During biaxial distensions, little relationship was seen between strain in either the circumferential and longitudinal axis and the change in firing rate; shown as scatter plots in **A** and **B**, respectively. The strongest relationship was observed between the total tissue strain and change in firing rate, shown in **C**. Data points in each scatter plot represent the mean strains and change in firing evoked under each combination of loads.

Direct effects of receptor agonists on viscerofugal neurons

Several other receptor agonists were tested on viscerofugal neuron firing following studies of isotonic distensions, during synaptic blockade by Ca^{2+} free Krebs solution (high Mg^{2+}, 1mM EDTA). Single units had a basal firing rate of 4.3 ± 2.9Hz (14 units, n=8). The nicotinic receptor agonist DMPP (10μM) evoked a significant increase in firing rate of 13.1 ± 2.4Hz (6 units, n=6, 30s samples, $p<0.05$, paired t-test); serotonin (10μM) increased firing rate by an average of 11.4 ± 5.1Hz (9 units, n=6, $p<0.05$, paired t-test, **figure 4.23B**); the selective NK_2 tackykinin receptor agonist peptide β-ala^8 neurokinin $A^{(4-10)}$ (10nM) increased firing rate by an average of 10.7 ± 12.3Hz (10 units, n=6, $p<0.05$, paired t-test, **figure 4.23A**); the selective P2X receptor agonist αβ methylene ATP (0.1μM) increased firing rate by an average 4.5 ± 5.1Hz (8 units, n=6, $p<0.05$, paired t-test); the non-selective muscarinic receptor agonist oxotremorine (1μM) increased firing rate on average 4.5 ± 4.1Hz (7 units, n=6, $p<0.05$, paired t-test); the glutamate receptor agonist, L-glutamate (100μM), showed no significant effect on firing rate (mean change 0.4 ± 0.8Hz, 8 units, n=6, NS, paired t-test); the adrenergic receptor agonist noradrenaline (10μM) showed predominantly inhibitory effects, significantly decreasing firing rate by an average of 5.1 ± 5.4Hz (10μM, 7 units, n=5, $p<0.05$, paired t-test), however occasional excitatory effects were also seen (see **figure 4.24**). These data are summarized in **figure 4.25**. Controls (see methods) had no significant effect on firing rate (DMSO in Ca^{2+} free Krebs solution, mean change -0.1 ± 0.3, 4 units, n=4, NS, paired t-test; ethanol Ca^{2+} free Krebs solution mean change 0.2 ± 0.3, 3 units, n=3, NS, paired t-test; water in Ca^{2+} free Krebs solution mean change -0.2 ± 0.2, 3 units, n=2, NS, paired t-test).

Spontaneous contractions and burst firing activity

Under isotonic conditions without applied loads, some preparations had regular spontaneous contractions of the circular muscle (7/13 preparations). The spontaneous contractions were small in amplitude (average amplitude: 1.3 ± 0.9mm, frequency 1.8 ± 0.7 per minute, 34 contractions, n=7) in normal Krebs solution. Overall, these contractions were not associated with detectable changes in viscerofugal neuron firing rate.

There were also intermittent periods of burst firing activity in viscerofugal neurons; observed in 4 of 42 preparations (**figure 4.26**). In a single preparation hexamethonium (500μM) was added on two separate occasions during burst firing activity; burst firing was abolished in each case without blocking ongoing firing (**figure 4.26B**).

