Chapter 2
Extracellular recording of viscerofugal neurons from colonic nerves trunks in guinea pig distal colon after organ culture

INTRODUCTION

Extrinsic reflex circuits from the intestine can bypass the central nervous system to modulate gut function (Kuntz and Saccomanno, 1944). Viscerofugal neurons form part of the afferent arm of this circuit, with cell bodies in enteric ganglia and axons that project outside the gut through extrinsic nerve trunks and synapse onto prevertebral sympathetic neurons (Crowcroft et al., 1971b, Peters and Kreulen, 1986). These, in turn, project back to the gut wall of the same, or more proximal regions, where they modulate gastrointestinal motility (Weems and Szurszewski, 1977) and secretion (Quinson and Furness, 2002). Localised gut distension activates this circuit, causing inhibition of gut contractility locally and orally (Kuntz and Saccomanno, 1944). Characterizing activity of individual viscerofugal neurons would help us better understand and model gastrointestinal motility and secretion.

In isolated preparations of intestine, with intact connections to decentralized prevertebral ganglia, converging synaptic inputs from viscerofugal neuron populations onto sympathetic neurons can be recorded (Crowcroft et al., 1971b). Luminal distension increases the frequency of nicotinic synaptic inputs to sympathetic neurons (Crowcroft et al., 1971b). Intestinal distension under synaptic blockade in the intestine reduces, but does not abolish synaptic input to sympathetic neurons – suggesting some viscerofugal neurons may be directly mechanosensory (Bywater, 1993, Parkman et al., 1993, Stebbing and Bornstein, 1993).

Intracellular recordings of retrogradely labelled viscerofugal cell bodies, showed that they receive fast nicotinic excitatory synaptic inputs, suggesting they may function as interneurons (Sharkey et al., 1998). However, mechanical stimuli cannot be adequately tested during intracellular recordings because microelectrodes are easily

dislodged. Thus, the mechanosensitivity of single viscerofugal neurons have not been investigated. Extracellular recordings of viscerofugal neuron axons, located in colonic or mesenteric nerve trunks, would allow investigation of the effects of mechanical and pharmacological stimuli at a single neuron level. Many extracellular electrophysiological recordings from mesenteric nerve trunks have been made to study vagal and spinal afferent neurons innervating the small and large intestines (Cervero and Sharkey, 1988, Blackshaw and Grundy, 1993, Lynn and Blackshaw, 1999, Lynn et al., 2003, Booth et al., 2008, Song et al., 2009). Despite this, no neuronal firing activity has been identified which could be unequivocally attributed to viscerofugal neurons. Thus, it is not known whether viscerofugal neurons are detectable in extracellular recordings of nerve activity in colonic/mesenteric nerve trunks. We hypothesized that: (a) selective ablation of severed extrinsic nerve fibres in colonic nerve trunks can be achieved in isolated gut preparations during organotypic culture, leaving viscerofugal axons intact; (b) that this would permit extracellular recordings from identified viscerofugal neurons, and; (c) individual viscerofugal neuron firing activity and responses to mechanical stimuli can be characterized in preparations after organ culture, without the confounding presence of extrinsic nerves.

METHODS

Dissection and extracellular recording setup

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

Organ-cultured preparations were maintained in sterile culture medium (Dulbecco's modified Eagle's [DME]/Han's F12, Sigma [1:1 ratio mix, supplemented with L-glutamine and 15 mM HEPES]; including 10% fetal bovine serum (Gibco, Life Technologies Corporation, USA), 100 IU/ml penicillin (Pen Strep, Gibco), 100 µg/ml streptomycin D (Pen Strep, Gibco), 10 µg/ml gentamycin (Gibco), 2.5 µg/ml amphotericin B (Sigma), and 1.8 mM $CaCl_2$. Preparations were slowly agitated for 4-6 days in a humidified incubator (36°C, 5% CO_2 in air) (Song et al., 1995). Culture medium was replaced every 24 hours. During electrophysiological recordings, preparations were superfused with Krebs solution (35°C). Acute control preparations

were set up for recording or biotinamide tracing immediately after dissection. **Figure 2.01** shows a basic process overview.

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

Biotinamide labelling

A drop of biotinamide solution (5% biotinamide (N-[2-aminoethyl] biotinamide hydrobromide), dissolved in artificial intracellular solution (150 mmol L^{-1} monopotassium L-glutamic acid, 7 mmol L^{-1} $MgCl_2$, 5 mmol L^{-1} glucose, 1 mmol L^{-1} ethylene glycolbis(ß-aminoethyl ether)-N,N,N=,N=-tetraacetic acid, 20 mmol L^{-1}

HEPES buffer, 5 mmol L^{-1} disodium adenosine triphosphate, 0.02% saponin, 1% dimethyl sulfoxide, 100 IU mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 20g mL^{-1} gentamycin sulphate) was placed on a dissected nerve trunk and the main chamber was filled with sterile culture medium (Tassicker et al., 1999a). Preparations were incubated overnight (12-16 hours; 36°C, 5% CO_2 in air), then fixed overnight in Zamboni's fixative (15% saturated picric acid, 2% formaldehyde in 0.1 M phosphate buffer, pH 7.0). Preparations were cleared in DMSO (3 x 10 minute washes) then washed in 0.1M phosphate-buffered saline (0.15 M NaCl, pH 7.2; 3 x 10 minute washes) followed by incubation for 3 hours in 3-1-*O*-(2-cyanoethyl)-(*N,N*-diisopropyl)indo-carbocyanine (CY3) conjugated to streptavidin. Subsequently, preparations were washed with PBS (3 x 10 minute) and equilibrated in a series of carbonate-buffered glycerol solutions (50, 70 and 100% solutions; 3 x 10 minutes) prior to mounting on glass slides in buffered glycerol (pH 8.6).

Image analysis

Biotinamide-labelled nerves were viewed and analysed on an Olympus IX71 epifluorescence microscope fitted with an appropriate dichroic mirror and filter. Images were captured with a Roper Scientific Photometrics digital camera operating with a HP Compaq dc7100 CMT computer with a Microsoft Windows XP operating system, running AnalySIS 5.0 software (build 1153). The micrographs of cell bodies in **figure 2.13** were acquired with a Leica SP5 scanning confocal microscope (Leica Microsystems, Mannheim, Germany). CY3 fluorophores were excited with 561nm laser light. Emitted photons (565-645nm) were captured with a photon multiplier tube, pinhole set to 1 Airy unit. Laser power, photon multiplier tube gain and offset were adjusted as required. Z-stacks were taken with a 63x oil-immersion lens at

0.5µm slices through the Z-axis. Brightness and contrast adjustments, cropping, pseudocolouring and photomontages of biotinamide labelled preparations were performed using Adobe Photoshop (CS5, Adobe Systems Inc, San Jose, CA).

Quantification of viscerofugal axons in nerve trunks

In organ-cultured and acute preparations, biotinamide-labelled viscerofugal cell bodies, as well as biotinamide-filled axons were visualized on an epifluorescence microscope and counted to obtain the percentage of viscerofugal axons among all labelled axons. Axons were counted at sites where bundles of mesenteric nerves spread out upon entering the myenteric plexus, taking care to focus throughout the depth of the tissue.

Immunohistochemistry

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

Drugs

Stock solutions of drugs were made as follows: 10^{-2}M nicardipine hydrochloride in water (Sigma; N7510), 10^{-3}M hyoscine hydrobromide in water (Sigma; S0929), 10^{-1}M hexamethonium chloride in water (Sigma; H2138), 10^{-1}M 1,1-dimethyl-4-

phenylpiperazinium iodide (DMPP) in water (Sigma; D5891), $3x10^{-4}$M tetrodotoxin (TTX) in water (Alomone; T-500), 10^{-2}M N-Vanillylnonanamide (synthetic capsaicin) in ethanol (Sigma; V9130). All drugs were kept refrigerated and diluted to working concentrations in Krebs solution, shortly before use.

Statistical analysis

Statistical analysis was performed by Student's two-tailed t-test for paired or unpaired data or by repeated measures analysis of variance using Prism v.5 software (GraphPad Software, Inc., San Diego, CA, USA). Chi-squared tests with Yates' correction for continuity were performed 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. Lower case "n" always indicates the number of animals used in a set of experiments.

Note to reviewers

This chapter includes some experimental data acquired prior to enrolment in PhD studies. This includes 10/22 electrophysiological recordings, 11/12 biotinamide labelling experiments, and 0/4 immunohistochemical experiments. Results sections that include any of these data have headings marked with asterisks (these can also be seen in the table of contents). However, considerable additional analysis was performed on this data during the course of the PhD, as new results suggested the need to re-examine previous recordings.

