Chapter 3
Identification of viscerofugal neurons in extracellular recordings from colonic nerve trunks in fresh preparations of guinea pig distal colon

INTRODUCTION

Viscerofugal neurons have cell bodies in the gut wall and project to prevertebral ganglia, forming a neural circuit with noradrenergic sympathetic neurons that control motility (Weems and Szurszewski, 1977) and secretion (Quinson and Furness, 2002). Localised distension of the gut potently activates this circuit, resulting in inhibition of gut contractility orally and locally (Kuntz, 1940, Kuntz and Saccomanno, 1944). Populations of viscerofugal neurons have been studied indirectly by recording their converging cholinergic inputs in sympathetic neurons (Crowcroft et al., 1971b). Intestinal distension increases the frequency of cholinergic synaptic potentials to sympathetic neurons (Crowcroft et al., 1971b) and may also evoke neuropeptide release (Ermilov et al., 2004b). To date, there have been no studies characterising the responses of single viscerofugal neurons to mechanical stimuli. Extracellular recordings from extrinsic nerve trunks to the intestine would be one way to achieve this, but it has not been possible to unequivocally identify the firing of viscerofugal neurons in such recordings (Cervero and Sharkey, 1988). Extrinsic nerve trunks to the gut contain the axons of enteric viscerofugal neurons as well as those of extrinsic primary afferent neurons. Over the last decade, it has become possible to correlate sensory transduction sites with neuroanatomical structures by combining extracellular recording with neuronal tracing. This has resulted in positive identification of recordings belonging to specific morphologically characterized classes of extrinsic sensory neurons, including vagal IGLEs (Zagorodnyuk and Brookes, 2000), rectal IGLEs (Lynn et al., 2003), and medium/high-threshold mechanoreceptors on blood vessels (Song et al., 2009). In this series of experiments,

we used a similar technique to identify the action potentials of viscerofugal neurons in extracellular recordings from colonic nerve trunks.

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, $NaH_2PO_42H_2O$ 1; $NaHCO_3$ 25; $MgCl_26H_2O$ 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. Mucosa and submucosa were removed by sharp dissection. Where specified, circular muscle was also removed, exposing the myenteric plexus. 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 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 shape using Spike Histogram and Scope View software (ADInstruments). During electrophysiological recordings, preparations were continuously superfused with normal Krebs solution (~1.6ml/min, 35°C).

Mapping procedure

Small fragments of carbon graphite (Sigma) were applied to preparations using a 100mg (1mN) von Frey hair tip that had been dipped into a concentrated sucrose solution and left to evaporate (Zagorodnyuk and Brookes, 2000). Preparations were photographed (Canon Powershot A650 IS) through an inverted bright-field microscope (Olympus CKX41) prior to recording. The photographs revealed the carbon fragments and the structure of myenteric plexus; both of which served as reliable markers later during electrophysiological recording and morphological analysis of biotinamide fills.

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, see **figure 3.01** for diagram). 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 site of drug ejection was revealed by the dye (see **figure 3.01B**, see also **chapter 4**, **figure 4.01** for micrographs of DMPP ejection); ejection of dye solution without DMPP was used as a control. Sites on the tissue at which drug ejection caused repeatable bursts of firing activity were marked on printed micrographs. The

sites were mapped with DMPP from 'downstream' to 'upstream', with respect to the flow of Krebs solution, in order to minimize spread of drug across the 'unmapped' areas. Focal mechanical sensitivity was also investigated using von Frey hairs (0.8-1mN). For both DMPP application and von Frey hair probing, a site was considered responsive if stimulation evoked a response on 3 successive occasions.

Biotinamide labelling

After electrophysiological recordings, a single drop of biotinamide solution (5% biotinamide (N-[2-aminoethyl] biotinamide hydrobromide), dissolved in artificial intracellular solution (150 mmol/L monopotassium L-glutamic acid, 7 mmol/L $MgCl_2$, 5 mmol/L glucose, 1 mmol/L ethylene glycolbis(ß-aminoethyl ether)-N,N,N=,N=-tetraacetic acid, 20 mmol/L HEPES buffer, 5 mmol/L disodium adenosine triphosphate, 0.02% saponin, 1% dimethyl sulfoxide, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 20g/mL gentamycin sulphate) was placed on the recorded nerve trunk and normal Krebs solution in the main chamber was replaced with 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 $CaCl_2$.. The preparations were incubated overnight (12-16 hours; 36°C, 5% CO_2 in air). After incubation, the preparations were fixed overnight in Zamboni's fixative (15% saturated picric acid, 2% formaldehyde in 0.1 M phosphate buffer, pH 7.0). Preparations were cleared in DMSO (3 x 10 minute washes) and then washed in 0.1M phosphate-buffered saline (0.15 M NaCl, pH 7.2; 3 x 10 minute washes) followed by

incubation for 3 hours in 3-1-*O*-(2-cyanoethyl)-(*N,N*-diisopropyl)indo-carbocyanine (CY3) conjugated streptavidin. Preparations were then washed with PBS (3 x 10 minute) and equilibrated in a series of carbonate-buffered glycerol solutions (50, 70 and 100% solutions; 3 x 10 minutes) before being mounted 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). Some images (**figure 3.06**) 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 20x multi-immersion lens at 1µm slices through the Z-axis and a 63x oil-immersion lens at 0.5µm slices through the Z-axis. Brightness and contrast adjustments, cropping, pseudocolouring and photomontages of biotinamide labelled preparations were performed using Adobe Photoshop (CS1, Adobe Systems Inc, San Jose, CA).

Mapping analysis

Photomontages of the mapped area in acute preparations were made with brightfield illumination (to visualize carbon spots) and CY3 fluorescence (to reveal biotinamide-

labelled fibres in extrinsic nerve trunks). DMPP-responsive sites (hotspots) were marked on montages using carbon spots as reference. In parametric analysis, the mapped area and hotspot sites were defined by X/Y coordinates. For each hotspot site, ten random X/Y coordinates were generated using a random number generator (Microsoft Office Excel). Random sites were confined to the mapping area and their distances to the nearest viscerofugal cell body were calculated. Mean distances of viscerofugal cell bodies from hotspot sites were compared to those from random sites and tested with Student's t-test.