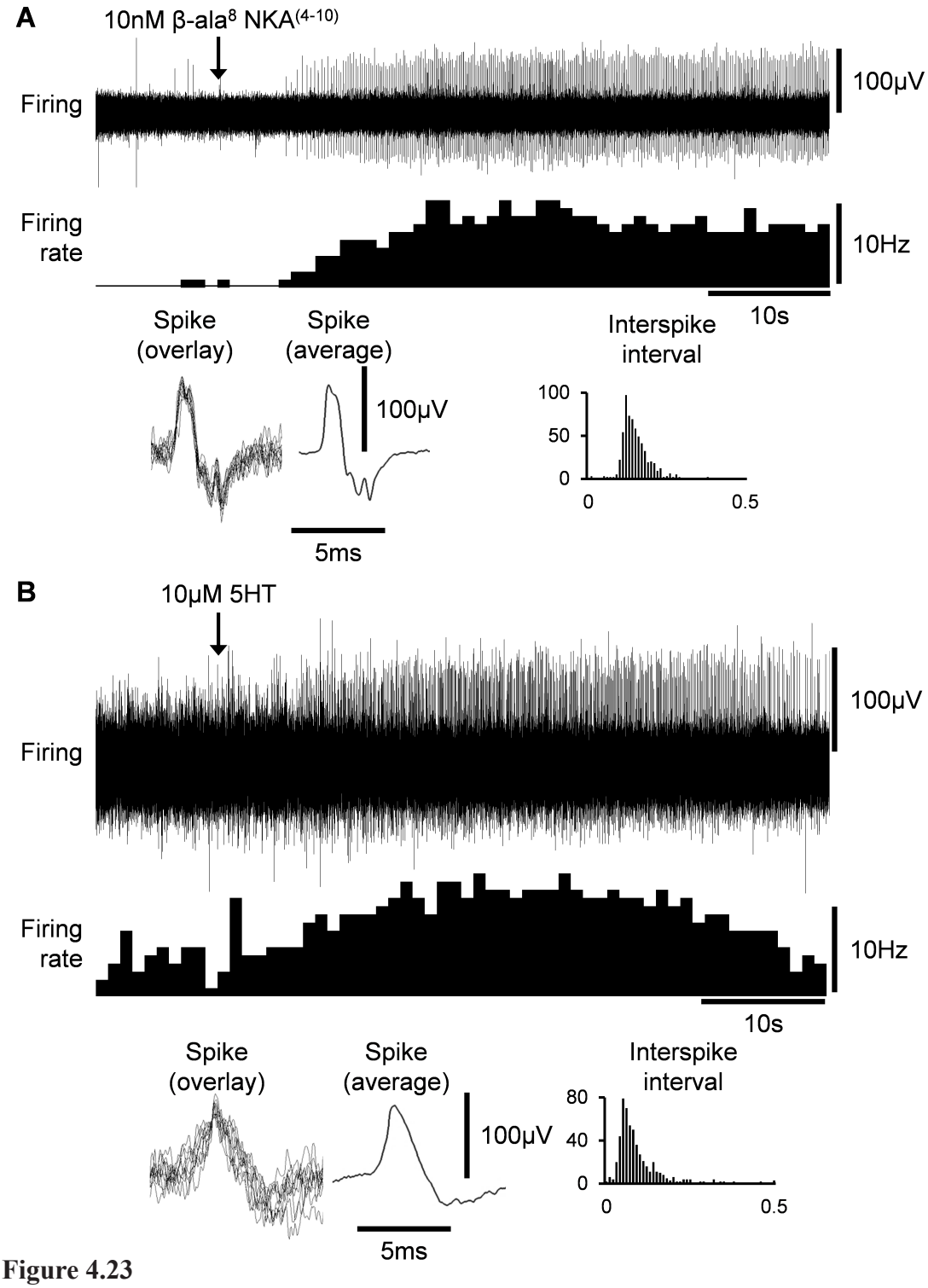

Figure 4.23

Activation of viscerofugal neurons by an NK_2 receptor agonist and serotonin. In preparation for studies of isometric and isotonic contractions, several pharmacological agents were tested after the isotonic stretch experiment in Ca^{2+} free Krebs solution (1mM EDTA). (**A**) The NK_2 receptor agonist β-ala^8 neurokinin A $^{(4-10)}$ has been used to evoke phasic contractions of circular smooth muscle. We found that in Ca^{2+} free Krebs solution, this peptide (10nM, applied direct to the preparation) potently evoked long trains of action potentials in viscerofugal neurons. Thus, we could not use this drug to investigate the mechanosensitivity of viscerofugal neurons. Shown in **B** is the direct effect of serotonin (10μM, final bath concentration) on viscerofugal neuron firing. Serotonin strongly activated viscerofugal neurons.