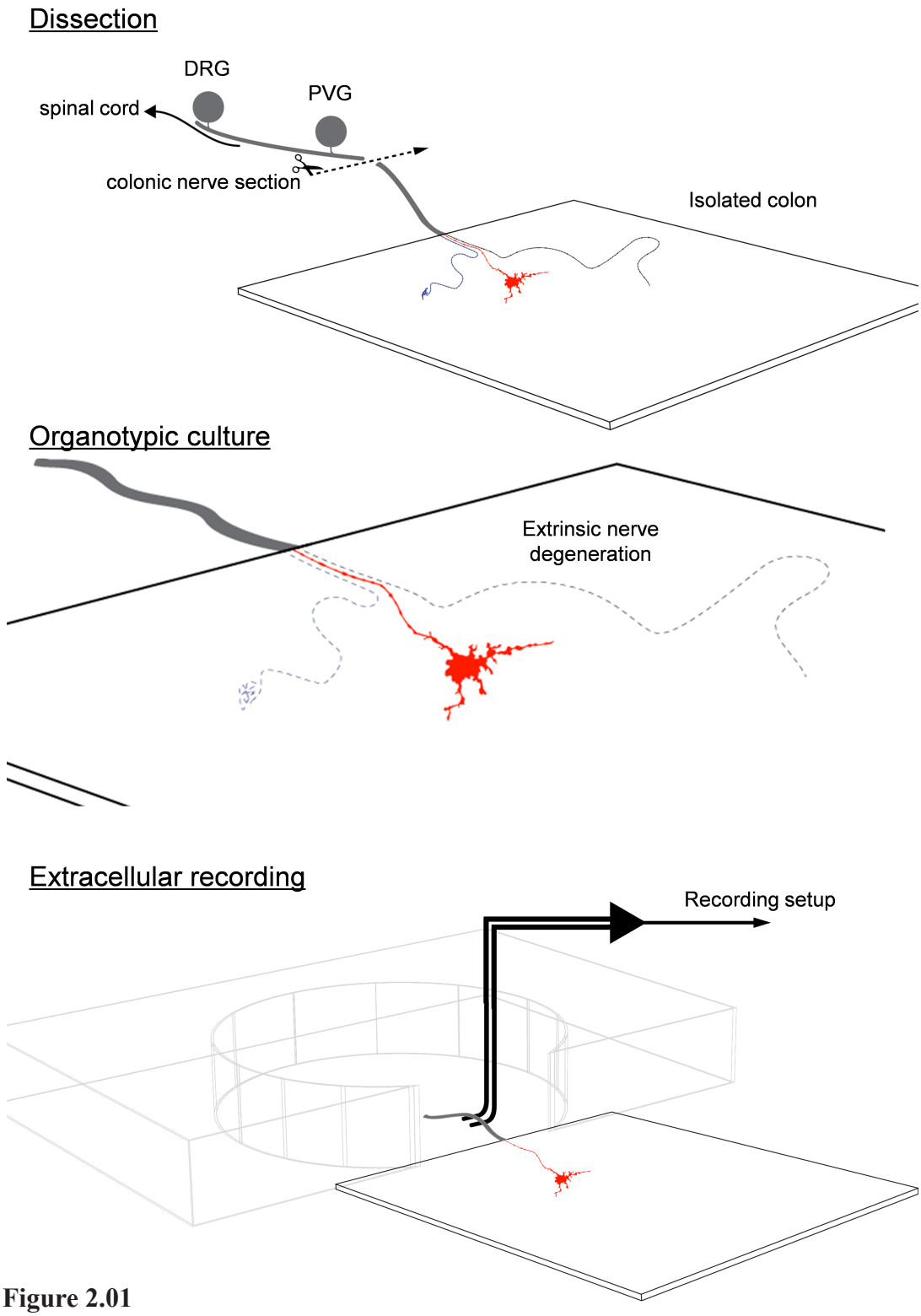

Figure 2.01

Schematic diagram of the preparation of organ cultured tissue in the present study. During dissections, all of the axons contained within colonic nerve trunks were severed. The isolated preparations of distal colon, with mucosa and submucosa removed, were maintained in organ culture for 5-6 days. Thus, within a colonic nerve trunk, only the axons of viscerofugal neurons remained attached to their cell bodies during organ culture. Preparations were removed from organ culture after 5-6 days and set up for electrophysiological recordings from colonic nerve trunks.

RESULTS

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

Flat sheet preparations of guinea-pig distal colon (1-2cm in length, mucosa and submucosa removed) were maintained in organotypic culture for 4-5 days to determine whether axons of spinal afferent and sympathetic efferent neurons, severed from their cell bodies, would degenerate during this period. As shown in small intestine (Tassicker et al., 1999a), biotinamide filling of extrinsic nerve trunks in acute preparations (freshly removed from animal, n=6) revealed viscerofugal cell bodies (see **figure 2.03C-F**) and dense labelling of the fine branching varicose fibres of extrinsic pathways, including spinal afferent and sympathetic neurons (**figure 2.02A and 2.03A**). The same protocol was then applied to 6 preparations after organ culture for 4-5 days. After organ culture, the density of biotinamide-labelled fibres was considerably reduced; most of the remaining fibres could be traced to viscerofugal neuron cell bodies (**figure 2.02B and 2.03B**). This suggested that viscerofugal neuron cell bodies and their axons persisted during organ culture, while extrinsic nerve fibres degenerated. To quantify this, we counted all biotinamide labelled axons, as well as viscerofugal neuron cell bodies (see methods), to obtain the proportion of axons that belonged to viscerofugal neurons. In acute preparations (n=6), viscerofugal axons were estimated to form a minority, comprising 8.6 ± 4.2% of all filled axons within labelled nerve trunks. After culture, the proportion of viscerofugal axons of labelled axons in colonic nerve trunks increased to a majority of 67.1 ± 13.8% (n=6, $p<0.001$), confirming that viscerofugal neurons and their axons persisted in organ culture and were considerably enriched as a proportion of all surviving axons.

Immunohistochemistry

The most likely explanation for the increased proportion of biotinamide-labelled viscerofugal neuron axons after organ culture was that the axons of spinal afferent neurons and sympathetic neurons had degenerated, while intrinsic neurons, including viscerofugal neurons, persisted (Song et al., 1995). To test this, preparations were immunohistochemically labelled for common neurochemical markers of sympathetic and spinal sensory axons: tyrosine hydroxylase (TH), and calcitonin gene-related peptide (CGRP), respectively. In control preparations, fixed shortly after removal from the animal, a dense network of TH-immunoreactive varicose branching axons was visualised within myenteric ganglia (**figure 2.04 and 2.05A**). There were also numerous CGRP-immunoreactive varicose axons (**figure 2.04 and 2.05C**), previously shown to belong to spinal afferent neurons (Gibbins et al., 1985). After organ culture, axons containing TH-immunoreactivity were nearly abolished (**figure 2.05B**). Likewise, CGRP-immunoreactivity was greatly diminished in organ-cultured preparations (**figure 2.05D**); except for occasional intrinsic CGRP-immunoreactive cell bodies seen in 2 out of 4 preparations (**figure 2.05E**). In organ-cultured preparations, no colocalization of biotinamide-labelled axons with either CGRP or TH-immunoreactivity was evident. These results suggest that 5 days in organ culture was sufficient to cause nearly-complete degeneration of extrinsic axons in these preparations.

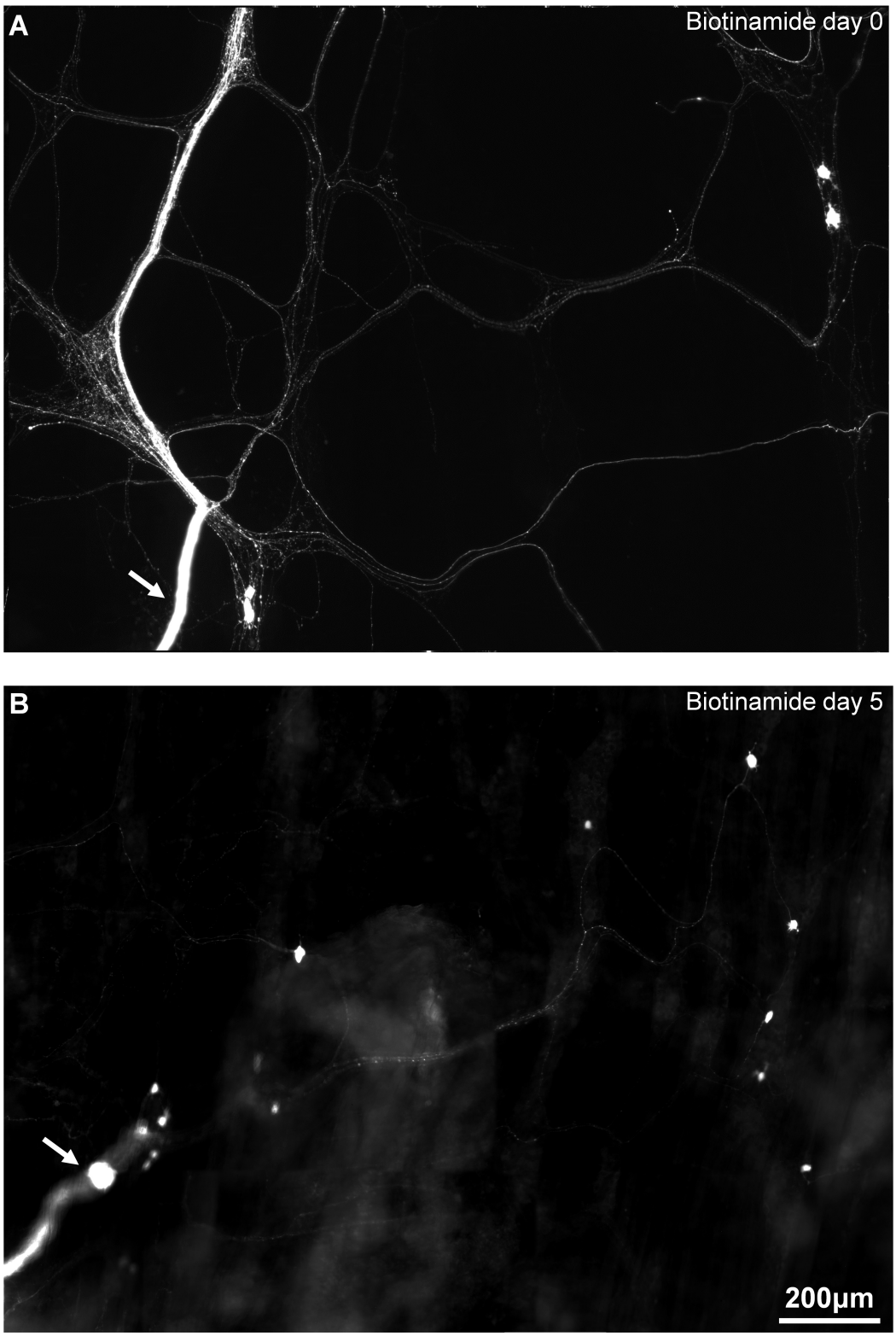

Figure 2.02

Biotinamide labelled colonic nerve trunks in fresh tissue and after 5 days organ culture. (**A**) Biotinamide labelling from a colonic nerve (arrow) in freshly dissected tissue revealed many fine varicose nerves that ramified throughout the myenteric ganglia, in addition to viscerofugal neuron cell bodies. (**B**) After 5 days culture, biotinamide labelling of colonic nerves labelled significantly fewer varicose fibres, consistent with degeneration of extrinisic fibres. However, viscerofugal neurons persisted, and were labelled