In addition, individual ganglia in the mapped area were defined as DMPP-sensitive or DMPP-insensitive depending on whether DMPP evoked bursts of action potentials. The same ganglia were subsequently defined as either containing or not containing retrogradely-labelled viscerofugal cell bodies following biotinamide filling of the recorded mesenteric nerve trunk. The resulting 2 x 2 contingency table was tested by a chi squared test.

Drugs

Stock solutions of drugs were made as follows: 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). 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 (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). Chi-squared tests with Yates' correction for continuity were performed using IBM SPSS. 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 ($P>0.05$).

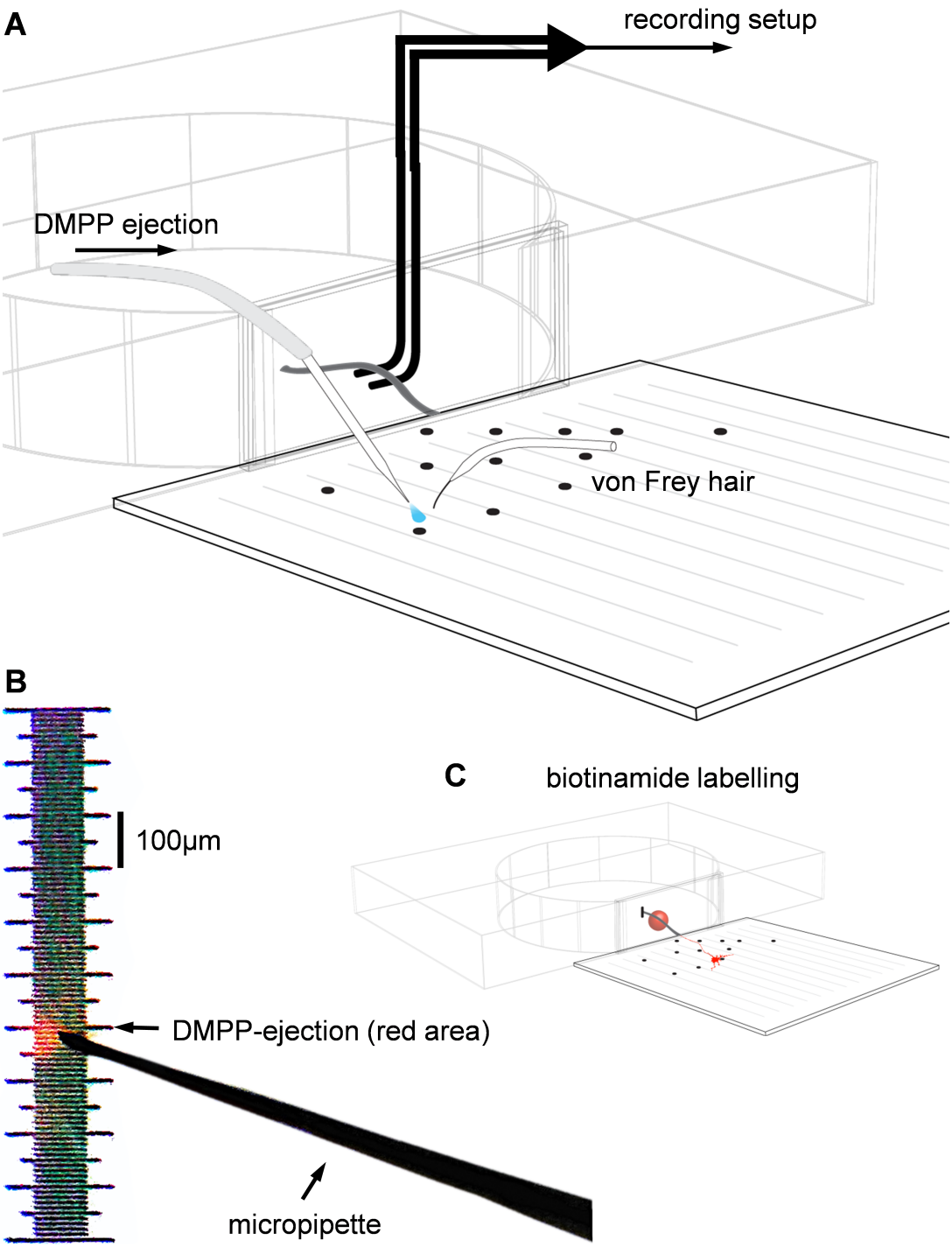

Figure 3.01

Combined physiological mapping and neuroanatomical tracing technique. (**A**) DMPP and von Frey hairs were applied to preparations during standard differential extracellular electrophysiological recordings from colonic nerves. Black carbon markers (graphite) are shown on the preparation. These were used as reference points to map sites on the tissue where DMPP and von Frey probes were applied. These sites were marked on printed micrographs of the preparation. DMPP application was focal, typically limited to an area of about 100µm in diameter immediately after ejection, before diffusing away from the tissue. A puff of DMPP is shown after it was ejected from a micropipette in **B**. The size of the region covered by DMPP is shown as an area coloured red, against a 1mm scale. After extracellular recording from colonic nerves, the same nerves were labelled with the neuronal tracer, biotinamide (**C**).

RESULTS

Localization of extracellularly recorded viscerofugal neurons

Our first aim was to develop a method to reliably distinguish action potentials of viscerofugal neurons from those of extrinsic sensory nerves that project in the same colonic nerve trunks. Capsaicin, which activates 85% of medium-high threshold afferent neurons (Song et al., 2009), was applied into the recording chamber to desensitize most extrinsic sensory axons. A single bolus of capsaicin (final bath concentration 0.3µM) evoked a large burst of action potentials in 8 of 9 freshly dissected preparations (**figure 3.02A**). Further applications of capsaicin failed to evoke additional firing (n=9, **figure 3.02B**).