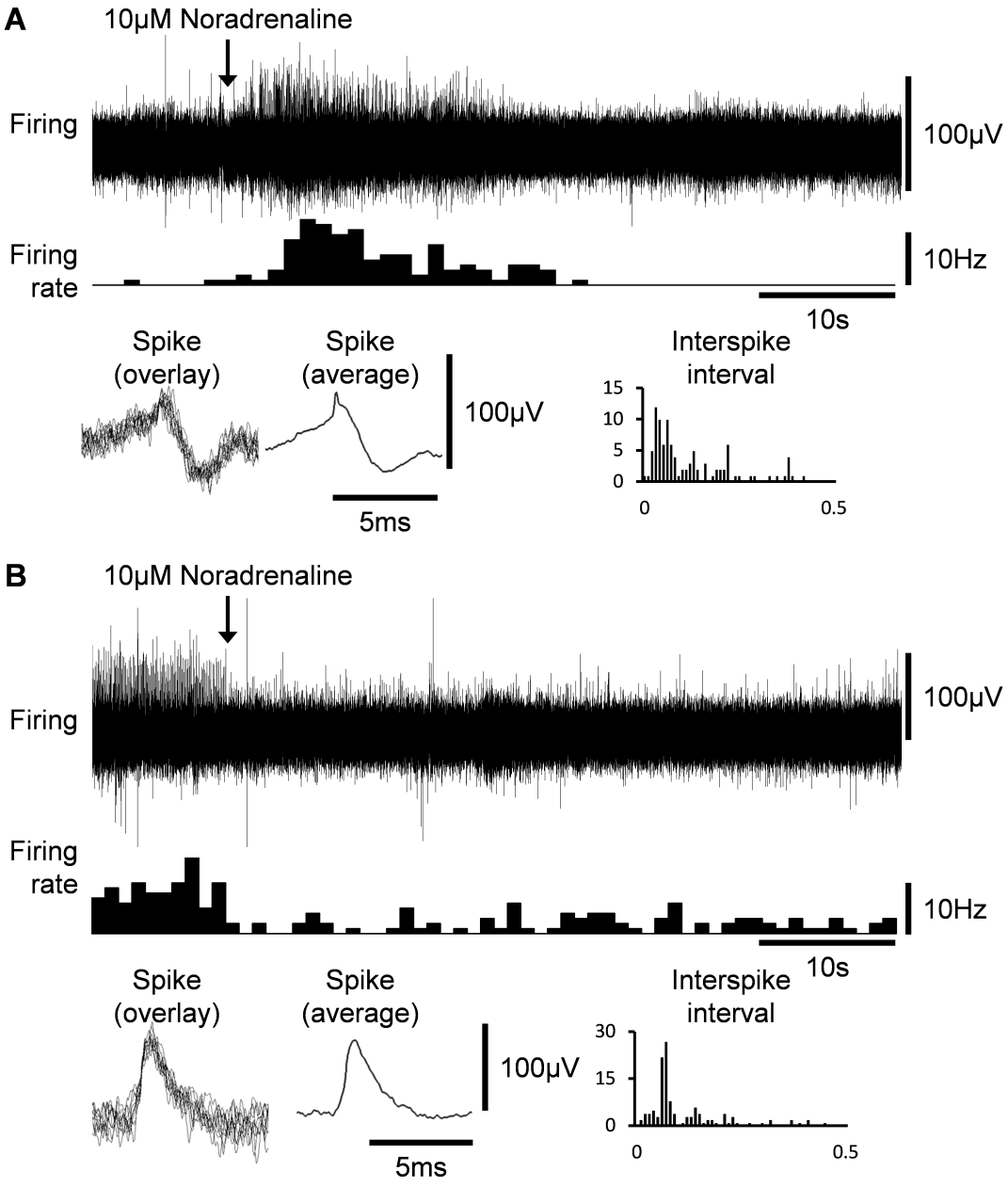

Figure 4.24

Excitation and inhibition of viscerofugal neuron firing by noradrenaline. This figure shows examples of the excitatory (**A**) and inhibitory (**B**) effects of noradrenaline (10μM, final bath concentration) on viscerofugal neuron firing. The inhibition of viscerofugal neuron firing was the most commonly observed effect of noradrenaline (7/10 units tested, n=8).

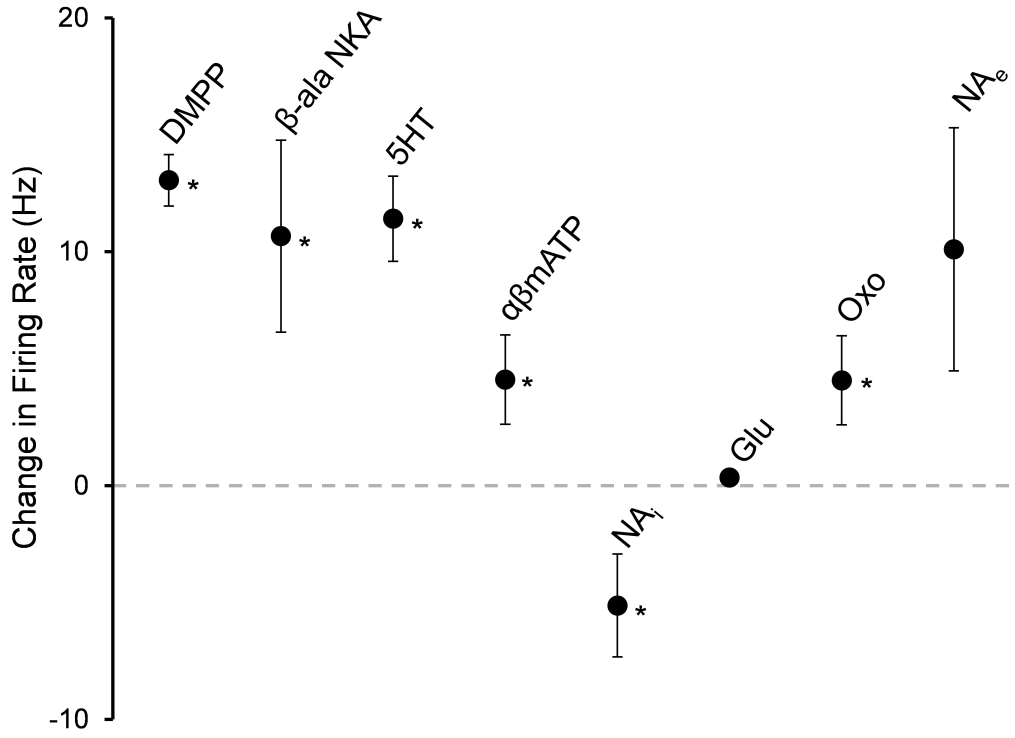

Figure 4.25

The effects of pharmacological agents on viscerofugal neuron firing rate. This graph summarizes the effects of drugs that were tested on viscerofugal neurons in Ca^{2+} free Krebs solution (1mM EDTA). Significant increases in firing rate were evoked by 10μM DMPP, 10nM β-ala^{8} NKA$^{(4-10)}$, 10μM serotonin (5HT), 0.1μM αβmethylene ATP, and 1μM oxotremorine (each n=6, $p<0.05$, paired t-tests); 10μM noradrenaline significantly decreased firing rate (NA_i, n=5, $p<0.05$, paired t-test), except in 3 cells, where excitation occurred (NA_e, n=3, NS, paired t-test). The inhibitory and excitatory effects of noradrenaline were separated into different groups, named "NA_i" and "NA_e", respectively. Lastly, 100μM L-glutamate had no significant effect on firing rate (n=6, NS, paired t-test).