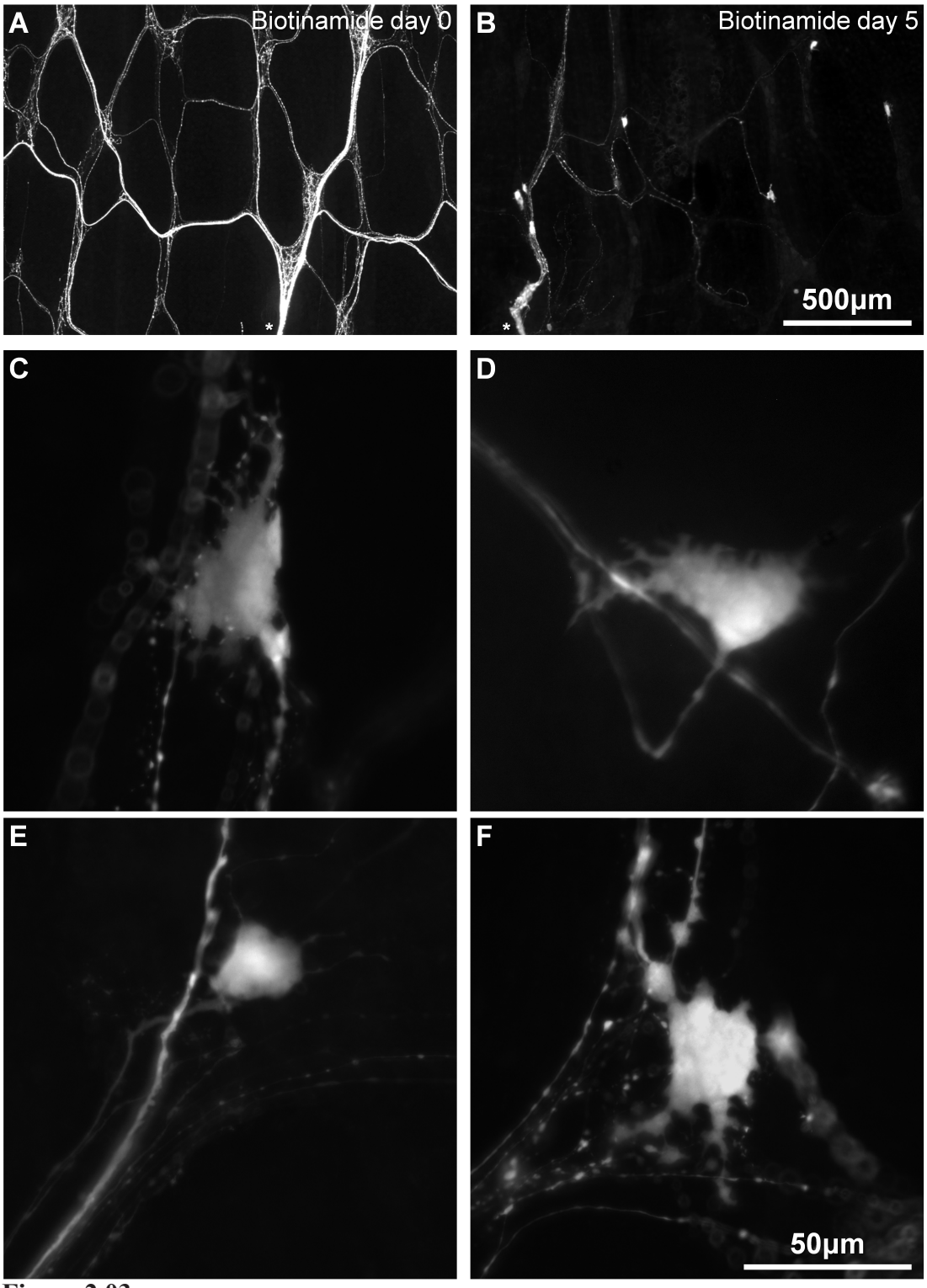

Figure 2.03

Photomontages of biotinamide labelling from colonic nerve trunks. (**A**) In "fresh" tissue, labelled immediately after dissection, there are dense networks of fine extrinsic nerve fibres that enter and prolifically ramify throughout the myenteric plexus. (**B**) shows a preparation labelled after 5 days in organ culture. In contrast to fresh tissue, labelled fibres are sparse and many were traceable back to cell bodies. Examples of the soma-dendritic morphology of retrogradely labelled viscerofugal neuron cell bodies in preparations that were labelled immediately after dissection are shown in (**C-F**).

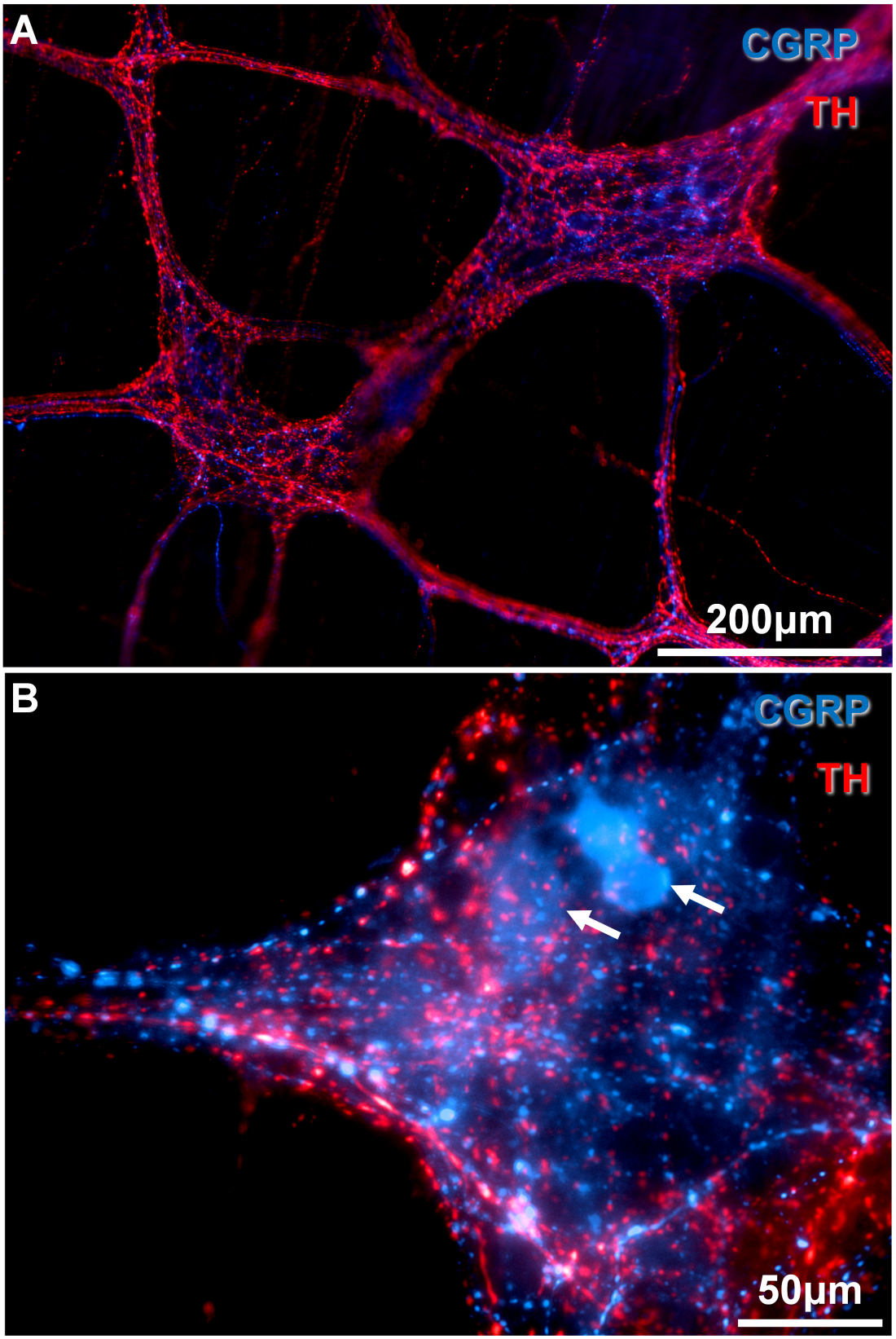

Figure 2.04

Immunoreactivity for CGRP (shown in blue) and TH (shown in red) in guinea pig distal colon (submucosa/mucosa removed). In the low power (**A**) and higher power (**B**) photomicrographs, prolific varicose fibres immunoreactive for CGRP or TH occur in the myenteric plexus and occasionally in the muscle layers. In addition to varicose fibres, there were occasional enteric nerve cell bodies that contained CGRP. Examples of two CGRP immunoreactive myenteric neurons are shown in **B** (marked with arrows).

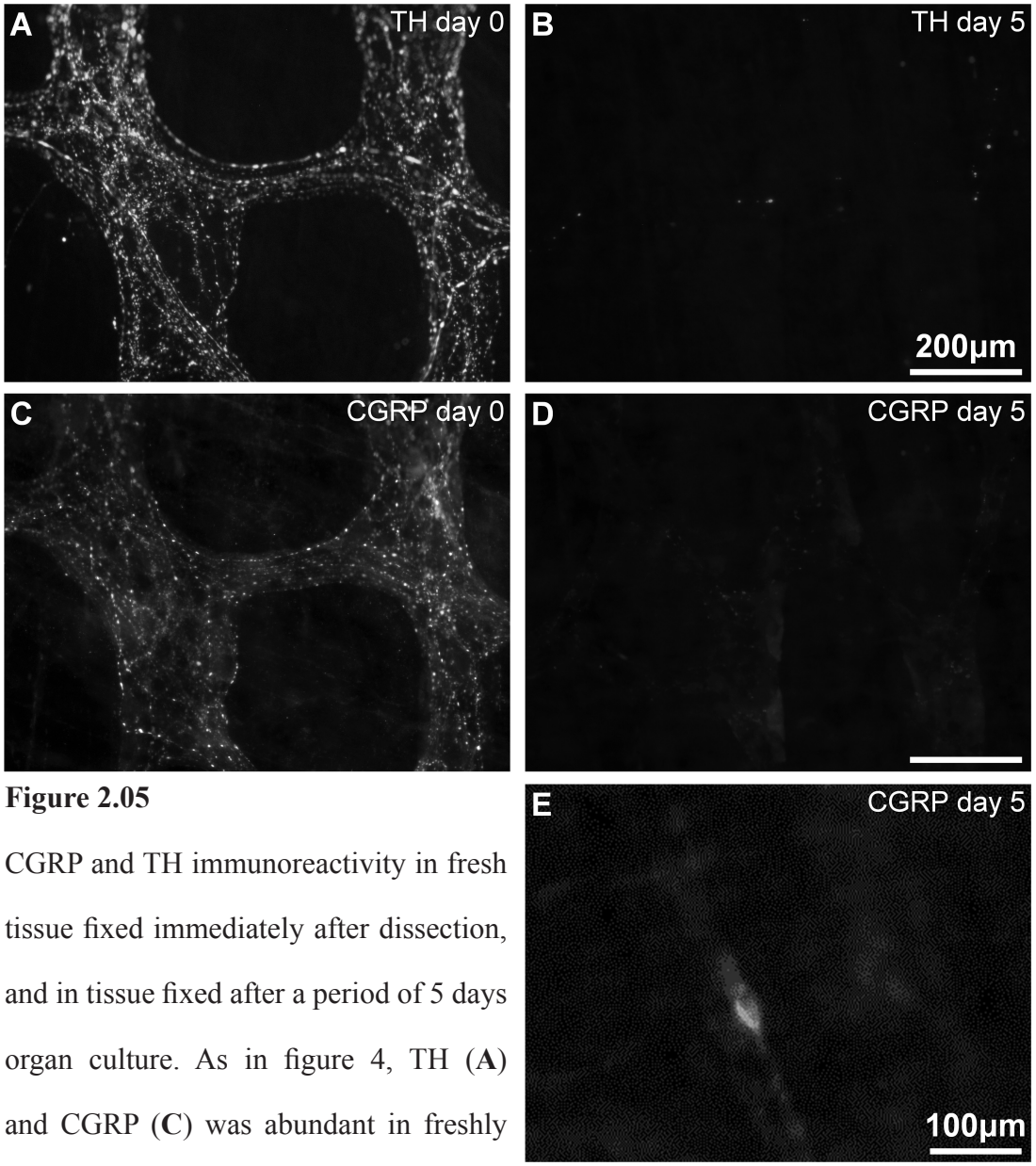

Figure 2.05

CGRP and TH immunoreactivity in fresh tissue fixed immediately after dissection, and in tissue fixed after a period of 5 days organ culture. As in figure 4, TH (**A**) and CGRP (**C**) was abundant in freshly fixed tissue (day 0); however after 5 days of organ culture, immunoreactivity to both antibodies was markedly reduced (**B** & **D**). The reduction of immunoreactivity to these markers is consistent with the degeneration of extrinsic nerve fibres, including spinal afferent (CGRP) and sympathetic (TH). Nevertheless, CGRP still labelled occasional myenteric neurons in preparations that had been maintained in organ culture - an example of a CGRP immunoreactive cell body is shown in **E**. This suggests the antibody was able to label CGRP in cultured tissue, but spinal afferent neurons had degenerated.