Viscerofugal neurons have nicotinic receptors and are potently activated by nicotinic receptor agonists (Crowcroft et al., 1971b, Ermilov et al., 2003). The nicotinic receptor agonist, DMPP (10^{-3}M) applied as a bolus directly to the tissue, evoked firing in 9 of 9 preparations (**figure 3.02C**), suggesting viscerofugal neurons were present in recordings. DMPP was applied focally to individual myenteric ganglia, from a micropipette (10-20µm tip diameter) by pressure ejection (140kPa pulses of Helium for 10 - 50ms). Most ganglia tested in this way were unresponsive (see **figure 3.06**, for example). However, at 1-3 sites in each preparation, bursts of action potentials were recorded in the colonic nerve, immediately after DMPP ejection. DMPP-responsive sites did not respond to pressure ejected Krebs solution mixed with blue food dye without DMPP (2 units, n=2). Responding and non-responding myenteric ganglia were marked on a printed micrograph of the preparation. A typical example of a response to DMPP is shown in **figure 3.03A**. Overall, 24 DMPP-

responsive sites were identified (**figure 3.03B**). These were located in 23 ganglia in 9 preparations (one large ganglion had 2 distinct DMPP-responsive sites). At 15 of these sites, single units could be clearly discriminated (mean firing rate 3.6 ± 2.1Hz; peak firing rate after DMPP 34.5 ± 10.6Hz, 15 units, n=9). In the other 9 sites we could not uniquely discriminate single afferent units, due to a low signal to noise ratio. None of the 15 discriminated units responded to capsaicin applied at the same site (15 units, n=9). After recordings, nerve trunks were filled with biotinamide and the distribution of labelled viscerofugal nerve cell bodies was compared to responsive sites marked on the printed micrograph.

The 23 ganglia that showed responses to locally applied DMPP were from a total sample of 213 ganglia tested (n=9); 19 of the 23 ganglia contained one or more biotinamide-labelled viscerofugal cell bodies. The other 4 ganglia (of the 23) had responses to DMPP but did not contain a labelled viscerofugal neuron. Of the 213 ganglia tested, the remaining 190 showed no response to DMPP; of these, 180 had no retrogradely labelled viscerofugal cell bodies but 10 contained one or more viscerofugal neurons, from which no DMPP response was recorded. **Figure 3.04A** shows an example of a mapped preparation; **table 3.01A** shows the data generated from that example and **table 3.01B** shows combined results (n=9). The association between DMPP responses and the presence of retrogradely filled viscerofugal nerve cell bodies within the same ganglia was highly significant (X^2=103.1, df=1, $p<0.001$; Yates' correction for continuity; standardized residual = 9). **Figure 3.05** shows a large photomontage of biotinamide labelling and superimposed carbon markers of the preparation shown in **figure 3.04**.

DMPP-responsive sites were 173±156μm from the nearest biotinamide-filled viscerofugal cell body (24 sites, n=9), which was significantly closer than randomly generated sites within the mapped area in the same preparations (872 ± 549μm, 240 sites, $p<0.001$). In one case, 2 DMPP-responsive sites were distinguished within a single ganglion. Taken together, these results demonstrate that viscerofugal units can be readily identified in extracellular recordings by their responses to DMPP, and their cell bodies can be reliably localized to individual myenteric ganglia. **Figure 3.06** shows a mapped preparation, including all DMPP-responsive and unresponsive sites. Consistent with neuroanatomical descriptions of viscerofugal neurons (Messenger and Furness, 1992), responsive sites were located close to the mesenteric attachment (see **figure 3.03B**).

DMPP-responsive sites were an average of 229±170μm away from the mesenteric border, defined the point at which the mesenteric nerve entered the myenteric plexus. Assuming that extrinsic fibres from a mesenteric nerve innervate half of the circumference of the intestine (a mean circumferential distance of 5250 ± 490 μm, n=9 in our preparations), viscerofugal neurons are significantly closer to the mesenteric border than would be expected by chance ($P<0.001$, paired t-test, df=23)