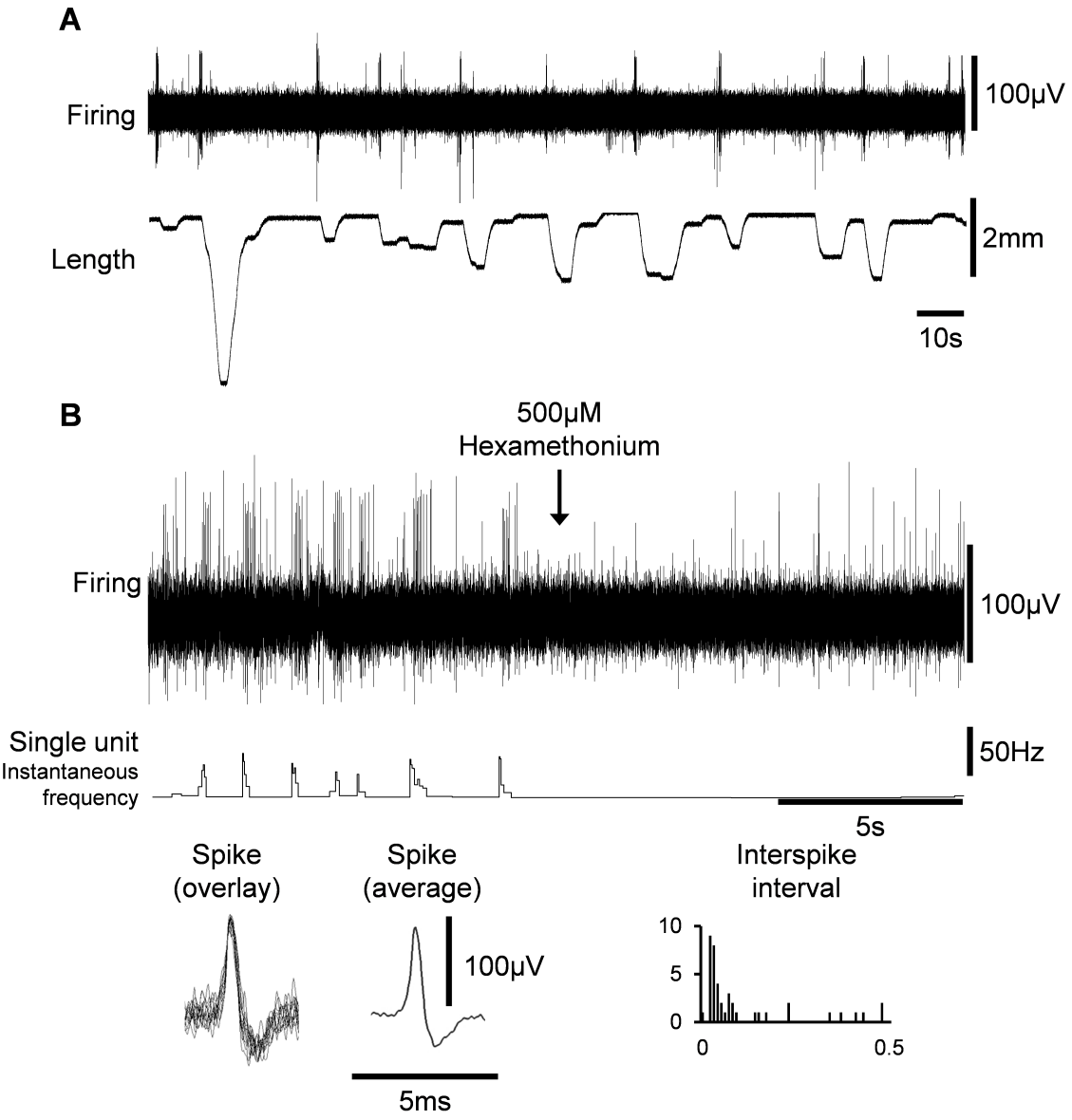

Figure 4.26

Examples of burst firing activity. An example of burst firing activity of viscerofugal neurons is shown in **A**. Note that the bursts of firing typically occurred just prior to the onset of a circular muscle contraction. This is similar to the behaviour observed in preparations that had been maintained in organ culture before recordings from colonic nerves (**chapter 2**). Burst firing activity was observed in 4 of the 19 preparations used in circumferential stretch and contraction studies. Burst firing was not observed during the periods in which preparations were perfused with Ca^{2+} free Krebs solution. When tested, hexamethonium blocked burst firing (2/2 separate occurrences, n=1); an example is shown in **B**.

DISCUSSION

Activation of viscerofugal neurons

Similar to many other classes of enteric neurons, viscerofugal neurons can be synaptically driven via cholinergic pathways (Crowcroft et al., 1971b). Evidence has been presented here that they may also be directly mechanosensory. Studies of recording synaptic input to prevertebral ganglion neurons showed that some synaptic drive persists when synaptic transmission within the gut wall is blocked by Ca^{2+}-depleted solution (Bywater, 1993, Parkman et al., 1993, Stebbing and Bornstein, 1993). These studies raised the possibility that direct responses arose from a distinct subset of viscerofugal neurons. The present study has shown that direct, responses to light von Frey hairs (1-2mN) occurred in every viscerofugal nerve cell localised with DMPP (18 of 18 units, n=6). Responses were of lower frequency and typically required more intense compression than those recorded from IGLEs or rectal IGLEs (Zagorodnyuk et al., 2001, Lynn et al., 2005). This suggests that viscerofugal neuron cell bodies are less sensitive to mechanical stimuli than low threshold vagal or sacral mechanoreceptors.

All viscerofugal neurons identified by DMPP were also directly sensitive to distension. Distension is a more complex stimulus than von Frey hair compression because it activates extensive enteric pathways, leading to a mixture of both passive and reflex-evoked responses, changes in muscle length and contractility. Viscerofugal neurons in the present study were activated by distension in both normal solution and in a Ca^{2+}-free solution, where synaptic activity and reflex responses were blocked. This demonstrated that a substantial proportion of