*Cell body morphology**

The degeneration of extrinsic axons in organ culture allowed examination of the detailed morphology of biotinamide-labelled viscerofugal nerve cell bodies, without the complication of a dense network of labelled axons surrounding the cell. A total of 78 retrogradely labelled cell bodies in organ-cultured preparations were examined in this way. In 36 cells, their axons could be unequivocally distinguished from all other labelled axons-of-passage. All of these cells were uni-axonal (examples are shown in **figure 2.13**) and either had lamellar dendritic Dogiel type I morphology or a few short filamentous and/or lamellar dendrites typical of "small simple cell" Dogiel type I morphology (Furness et al., 1988). No cells had two axons emerging from the cell body; thus none had Dogiel type II morphology. The remaining 42 cells could not be unequivocally classified due to the proximity of processes from other neurons or due to faint labelling. It is worth noting that viscerofugal neuron cell bodies were always labelled by biotinamide applied to colonic nerves after organ culture (average: 27 ± 22 per preparation). Variability between preparations in the number of retrogradely traced cell bodies (range 4-61) probably reflects an uneven distribution between extrinsic nerve trunks (Ross, 1958).

*Electrophysiology**

Extracellular recordings of colonic nerve trunks were made from 12 preparations maintained for 5-6 days in organ culture (n=8). Spontaneous action potentials were recorded in all preparations, from which 14 single units could be readily discriminated by spike amplitude and duration (mean firing rate 4.0 ± 1.9Hz, 14 units, n=7). Units in 10 of 12 preparations showed regular spontaneous bursts of firing (see **figure 2.06A and 2.06B**; inter-burst interval 2.4 ± 0.2s, burst duration

190±6ms). Bursts of firing involved several single units, distinguishable by spike amplitude and duration, suggesting that firing was synchronized between neurons, probably by a common underlying neuronal pathway. Single units fired an average of 5.4 ± 2.3 action potentials within a burst. In 4 units from 4 preparations, firing occurred regularly, without bursts (**figure 2.06C**; 4/12 preparations). Consistent with a neuronal origin of firing activity, all the recorded firing activity from colonic nerve trunks was abolished when the voltage-gated sodium channel blocker tetrodotoxin was added to the organ bath (1μM, 3/3 preparations tested, see **figure 2.08D**).

Capsaicin activates 85% of medium-high threshold spinal afferent neurons innervating the gut wall (Song et al., 2009). Capsaicin (0.4μM) reliably evoked robust firing in colonic nerves of freshly dissected, control preparations (5/5 preparations, an example is shown in **figure 2.07A**). In contrast, the same concentration of capsaicin evoked no change in firing in preparations that had been cultured (8/8 tested; see **figure 2.07B** and **2.07C**). These results are consistent with the complete degeneration of the severed axons of extrinsic sensory neurons during 5-6 days of organ culture.

Viscerofugal neurons in the guinea-pig colon have been reported to express nicotinic receptors (Crowcroft et al., 1971b, Sharkey et al., 1998, Ermilov et al., 2003). Application of the nicotinic receptor agonist, DMPP (10^{-4}M) in the recording chamber increased firing up to 50Hz in colonic nerves from organ-cultured preparations (mean 37.9 ± 8.6Hz; 10 units, n=6, **figure 2.07D**). Responses persisted when smooth muscle was paralysed with nicardipine (1μM) and hyoscine (1μM), indicating that increases in firing rate was not a result of DMPP-evoked muscle

contraction (6/6 units, n=3). Likewise, DMPP evoked increased firing in Ca^{2+} free Krebs solution (4/4 units, n=3), indicating a direct effect of DMPP on viscerofugal neurons. All responses were blocked by pre-administration of 400μM hexamethonium (12 units, n=7).

The nicotinic receptor antagonist, hexamethonium (400μM) significantly reduced spontaneous firing rate in 7 organ-cultured preparations (12 units, n=7, $p<0.01$; **figure 2.08**). In addition, hexamethonium always abolished burst firing activity in viscerofugal neurons (5/5 preparations; example **figure 2.08A**). This indicates that the synchronous firing of multiple viscerofugal neurons was partly driven, but entirely coordinated by, nicotinic synaptic inputs; suggestive of enteric interneurons.

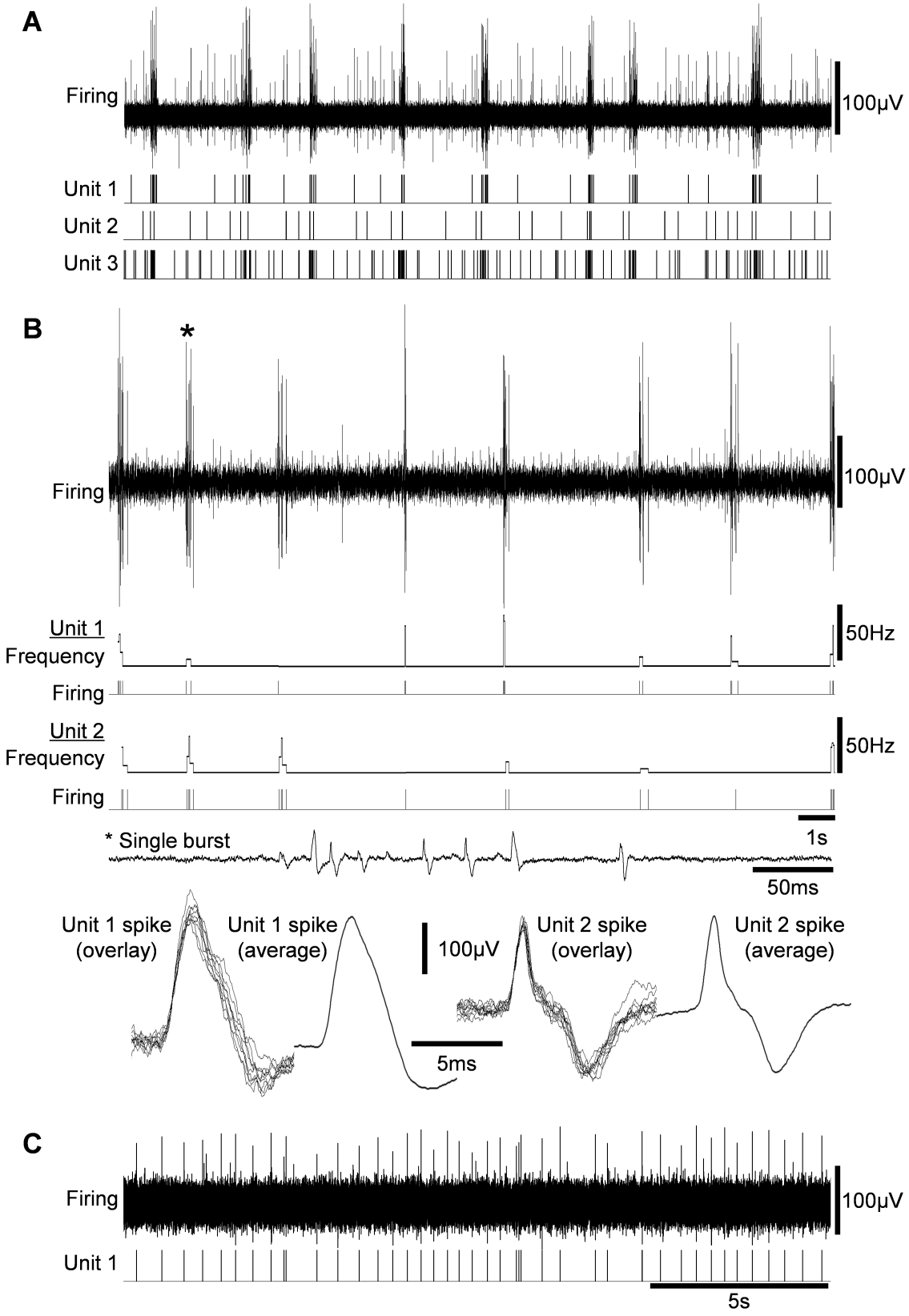

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Figure 2.06

Ongoing spontaneous burst firing activity and repetitive firing recorded from colonic nerve trunks to preparations of guinea pig distal colon that had been maintained in organ culture for 5 days. Single units were discriminated by amplitude, duration and shape. **A** & **B** show burst firing activity that was synchronized between units; the upper trace (**A**) shows units that fired with, and between bursts. The lower trace (**B**) contains units that tended to fire only within bursts, up to about 50Hz. Another unit, of lower amplitude, could not be adequately discriminated but can also be seen firing repetitively. Below is shown an expanded version of a single burst marked above with an asterisk. The individual spikes of unit 1 and unit 2 (spike shapes are shown below) can be seen within this individual burst of firing. The spike shapes of each discriminated unit are shown as overlays (10 spikes), and as an average (60 spikes). The firing activity shown in **C** is an example of the repetitive firing activity that was recorded in colonic nerve trunks to organ cultured preparations, in addition to burst-type firing.