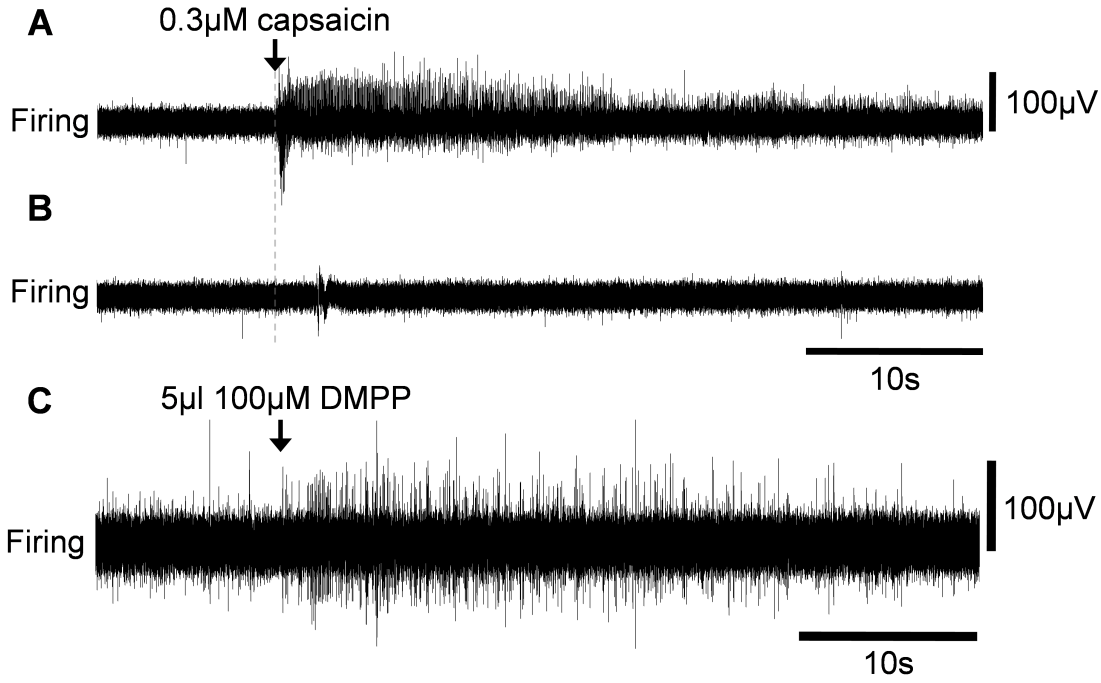

Figure 3.02

The effect of capsaicin and DMPP on firing activity recorded from colonic nerve trunks. A large bolus of capsaicin was applied to each preparation at the beginning of a recording. A bolus of capsaicin evoked strong firing responses on the first application in 8 of 9 preparations (**A**). All subsequent applications failed to evoke firing. An example of this is shown in **B**. This suggests that capsaicin-sensitive spinal afferent neurons had been desensitized. The nicotinic receptor agonist, DMPP, was applied directly to preparations. Bursts of firing was evoked by DMPP in all preparations, consistent with the presence of viscerofugal neurons, which have nicotinic receptors.

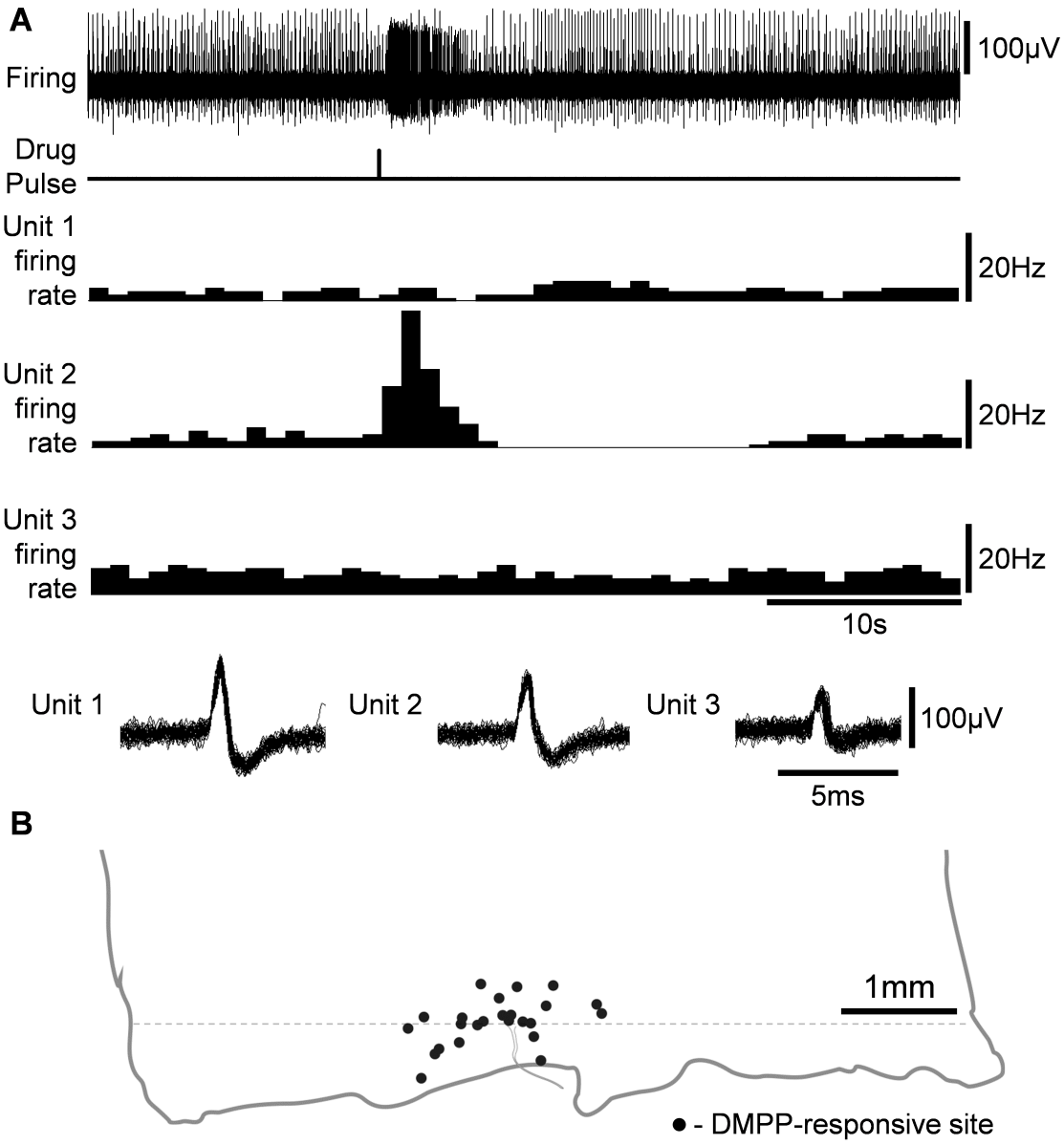

Figure 3.03

Focal application of DMPP. Shown in **A** is an example firing response to DMPP ejected from a micropipette onto part of a single myenteric ganglion. DMPP-ejection promptly evoked an increase in firing rate, up to 40Hz, from a single unit (unit 2). Note that DMPP-ejection did not affect the firing rate of the other units, probably because the DMPP affected a small area of tissue (see figure **3.1B**). Subsequent biotinamide filling of the recorded nerve trunk revealed a viscerofugal nerve cell body located 50-60µm from this activation site. The spike shapes of the 3 discriminated single units (shown below) have clearly distinguishable waveforms. (**B**) Composite map showing the locations of all sites at which DMPP evoked firing in all 9 preparations (24 sites, n=9). The activation sites were typically located close to the mesenteric border (dashed line).