viscerofugal neurons have intrinsic mechanosensitivity. Interestingly, viscerofugal neuron activation was relatively unaffected by Ca^{2+}-free solution, even though intramural tension was significantly reduced. Previous work in mouse also suggested that circumferential length, not tension, was the adequate stimuli for viscerofugal neurons. Contractions that emptied tubular preparations of murine intestine increased intramural tension, but decreased circumference, and this was associated with reduced viscerofugal neuron firing activity (Miller and Szurszewski, 2003). In the present study, pharmacological activation of contractility, under conditions of constant load or constant length, suggested that there is a near-linear relationship between circumferential length and viscerofugal neuron firing, independent of intramural tension. Viscerofugal neurons typically had low thresholds to distension. They showed bursts firing at low loads (>1g), which is comparable to low threshold vagal and sacral mechanoreceptors (Zagorodnyuk and Brookes, 2000, Zagorodnyuk et al., 2001, Lynn et al., 2003), but in contrast they showed only small increases in firing frequency. Their small increases in firing rate are consistent with previous reports based on viscerofugal synaptic inputs to prevertebral ganglia (Anthony and Kreulen, 1990, Bywater, 1993, Parkman et al., 1993, Stapelfeldt et al., 1993, Ermilov et al., 2004b).

In the present study on the relationship between tissue strain and viscerofugal neuron firing, smooth muscle was paralysed using Ca^{2+} free Krebs solution. Preparations were distended either uniaxially (in the circumferential or longitudinal axis), or biaxially. The reduced [Ca^{2+}] prevented local smooth muscle contractility, which can affect sensory neuron firing (Lynn et al., 2005). Both longitudinal and circumferential loading evoked firing, however, there was significant variability

between preparations (see **figure 4.18**, for example). We speculate that this may reflect discrepancies between the local mechanical conditions in the tissue immediately surrounding viscerofugal neurons and the overall mechanical state of the entire preparation that we measured with the isotonic transducers.