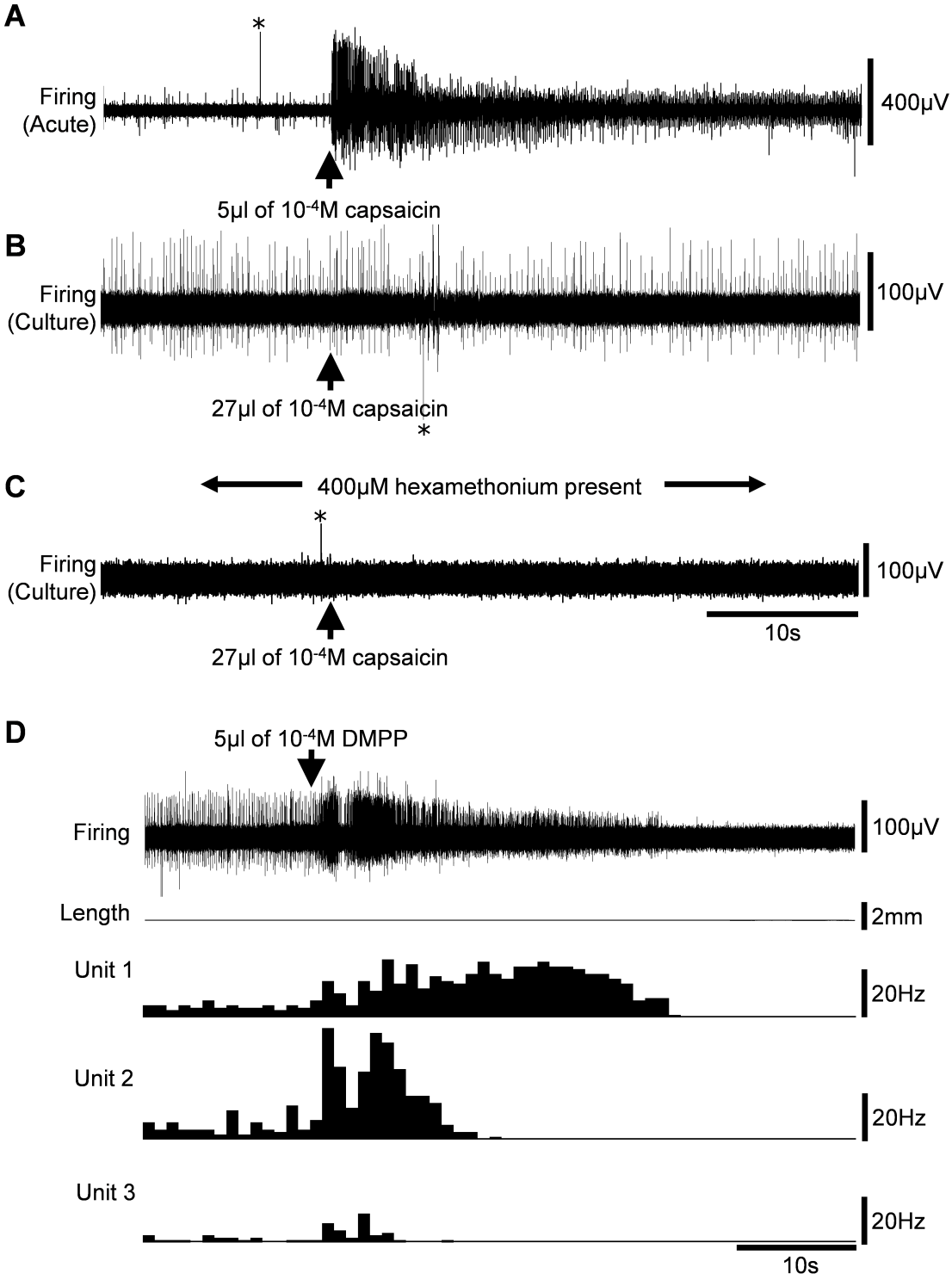

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Figure 2.07

Effect of capsaicin and DMPP on firing in colonic nerve trunks recorded immediately after dissection and after 5 days in organ culture. The recording shown in **A** is a typical example of a firing response evoked by capsaicin in a preparation set up for recording immediately after dissection. Strong firing discharges were evoked in all preparations of this type (5/5 preparations). This is consistent with the presence of functional spinal afferent neurons, which are activated by capsaicin. After 5-6 days in organ culture, firing responses could not be evoked by capsaicin, added directly onto the preparation. This suggests that organ cultured preparations lacked functional spinal afferent neurons. Shown in **B** is a typical example of this, when ongoing firing is present. This is seen more clearly in **C**, in which hexamethonium had been added prior to administration of capsaicin, abolishing firing in the period shown. When capsaicin was added under these conditions, the lack of an effect on firing is clearly apparent. Artefacts resulting from movements of the pipette in the recording chamber are marked with asterisks in **A – C**. In contrast to capsaicin, the addition of a nicotinic receptor agonist, DMPP, evoked strong firing in organ cultured preparations. This is consistent with viscerofugal neurons since, nicotinic receptors are expressed on viscerofugal nerve cell bodies (Ermilov et al., 2003). Shown in **D** is an example of DMPP-evoked firing in a preparation that had been maintained in organ culture for 5 days. This preparation contained 3 discriminable units that were activated by DMPP. In this example, the preparation was perfused by a Krebs solution containing hyoscine (1μM) and nicardipine (1μM) to paralyse the smooth muscle. Thus, note that smooth muscle contractions were not evoked by the addition of DMPP onto the preparation. This precluded the possibility of mechanically evoked firing.

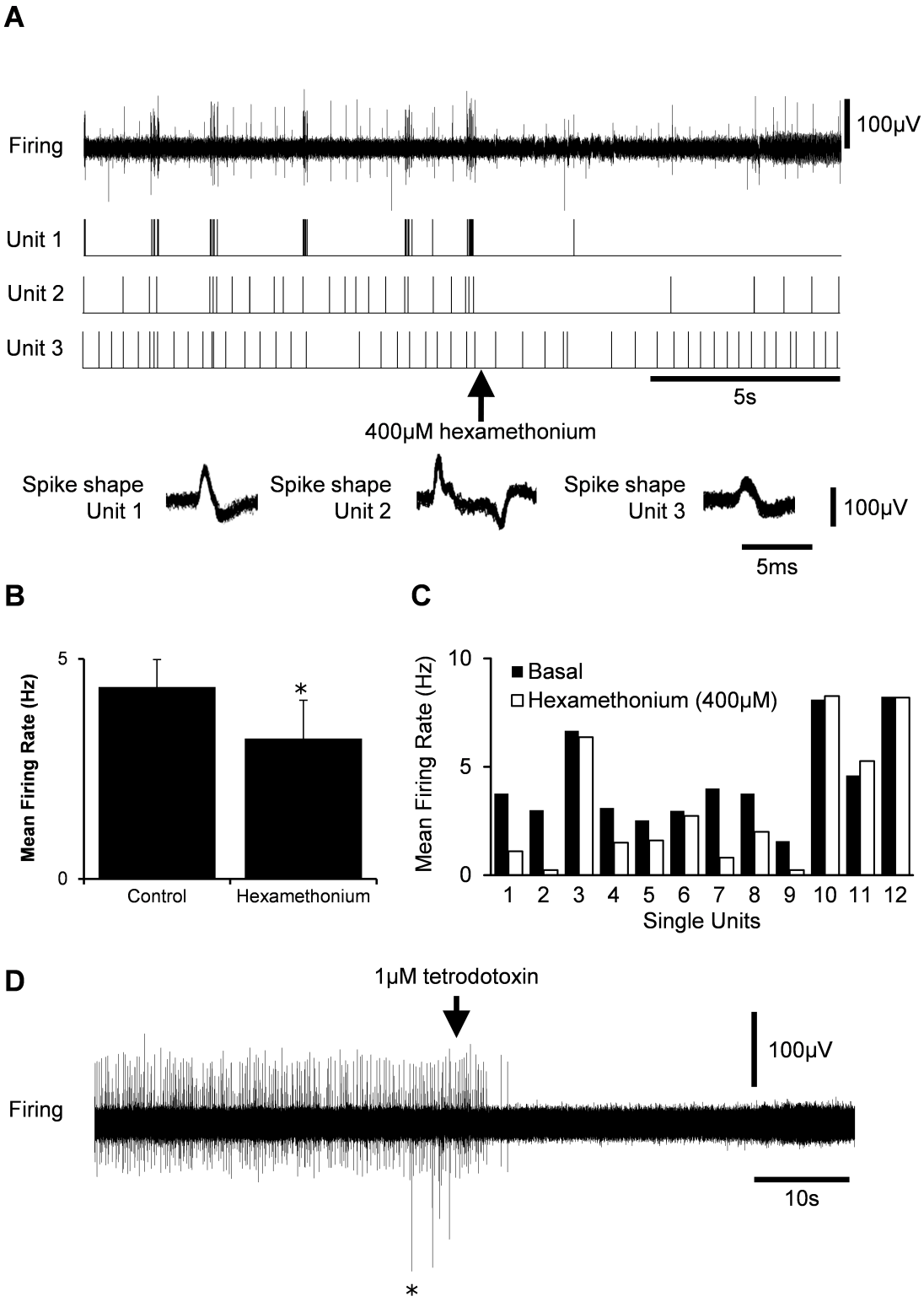

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Figure 2.08

The effects of hexamethonium and tetrodotoxin on firing recorded from colonic nerve trunks in preparations that had been maintained in organ culture for 5-6 days. Burst firing behaviour was always abolished by nicotinic receptor blockade with hexamethonium (400μM; **A**). This suggests that the coordination of firing among several neurons was mediated by nicotinic synaptic transmission. However, nicotinic blockade also reduced the ongoing firing rate (**B**), suggesting that the recorded firing was partly driven by nicotinic inputs. This can be seen in **C**, where the effect of hexamethonium on firing rate on single units is illustrated. The effect of hexamethonium ranged from no apparent change, to a near-complete abolition of firing rate. However, the addition of the voltage-gated sodium channel blocker tetrodotoxin (1μM) to the organ bath always completely abolished firing. An example is shown in **D**. Note that some noise artefacts can be seen when the hand-held pipette tip containing tetrodotoxin entered the Krebs solution, before it was ejected (this is indicated by the asterisk).

*Spontaneous contractions**

Under isotonic conditions, preparations that were recorded after 5-6 days of organ culture showed irregular spontaneous contractions of the circular smooth muscle (Δlength 2.2 ± 1.4mm, 23 contractions, mean frequency 0.8 ± 0.2 per min during active periods, n=5). Large bursts of viscerofugal neuron firing preceded the onset of all spontaneous contractions by an average of 2.3 ± 2.1s, (23/23 contractions, 7 units, n=5, see **figure 2.09**). These bursts were significantly longer than regular bursts (burst duration 1.91±0.3s, $p<0.001$ paired t-test). Overall, average firing rate in the 5 seconds preceding spontaneous contractions (11.5 ± 5.0Hz) was significantly greater than both mean basal firing (5.2 ± 3.2Hz) and mean firing during contractions (4.0 ± 3.0Hz, 7 units, n=5, $p<0.001$ 1 way ANOVA). The timing of these bursts of firing suggests that viscerofugal neurons are activated, prior to contraction, by enteric neuronal circuits that subsequently cause spontaneous smooth muscle contractions.

*Stretch**

The effects of circumferential stretch on firing in organ-cultured preparations were examined (1-3g, 8 preparations, 9 units, n=6). In some cases (10/35 stretches), distension evoked reflex contractions of the circular muscle (**figure 2.10B** and **2.11A**). On these occasions, large bursts of firing preceded contractions by an average of 2.75±0.52s. There was a significant association between these large bursts of firing (defined by being more than 1s long with firing rates exceeding 10Hz) and stretch-evoked contractions ($X^2=11.8$, df=1, $p<0.001$, Yates' continuity correction). A summary of the effect of distension is shown in **table 2.01**. Firing was modestly increased when distension failed to evoke a measureable reflex response (**figure 2.10B**). Overall (including all stretches, regardless of whether a contraction was

evoked), stretch caused a significant load-dependent increase in firing rate (9 units, n=6, $p<0.05$, 1 way ANOVA; **figure 2.11B**). Previous work suggested a lack of sensitivity to longitudinal distension among viscerofugal neurons (Miller and Szurszewski, 2003). Consistently, we found longitudinal stretch by loads up to 4g did not significantly affect firing rate ($p=0.4$, 1 way ANOVA, 4 units, n=3, **figure 2.12**).