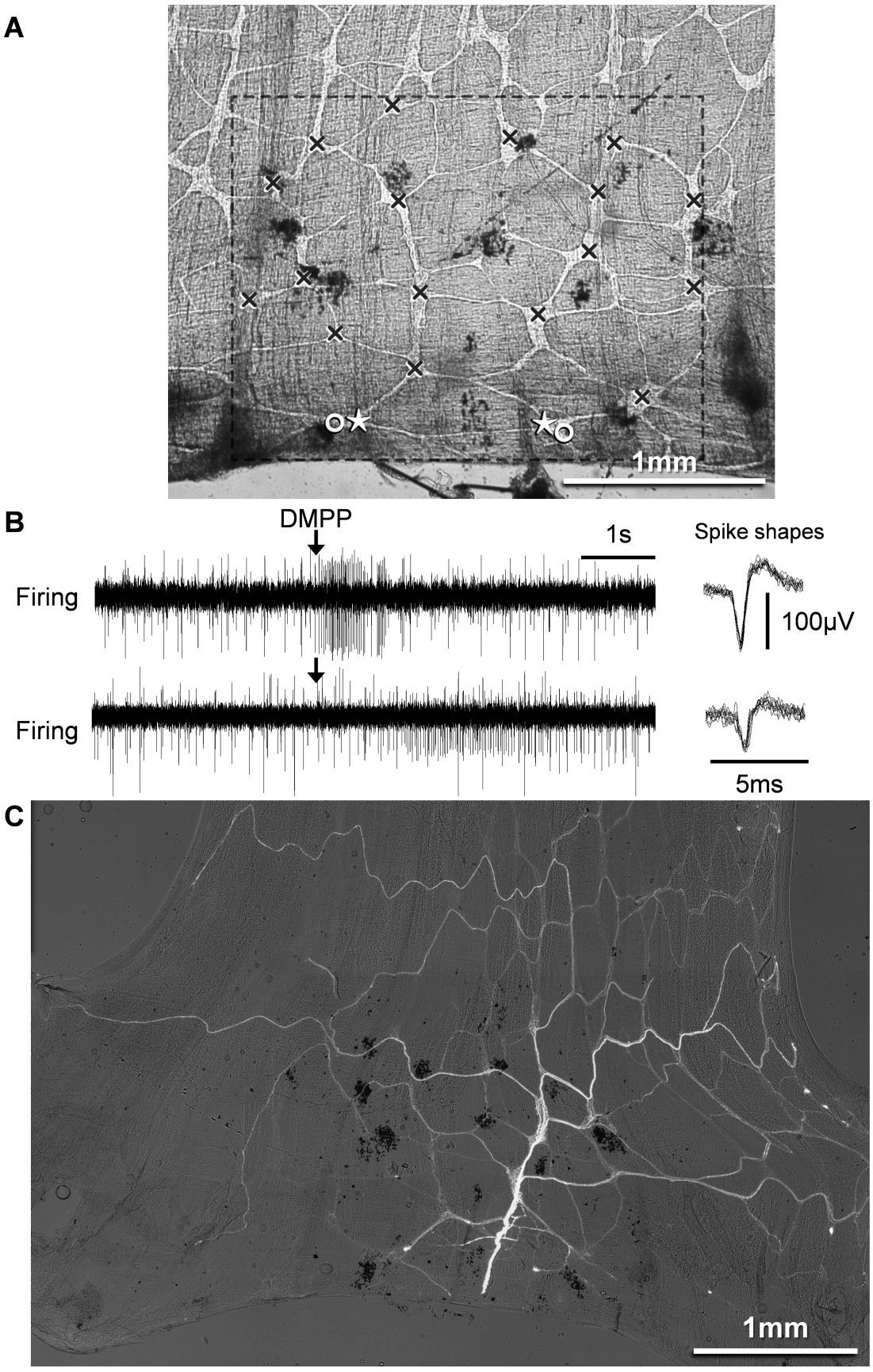

Figure 3.04 see next page for figure legend

Figure 3.04

(**A**) Photomicrograph of a live preparation, showing the myenteric ganglia where DMPP application evoked firing (open white circles) or no detectable response (black crosses) in recordings from colonic nerves. After biotinamide labelling, filled viscerofugal nerve cell bodies within the tissue were mapped (white stars; also see **C**, below, and **figure 3.5**). The results of this preparation are summarized in **table 3.1A**; combined results from 9 preparations are shown in **table 3.1B**. Firing responses to DMPP were associated with biotinamide filled viscerofugal cell bodies in the same ganglia. Shown in **B** are responses to DMPP applied at the locations shown in **A**. The upper and lower traces show the effect of DMPP applied to the ganglia at the left and right open white circles, respectively. The upper trace shows a burst of firing to DMPP, lasting approximately 1 second (larger unit, shown adjacent); DMPP in the lower trace evoked more prolonged firing discharge after a small delay (smaller unit, shown adjacent). (**C**) Photomontage of the preparation in **A** after fixation and biotinamide labelling.

The association between DMPP-evoked firing and biotinamide-filled nerve cell bodies - single preparation

Ganglia	DMPP (+)	DMPP (-)
Viscerofugal nerve cell body (+)	2	0
Viscerofugal nerve cell body (-)	0	17

Table 3.01A

These results indicate whether an individual myenteric ganglion contained (+) or did not contain (-) a retrogradely labelled viscerofugal nerve cell body, and whether focal DMPP application at that same ganglion had evoked (+) or did not evoke (-) a detectable burst of firing. This table refers to the example shown in **figure 3.04A**. In this example, there was a perfect match between the ganglia that contained viscerofugal nerve cell bodies, and where DMPP had evoked firing. This strongly suggests that the recorded firing arose from viscerofugal neurons. The pooled data from all preparations are shown below.

The association between DMPP-evoked firing and biotinamide-filled nerve cell bodies - pooled data

Ganglia	DMPP (+)	DMPP (-)
Viscerofugal nerve cell body (+)	19^{α}	10^{β}
Viscerofugal nerve cell body (-)	4^{δ}	180

Table 3.01B

Combined results showing whether individual myenteric ganglion either contained (+) or did not contain (-) a retrogradely labelled viscerofugal nerve cell body, and whether focal DMPP application applied at the same ganglion caused (+) or did not cause (-) an immediate firing response. This table includes results from all DMPP-mapped preparations (n=9) and shows that DMPP-evoked firing at a myenteric ganglion was strongly associated with a viscerofugal nerve cell body later revealed in the same ganglion. This supports the conclusion that the detected firing arose from viscerofugal neurons. X^2 test: $p<0.001$, $X^2=103.1$, df=1 with Yates' correction for continuity. Post hoc analyses: α: significantly more DMPP+ ganglia contained biotinamide-labelled viscerofugal nerve cell bodies than expected (standardized residual = 9); β: significantly less DMPP- ganglia contained biotinamide-labelled viscerofugal nerve cell bodies than expected (standardized residual = -3.1); δ: significantly less DMPP+ ganglia lacked biotinamide-labelled viscerofugal nerve cell bodies than expected (standardized residual = -3.6).