The effect of biaxial loading in the present study suggested that viscerofugal neurons lack a specific directionality in their mechanosensitivity. If viscerofugal neurons were direction sensitive, it would be expected that firing responses would preferentially reflect the changes in tissue strain in a single axis. However, this was not the case and mechanosensory firing of viscerofugal neurons increased by the total tissue strain (circumferential + longitudinal strains). The apparent anisotropic responses between longitudinal and circumferential loads were explicable by the increased compliance of the gut in the circumferential axis. This allowed greater strain in that direction, stimulating mechanosensory firing. When firing was plotted against strain in the 2 axes, the differences disappeared. The finding that both circumferential and longitudinal distensions evoke firing in viscerofugal neurons contrasts with the study of Miller & Szurszewski (2003) who recorded viscerofugal synaptic inputs to prevertebral ganglia in the mouse (Miller and Szurszewski, 2003). In their study, tube and flat sheet preparations were stretched circumferentially up to 15% of resting length (a strain of 0.15), resulting in an increase in the frequency of synaptic input from viscerofugal neurons. However, when the same preparations were stretched up to 20% of resting length longitudinally (a strain of 0.2) there was no increase in the rate of synaptic inputs. The strains applied by Miller & Szurszewski (2003) are comparable to the resultant strains in the present study. An important difference between studies is that preparations were distended under

synaptic blockade with a Ca^{2+} free Krebs solution in the present study. Enteric neural circuits in the colon may be strongly activated by circumferential distension, leading to activation of viscerofugal neurons via nicotinic inputs (Crowcroft et al., 1971b). However, longitudinal distension may extensively inhibit enteric neurons by release of nitric oxide (Dickson et al., 2007, Dickson et al., 2008). It is possible that such a mechanism may directly or indirectly inhibit viscerofugal neurons during gut elongation and mask their intrinsic mechanical excitation.

In the present study, spontaneous firing of viscerofugal neurons continued in Ca^{2+}-free solution, under conditions of minimal distension. This may reflect residual strain in the tissue (Gregersen, 2000), a threshold for action potential generation that is close to resting membrane potential, or the presence of pacemaker currents. Intramural tension activates "AH/Type-II" intrinsic primary afferent neurons in the small intestine (Kunze et al., 1995). Similar neurons exist in the colon (Neunlist et al., 1999, Nurgali et al., 2003b). The lack of effect of increasing intramural tension on firing rate in the present study suggests it is unlikely that AH/Type II neurons synaptically drive viscerofugal neurons. Interestingly, another class of mechanosensitive 'Dogiel type-I' S-neurons, identified by Spencer and Smith (2004), are also sensitive to length rather than tension (Spencer and Smith, 2004).

In enteric "intrinsic primary afferent neurons" ("IPANs" which correspond to AH/Type 2 neurons), focal mechanical distortion of cell processes caused generator potentials and excitation, whereas distortion of the soma led to inhibition of firing activity (Kunze et al., 2000). Only excitatory responses to von Frey probing were observed in our preparations. Von-Frey hair probing and direct, stretch-evoked

activation of viscerofugal neurons probably cause mechanical distortion of both cell bodies and their neurites, which are very short. DEG/ENaC channels may mediate some of this mechanosensitivity, since amiloride inhibits ongoing- and distension-evoked firing, but other unidentified mechanosensitive channels are also likely to contribute (Ermilov et al., 2004a).