Focal tissue compression

In a further series of experiments, single units were assessed for sensitivity to focal tissue compression by von Frey hairs (1-3mN). These were applied to numerous sites on the surface of the circular muscle layer, which in most cases evoked no detectable response in firing rate. However, probing at a few sites in all preparations tested gave rise to bursts of firing activity (5 preparations, n=5). All eleven discriminated units in these preparations promptly discharged a train of action potentials to focal probing at a single site of about 200μm diameter on the preparation in normal Krebs (**figure 2.11C**, 11/11 units tested). Firing responses to probing persisted in 400μM hexamethonium (4/4 units tested, n=1), and in Ca^{2+}free Krebs solution (7/7 units tested, n=4). Thus, probing resulted in direct mechanosensory firing responses, probably transduced at the cell body. Von Frey hair probing during extracellular recording was combined with biotinamide tracing from the recorded nerve trunk in a single preparation (**figure 2.13**). Sites where probing evoked firing appeared to selectively correspond to viscerofugal neuron cell bodies, however this was not studied further in this series of experiments. Detailed studies using this method in acute preparations were performed in later experiments (**chapter 3**).

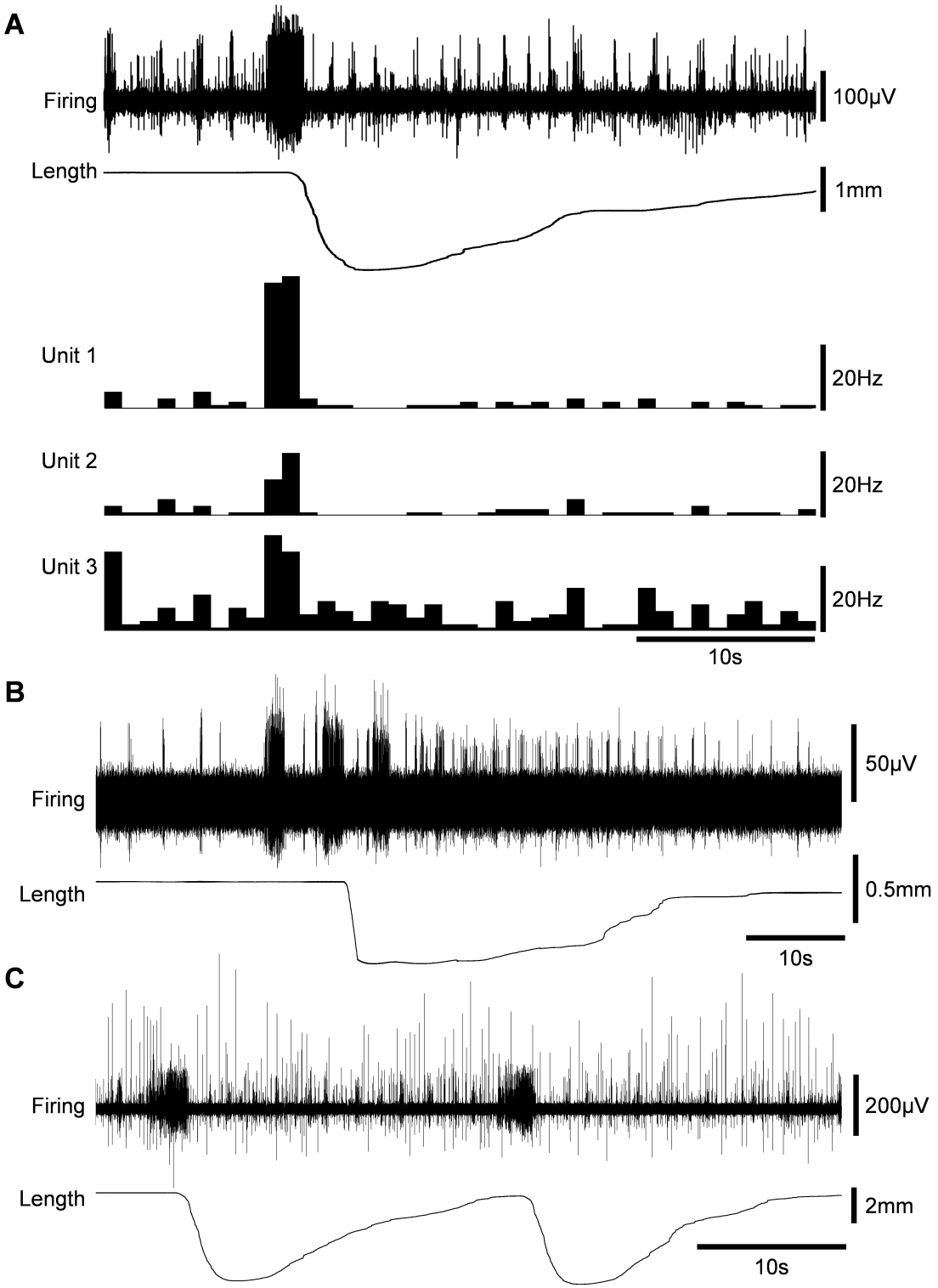

Figure 2.09

Spontaneous contractions of the circular smooth muscle were preceded by large bursts of firing activity in organ-cultured preparations. Shown in **A** is an example of a large burst of firing that occurred among 3 discriminated units prior to a contraction of the circular muscle. The large burst of firing occurred among regular burst and repetitive firing activity. Several large bursts of firing accompany the circular muscle contraction shown in **B**. These begin about 8 seconds before the shortening of the muscle is detected. In **C**, a series of two circular muscle contractions occur, each accompanied by a preceding burst of firing.

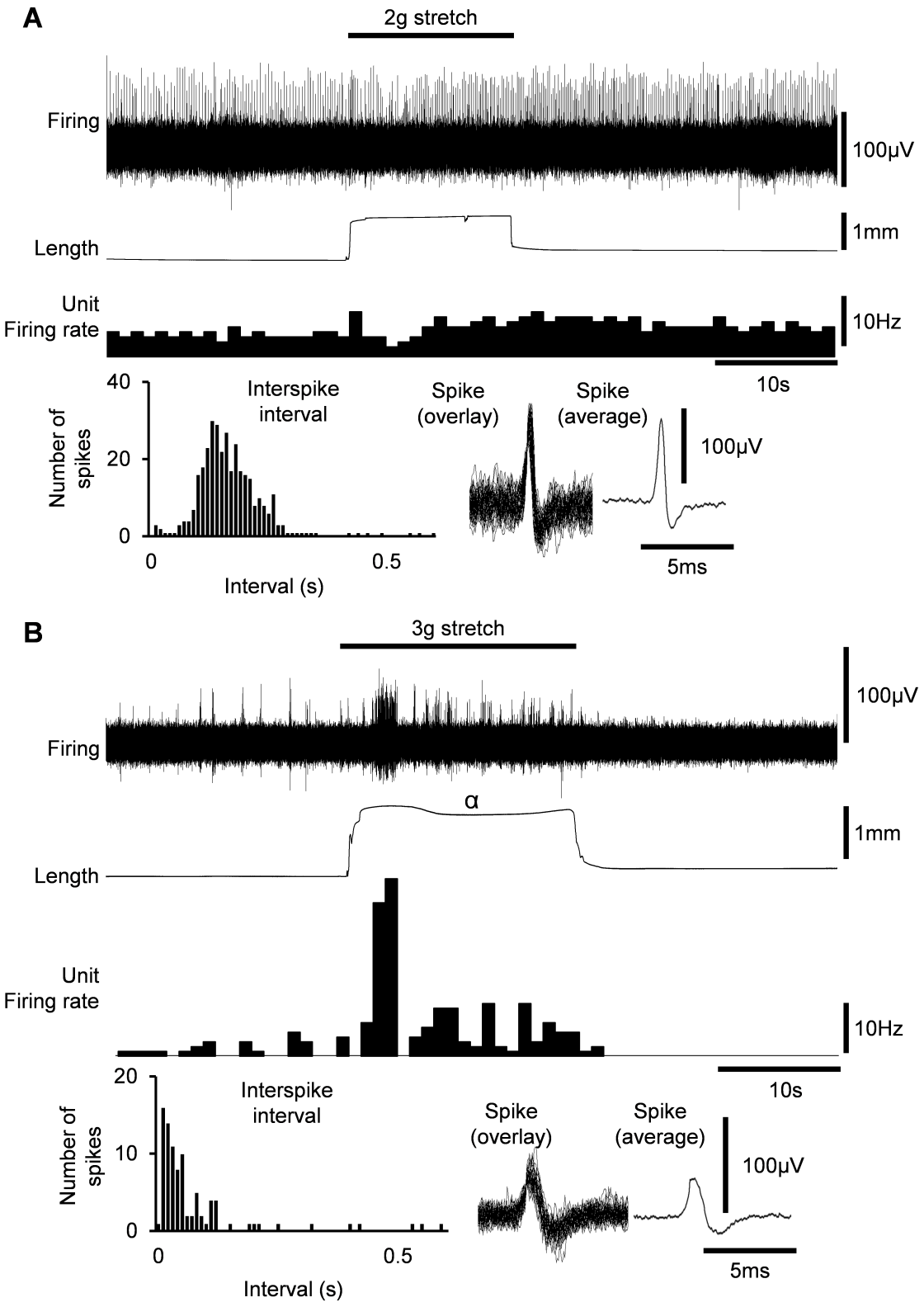

Figure 2.10 see next page for figure legend

Figure 2.10

Firing in colonic nerve trunks of organ-cultured preparations during circumferential isotonic stretch. Some stretches evoked reflex contractions, shown as a decrease in length during stretch (marked "α" in **B** and in **figure 11A**). Instances where stretch did not evoke contractions were accompanied by modest changes in firing rate. Shown in **A** is an example of a 2g distension that did not evoke a detectable muscle contraction. In **B**, a reflex contraction of the circular muscle is evoked. As with spontaneous circular muscle contractions, these contractions were associated with large bursts of firing. As a result, a large increase in firing rate (up to 50Hz) is recorded in the example shown. Note that firing was abolished for a period after removal of the applied load in **B**, which later recovered (not shown). This suggests some adaptation had occurred.

Below the graphs of firing rate in **A** and **B** is the spike shape of the discriminated units, shown as overlays of single spikes, which demonstrates the variability in the recorded waveforms, as well as the average spike shape (60 spikes). Also shown is the interspike interval histogram of the unit binned into 10ms intervals. Interspike intervals were used in conjunction with discrimination by spike shape, amplitude and duration as an indication of whether the recorded firing was consistent with a single neuron. Single neurons rarely fire faster than 50Hz. Thus, few or no spikes occurring within 10ms (first bin on the left) is consistent with firing rate of a single neuron.