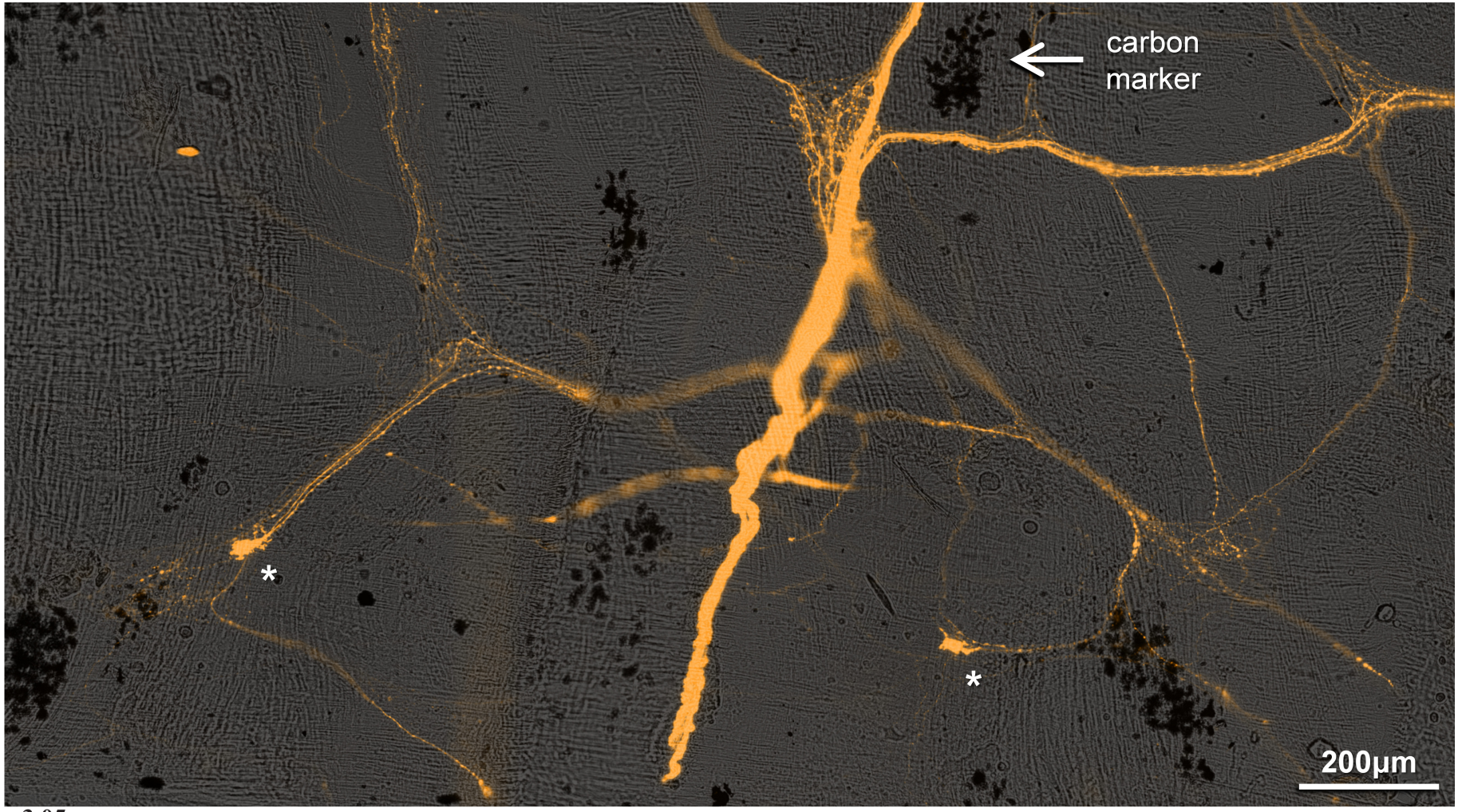

Figure 3.05

Biotinamide labelling with superimposed carbon markers. This photomontage shows the preparation presented in **figure 3.4** which had 2 DMPP-responsive ganglia; the two viscerofugal nerve cell bodies contained within the ganglia are clearly visible (adjacent asterisks).