The data presented in the current study shows that viscerofugal neurons are clearly capable of generating primary sensory responses to mechanical stimuli. However, it should be noted that viscerofugal neurons normally receive powerful excitatory synaptic input from enteric neuronal pathways (Sharkey et al., 1998). It is likely that in vivo, firing by viscerofugal neurons to mechanical stimuli represents combined mechanosensory and synaptically-driven responses. It should also be noted that in the present study, the mucosa and submucosal plexus were removed from preparations. This would have removed some neuronal or other cellular elements that might contribute directly, or indirectly, to viscerofugal neuron excitability, such as entero-endocrine cells, mast cells and submucosal sensory neurons. Viscerofugal neurons can be classified as interneurons: they receive synaptic input that can drive firing and they provide synaptic outputs onto other (sympathetic) neurons. However, our data demonstrate that they are also directly mechanosensitive. The idea that some enteric neurons can have primary mechanoreceptive functions as well as interneuronal functions has been suggested previously (Wade and Wood, 1988, Spencer and Smith, 2004, Mazzuoli and Schemann, 2009).

Direct effects of receptor agonists on viscerofugal neuron firing

The positive results of direct pharmacological excitation or inhibition viscerofugal neurons in the present study suggest they have receptors to several of the common

neurotransmitters used by neurons in the gut. In addition to DMPP, viscerofugal neurons were potently activated by serotonin and P2X receptor agonist αβmethylene ATP. Serotonin- and P2X receptors contribute to synaptic transmission in the enteric nervous system (Galligan and Bertrand, 1994, LePard et al., 1997, Zhou and Galligan, 1999, Nurgali et al., 2003a). The non-selective muscarinic receptor agonist oxotremorine significantly, but modestly increased firing rate in viscerofugal neurons. In S-neurons, muscarinic receptors mediate excitation by supressing K conductance (Galligan et al., 1989). No effect of L-glutamate on the firing rate of viscerofugal neurons was observed at the concentration used (100μM). There are conflicting reports that glutamatergic neurotransmission occurs among enteric neurons (Liu et al., 1997, Wang et al., 2014).

Adrenergic receptors are abundant on nerve fibres in the myenteric plexus of guinea pig colon (Nasser et al., 2006), and mediate presynaptic inhibition in enteric neurons (Hirst and McKirdy, 1974). Noradrenaline applied directly onto enteric neurons acts via the α_2 receptor to evoke increases in potassium ion conductance, leading to membrane hyperpolarization and decreased input resistance (Morita and North, 1981, Slack, 1986, Tatsumi et al., 1990). These mechanisms may underlie the significant inhibition of firing exerted by noradrenaline on viscerofugal neurons in the present study. However, noradrenaline caused excitation of some viscerofugal neurons in the present study (3/10 neurons). Excitation evoked by noradrenaline was also observed in a minority of gastric enteric neurons, possibly mediated by α_1 receptors (Tack and Wood, 1992).

Immunohistochemical studies have shown that NK_2 tachykinin receptors in the gut are primarily located on smooth muscle cells (Portbury et al., 1996). However, low levels of NK_2 receptor immunoreactivity also occur on a minority of myenteric

neurons in guinea pig distal colon (Portbury et al., 1996). Sucrose gap recordings of smooth muscle cells showed indirect functional evidence that NK_2 tachykinin receptors may occur on enteric inhibitory motor neurons (Zagorodnyuk and Maggi, 1995). In the present study viscerofugal neurons were strongly activated by the selective NK2 receptor agonist β-ala^8 neurokinin $A^{(4-10)}$ (Rovero et al., 1989), supporting the conclusion that enteric neurons express functional tachykinin NK_2 receptors.

Conclusion

The present study has demonstrated that all identified viscerofugal neurons were directly mechanosensitive, suggesting that they have combined roles, acting as both primary afferents and as interneurons. Unlike extrinsic spinal and vagal mechanoreceptors, their adequate stimulus appears to be changes in strain (length), rather than intramural tension. Furthermore, we suggest that direct mechanical activation of viscerofugal neurons may not be direction selective, as they can be activated by both circumferential and longitudinal distensions, and overall strain is the best predictor of firing.