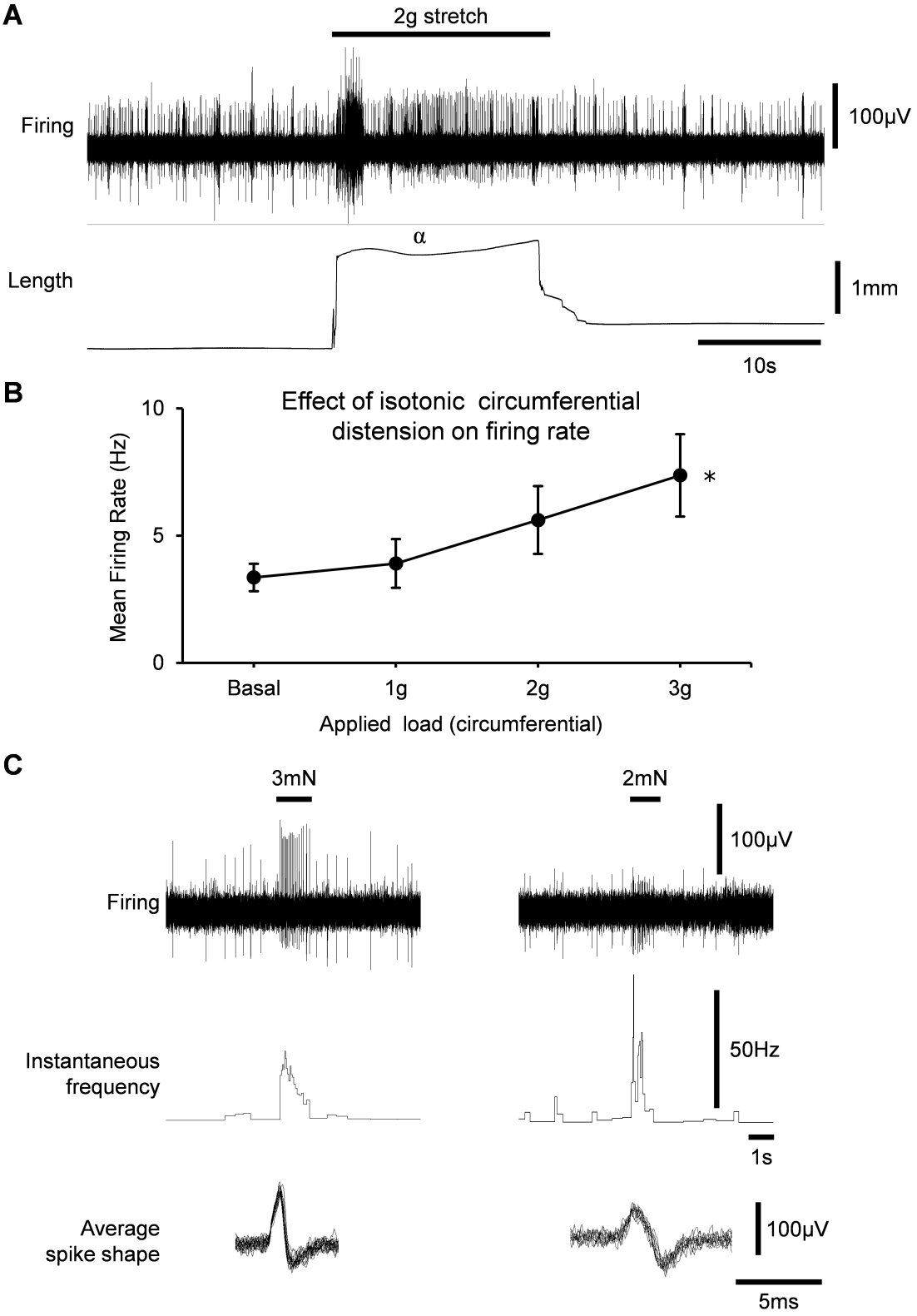

Figure 2.11 see next page for figure legend

Figure 2.11

Mechanically evoked firing. A further example of a circumferential stretch that evoked a reflex contraction of the circular muscle and was accompanied by a large burst of firing is shown in **A**. The overall effect of isotonic distensions of the circular muscle up to 3g on the firing rate recorded from colonic nerve trunks in organ-cultured preparations is shown in **B**. There was a significant effect of stretch on firing rate when all cases were included, regardless of whether reflex contractions were evoked by the stretch (9 units, $n=6$, $p<0.05$, 1-way ANOVA). Firing rates in **B** are shown as the mean ± standard error of the mean. Another type of mechanical stimulation tested was focal compression of the tissue by application of calibrated von Frey hairs to the surface of the circular muscle layer. Sites could be found in each preparation tested with focal probing that evoked bursts of firing when probed. Examples of single units in different organ-cultured preparations that were activated by a focal mechanical stimulus are shown in **C** and **D**. The burst of firing shown in **C** was evoked in the presence of 400μM hexamethonium; firing in **D** was evoked during perfusion with a Ca^{2+} free Krebs solution.

The association between large bursts of firing and reflex contractions of the circular muscle during distension

Distensions	'Large-burst' firing (+)	'Large-burst' firing (-)
Contraction (+)	8*	3
Contraction (-)	2	21

Table 2.01

The effect of circumferential stretch on evoked contractions of the circular muscle and occurrence of 'large burst' firing. The large bursts of firing were significantly associated with detectable contractions of circular muscle under load (X^2=11.8, df=1, p<0.001, Yates' continuity correction, *standardised residual=2.6).

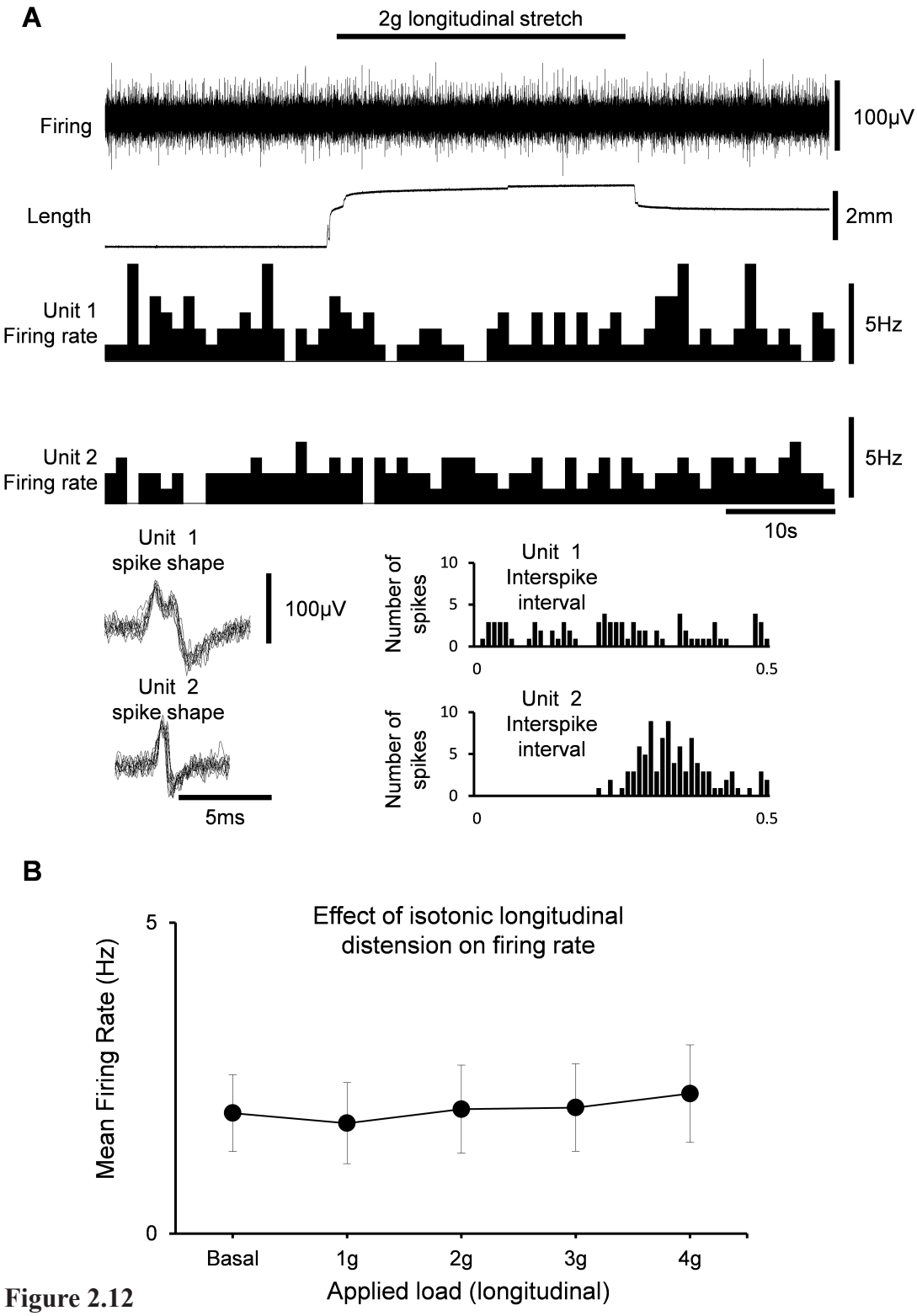

Figure 2.12

Isotonic longitudinal distension of organ-cultured preparations. Distending loads of up to 4g were applied in the longitudinal axis of preparations, without any significant effect observed on the firing rate recorded from colonic nerve trunks. An example of a 2g longitudinal stretch applied to a preparation with 2 discriminable units is shown in **A**. The overall relationship observed between longitudinal distensions and the rate of firing is shown graphically in **B**. This indicates there were no significant changes in firing rate within the range of longitudinal distensions tested. Firing rates in **B** are shown as the mean ± standard error of the mean.