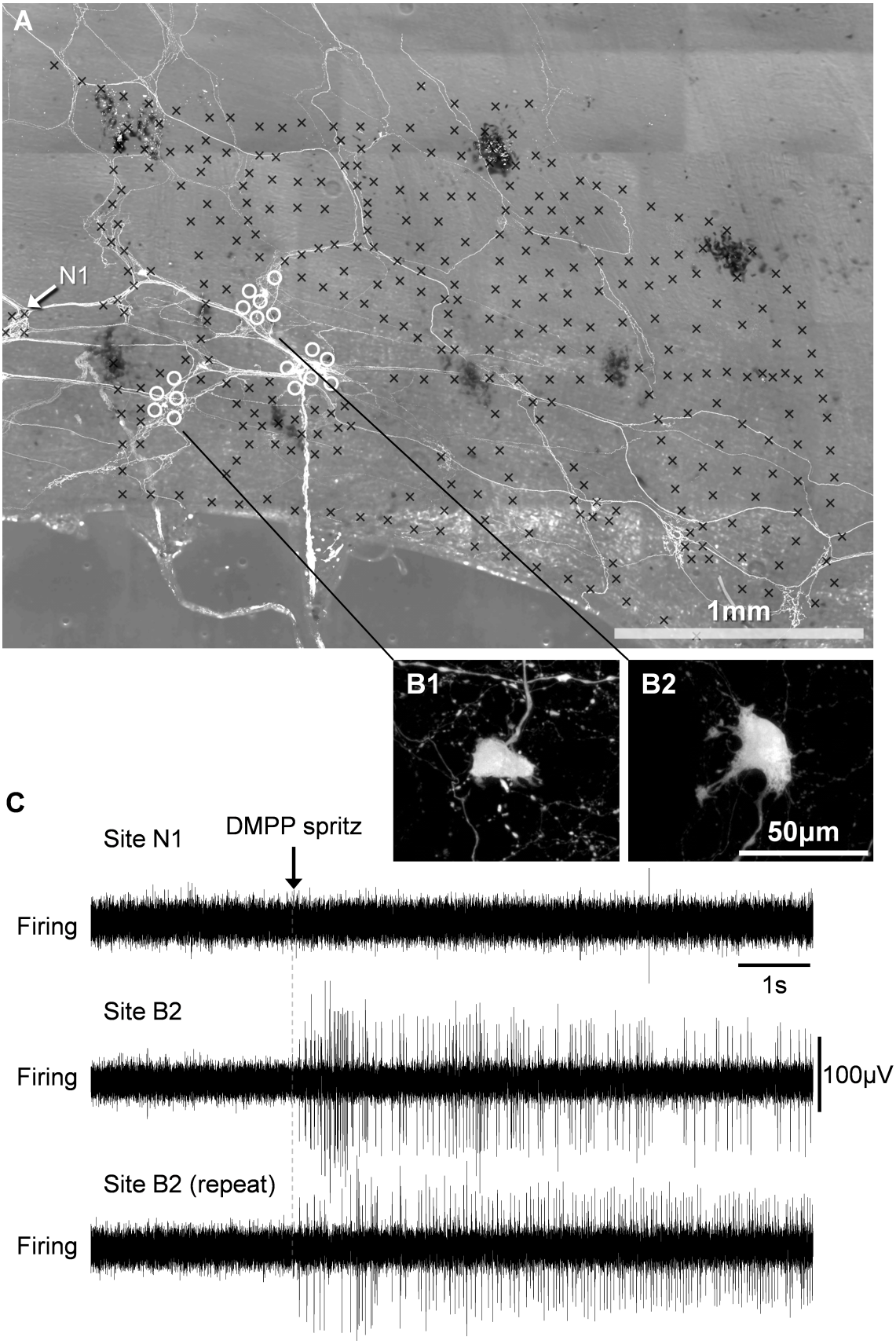

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Figure 3.06

Map of responses to DMPP superimposed on a photomontage of dye filling (**A**) Photomontage of a biotinamide-filled preparation showing site where DMPP was focally applied. Sites that evoked firing are shown by open white circles. Sites where DMPP evoked no detectable response are shown by black crosses. Myenteric ganglia and carbon marks on the preparation were used as landmarks. (**B1** & **B2**) Confocal micrographs of biotinamide labelled viscerofugal cell bodies adjacent to two DMPP-responsive sites. (**C**) Effect of DMPP ejection onto a myenteric ganglion (see the site marked **N1** in photomontage **A**) which did not evoke a firing response, and repeated DMPP pulses at sites near the cell body in **B2**.

Focal mechanical stimulation

Focal compression of the tissue with light von Frey hairs (0.8-1mN) at DMPP-responsive sites evoked repeatable bursts of action potentials (mean peak firing rate; 20.0±9.0Hz, 10 units, n=6, **figure 3.07**). Thus, about half the units identified using focal DMPP were also sensitive to mechanical stimulation at the force tested (0.8-1mN). A more detailed study of the effects of focal mechanical stimulation, using a greater range of probe strength, follows in **chapter 4**.

Viscerofugal neuron morphology

Biotinamide tracing of colonic nerve trunks in DMPP mapping studies revealed a total of 62 viscerofugal neuron cell bodies. Cell bodies were 35.5 ±10.2 μm and 19.8 ± 3.8μm along their major and minor axes, respectively. Two types of cell body morphology were observed – smooth, spherical- or fusiform-shaped cell bodies resembling 'simple cells' that had no discernible dendrites (23/62 cells), and ovoid- or irregular-shaped cell bodies with short flat irregular shaped dendrites ("lamellar" Dogiel type I cells; 39/62 cells). Biotinamide filling usually labelled many fine ramifying axons (largely sympathetic) some of which ran close to filled viscerofugal nerve cell bodies. In many cases it was impossible to be sure whether these connected with viscerofugal cell bodies or not. However, 7 of 62 viscerofugal neurons were not surrounded by other labelled fibres, so that their axonal processes could be unequivocally identified. All were uni-axonal. No definite multi-axonal viscerofugal neurons were observed in the present study, unlike one previous report (Ermilov et al., 2003). Viscerofugal neuron morphology is covered in more detail in **chapter 5**.