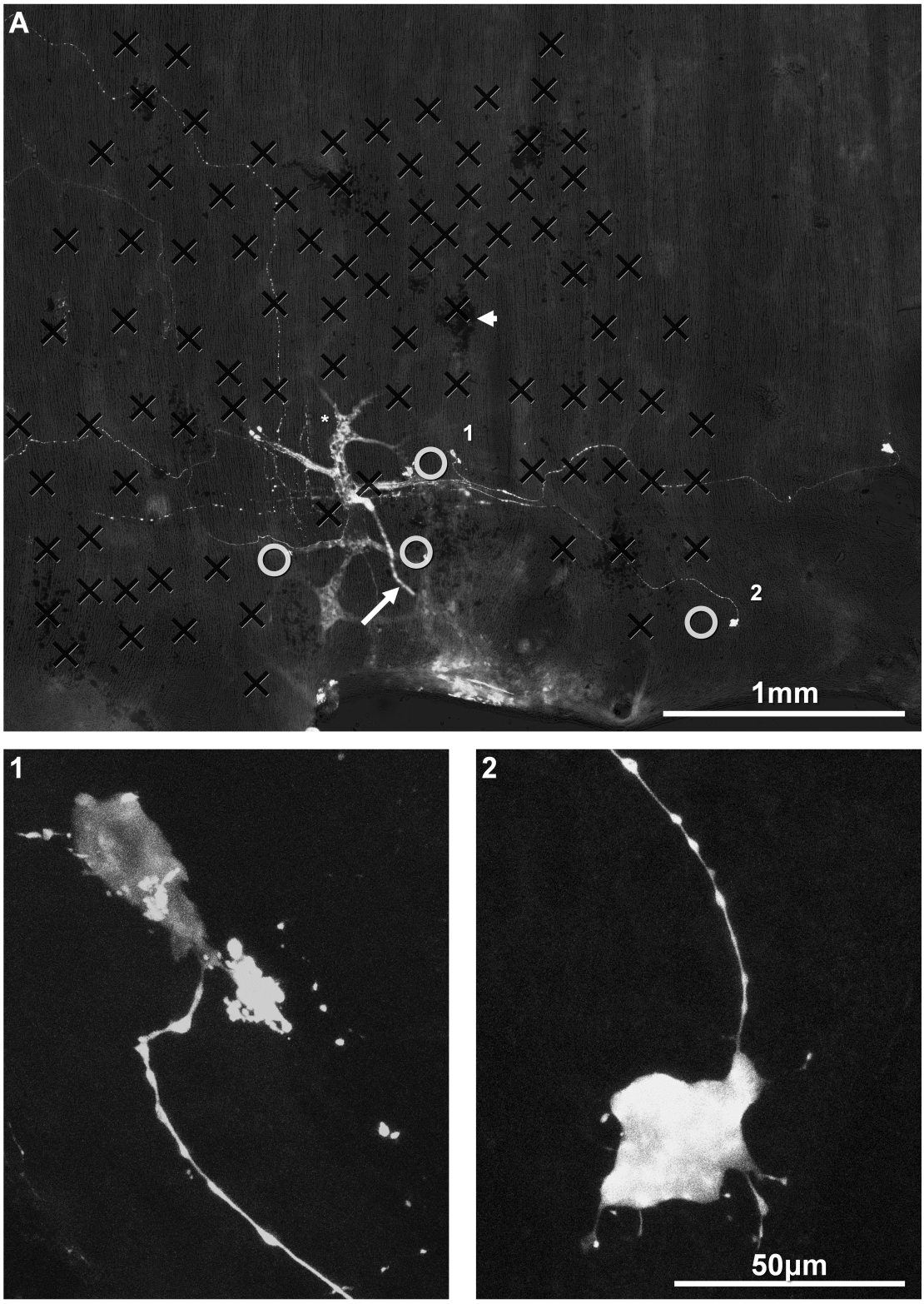

Figure 2.13 see next page for figure legend

Figure 2.13

Combined von Frey probing and biotinamide tracing from a colonic nerve trunk, in an organ-cultured preparation of guinea pig distal colon. **A** shows a photomontage of a preparation labelled with biotinamide from the recorded nerve trunk. This indicates all the sites where a von Frey hair was applied to the surface of the circular muscle – these are shown as black crosses and open white circles. The irregularly shaped black spots of carbon graphite can be seen on the circular muscle layer (example adjacent the arrowhead); carbon markings were used as reference points to mark the locations that were probed with von Frey hairs. Each of the superimposed black crosses indicates sites that were probed with a von Frey hair (2-3mN) and no measurable change in firing rate was detected. The open white circles indicate sites where probing with a von Frey hair promptly evoked a burst of firing (as in **figure 11C**). Some biotinamide leakage into myenteric ganglia adjacent the entry of the colonic nerve trunk can be seen (asterisk). Despite this, viscerofugal nerve cells bodies are clearly apparent. Note that each of the marked activation sites (open white circles) were in close proximity to viscerofugal nerve cell bodies. This suggests that viscerofugal nerve cell bodies were the site of mechanotransduction. Two of the cell bodies, marked **1** and **2** in the photomontage, are shown in the confocal z-stacks (maximum intensity) correspondingly marked below. They are uniaxonal with a simple soma-dendritic morphology.

DISCUSSION

In this study, we have developed a preparation of guinea pig distal colon in which viscerofugal neurons, including their axons, are preserved and functional, while CGRP immunoreactive afferent neurons and TH immunoreactive sympathetic neurons are degenerated. Several lines of evidence indicate that viscerofugal neurons were the source of action potentials recorded from colonic nerve trunks in these preparations. First, viscerofugal cell bodies were always revealed by biotinamide applied to colonic nerves in both cultured and acute preparations. After organ culture, viscerofugal axons constituted the majority of all surviving axons in colonic nerve trunks. The common markers for extrinsic spinal afferent neurons and sympathetic efferent neurons (CGRP and TH, respectively) were almost entirely depleted by 5 days organ culture apart from a few intrinsic neurons containing CGRP. This strongly suggests that degeneration of severed extrinsic fibres had occurred, including the axons of spinal afferent neurons. Capsaicin evokes action potential discharge of spinal afferent neurons (Holzer, 2004). In the present study, all preparations recorded immediately after dissection fired strongly to capsaicin, consistent with the presence of functional spinal afferent neurons. After 5-6 days of organ culture, no firing responses to capsaicin could be evoked. This provides functional evidence that extrinsic mechanosensitive nerves were depleted after organ culture. Sensitivity to nicotinic receptor agonists, regardless of the contractile state of the gut, is consistent with previous reports of viscerofugal neuron pharmacology (Crowcroft et al., 1971b), which have been shown immunohistochemically to express nicotinic receptors (Ermilov et al., 2003). Finally, the punctate mechanosensitive sites revealed by von Frey hairs in this project are consistent with responses by

isolated viscerofugal neurons but not with the extensive fields of innervation of extrinsic sensory neurons in the gut wall. Based on these data, it is reasonable to conclude that viscerofugal neurons were the major, if not the sole source of action potentials recorded from colonic nerve trunks after 5-6 days of organ culture.

Viscerofugal neuron firing and motor activity

Burst firing patterns in viscerofugal neurons have not previously been reported in any electrophysiological studies. However, burst firing activity by enteric neurons has been reported in small intestine (Wood, 1970) and colon (Ohkawa and Prosser, 1972). Viscerofugal neurons are present in both regions, but in greater numbers in the latter (Messenger and Furness, 1992, 1993). Wood (1970) identified "burst" and "single spike" units. Burst-type units were sub-classified into "steady" and "erratic" bursters based on the variability of their inter-burst interval (Wood, 1975). The activity of the latter type was blocked in Ca^{2+}-free solution while the former was not (Wood, 1975). From the present study, viscerofugal neurons are similar to 'erratic bursters' with inter-burst interval and the number of action potentials within each burst similar to Wood's report (2.9 ± 1.4s and 4.2 ± 1.4 action potentials per burst (Wood, 1989), and 2.4 ± 0.2s inter-burst interval and 5.4 ± 2.3 action potentials in the present study).

In vitro, the guinea pig colon demonstrates irregular ongoing activation of motor pathways to both circular and longitudinal muscle layers (Spencer and Smith, 2001). The synchronized output from these myenteric pathways to circular muscle cells occurred at comparable frequencies to the burst firing activity recorded in the present study (Spencer et al., 2001). We speculate that burst firing of viscerofugal neurons

may reflect synaptic input from the same motor circuits. This would also explain why viscerofugal neuron firing typically increased before the shortening of circular muscle commenced in spontaneous contractions (**figure 2.09**). It may also explain why the peak response to distension was reached after the passive lengthening caused by rapidly applied load and just before reflex contractions (see **figure 2.10B** and **2.11A**). Compatible with this, Miller and Szurszewski (2002) showed that fast synaptic inputs (from viscerofugal neurons) to sympathetic ganglion neurons peaked prior to phasic contractions of the intestine with firing at a lower frequency during the peak of contraction (when the intestine was empty and the circumference was minimized). In some cases, distending loads of circular muscle did not evoke reflex contractions of the circular muscle. In these cases, viscerofugal neuron firing responses were modest, rarely doubling above the basal firing rate. This modest response amplitude is comparable to the small increase in frequency of synaptic fast potentials recorded in inferior mesenteric ganglion of the guinea pig in response to colonic distension (Anthony and Kreulen, 1990, Bywater, 1993, Parkman et al., 1993, Stapelfeldt et al., 1993, Ermilov et al., 2004b). In the present study, with the largest stimulus applied, peak firing frequency was less than 8Hz, compared to basal ongoing firing close to 4 Hz in the absence of distension.

Dual roles of viscerofugal neurons

Distension activates robust cholinergic pathways from the gut to sympathetic pre-vertebral ganglion neurons, mediated via viscerofugal neurons (Crowcroft et al., 1971b). When nicotinic receptors in the gut wall are pharmacologically blocked, distension-evoked viscerofugal synaptic input to sympathetic neurons is significantly depressed (Crowcroft et al., 1971b). This suggests that viscerofugal neurons may be

synaptically driven via cholinergic pathways, similar to many other classes of enteric neurons (Gershon, 1967, Nishi and North, 1973, Brookes et al., 1987, Brookes et al., 1997b). However, some viscerofugal output from the gut persists, even when synaptic transmission in the gut is entirely blocked by Ca^{2+}-depleted solution (Bywater, 1993, Parkman et al., 1993, Stebbing and Bornstein, 1993). This has been interpreted as evidence that viscerofugal neurons may also be directly mechanosensitive. It has also been suggested that fast cholinergic synaptic inputs in prevertebral ganglia may arise from collaterals of spinal afferent neurons (Keef and Kreulen, 1990). The present study suggests that viscerofugal neuron cell bodies are directly mechanosensory. When tested, direct, localised responses to von Frey hairs (1-3mN) occurred in all viscerofugal units identified in our recordings at a single restricted site on the preparation. The observation that some enteric neurons are both mechanosensitive and receive synaptic input has a precedent. Recordings using voltage-sensitive dyes suggest that many enteric interneurons and motor neurons with fast synaptic inputs (S/Type I cells) may function as rapidly adapting mechanosensors to physiological stimuli (Mazzuoli and Schemann, 2009). In addition, slowly-adapting Dogiel type I "sensory interneurons" in the guinea pig distal colon are both mechanically and synaptically activated by ongoing distension of the gut wall (Spencer and Smith, 2004).

Subtypes of viscerofugal neurons

Retrograde tracing studies, using biotinamide or fast blue, or intracellular dye filling with biocytin, suggest that viscerofugal neurons are uniaxonal with smooth or irregular shaped cell bodies and short lamellar or filamentous dendrites (Kuramoto and Furness, 1989, Furness et al., 1990c, Messenger and Furness, 1992, Furness and

Anderson, 1993, Messenger and Furness, 1993, Sharkey et al., 1998, Tassicker et al., 1999a, Lomax et al., 2000, Olsson et al., 2004). A study using DiI and intracellular filling with Lucifer Yellow in guinea pig colon revealed a subset (30%) of multiaxonal viscerofugal neurons, although the majority were uniaxonal (Ermilov et al., 2003). In the present study, degeneration of sympathetic and sensory fibers in organ culture allowed observation of large numbers of biotinamide-filled viscerofugal neurons uncomplicated by surrounding nerve fibres. All appeared to be uni-axonal with either simple or lamellar Dogiel type I soma-dendritic morphology. The discrepancy in morphological identification may be related to the type of tracer used (DiI labelling tends to be punctate and Lucifer Yellow gives less complete fills than biotin derivatives; Bornstein et al., 1991b), or differences between strains of guinea pigs.

Conclusion

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