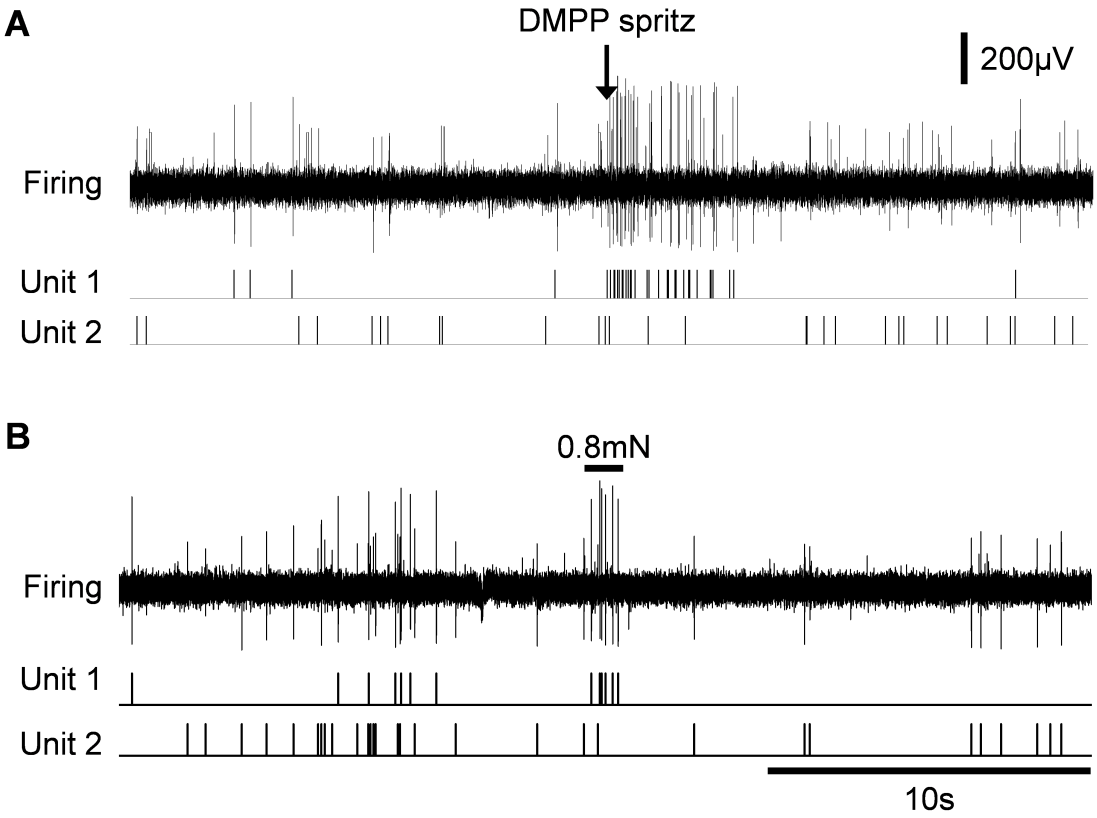

Figure 3.07

Mechanical activation of viscerofugal neurons using von Frey hairs. (**A**) Focal application of DMPP onto the preparation promptly evoked firing from a single viscerofugal neuron. In this recording there were 2 discriminable units; DMPP at this particular site evoked firing of a single unit. (**B**) A light von Frey hair (0.8mN) applied at the same site evoked a burst of firing from the same unit that was activated by DMPP.

DISCUSSION

Morphological identification of afferent neurons

In studies of sensory innervation of the skin, the morphological identity of many physiologically–identified cutaneous afferents has been established, providing a firm foundation for the understanding of cutaneous sensation (Iggo, 1960). Equivalent accounts of morphology and physiology for identified visceral afferents have been reported since the development of rapid anterograde tracing techniques (Tassicker et al., 1999b) combined with close-to-target in vitro afferent recordings. Intraganglionic laminar endings (IGLEs) correspond to the transduction sites of low threshold vagal mechanoreceptors in the stomach and oesophagus. (Zagorodnyuk and Brookes, 2000, Zagorodnyuk et al., 2001). Rectal IGLEs correspond to a distinct class of sacral mechanoreceptors to the distal bowel (Lynn et al., 2003). Varicose branching axons on extramural and intramural blood vessels have been morphologically identified as the transduction sites of putative mechanonociceptors in the guinea-pig small intestine (Song et al., 2009). The results of the present study have shown that DMPP-responsive viscerofugal neurons in guinea pig colon have uni-axonal cell bodies, mostly with "simple" or Dogiel type I morphology. These findings are consistent with previous reports in the guinea pig (Kuramoto and Furness, 1989, Furness et al., 1990c, Messenger and Furness, 1992, 1993, Sharkey et al., 1998, Tassicker et al., 1999b, Lomax et al., 2000, Olsson et al., 2004) and in a recent study of organ-cultured preparations in which extrinsic neurons had degenerated (Hibberd et al., 2012b).

In the present study, we have further developed this combination of techniques to allow us to establish the relationship between labelled viscerofugal nerve cell bodies and recordings from colonic nerve trunks. The majority of sites in the myenteric plexus that responded to locally-applied DMPP (19 of 23 sites) contained retrogradely-labelled viscerofugal neuron cell bodies. It is possible that in the other 4 ganglia biotinamide fills were incomplete and failed to fill neurons that were present. Alternatively, DMPP might act indirectly in a few cases, by activating other myenteric neurons which synapse onto viscerofugal neurons elsewhere in the preparation. Viscerofugal cell bodies are known to receive fast excitatory inputs from other myenteric neurons (Sharkey et al., 1998).

Theoretically, the fact that in 4 of 23 ganglia DMPP evoked colonic nerve firing but viscerofugal neurons were not filled by biotinamide raises the possibility that some DMPP-responsive sites may correspond to extrinsic sensory nerve endings. Consistent with this, some extrinsic vagal afferents in the rat duodenum, and spinal afferents in the mouse rectum, are activated by DMPP (Jiang et al., 2001). About 15% of medium-high threshold spinal afferent neurons with intramural transduction sites in the gut are insensitive to capsaicin (Song et al 2009) and thus may have remained active after the desensitising dose of capsaicin. We ruled out that capsaicin-sensitive extrinsic afferents recovered their excitability over the duration of our experiments by applying capsaicin again at the end of the recording period. In none of 7 preparations had responses recovered. The possibility that some spinal afferents may be activated directly by DMPP can only represent a small source of contamination, since dye filling showed such a strong association between DMPP-responsive sites and viscerofugal nerve cell bodies. Collaterals of spinal afferents are

found in all myenteric ganglia whereas DMPP-responsive sites and viscerofugal nerve cell bodies had a very sparse, punctate distribution.

There are other possible explanations for the small discrepancy between DMPP responses and labelling of viscerofugal nerve cell bodies. Viscerofugal axons typically have small amplitude action potentials; it is possible that some viscerofugal neuron action potentials were simply undetectable in the noise of recordings. Alternatively, some viscerofugal neurons may not respond to nicotinic agonists, because of low expression of nicotinic receptors (Ermilov et al., 2003). This seems unlikely since intracellular recording of retrogradely labelled viscerofugal nerve cell bodies showed that 19 of 19 neurons tested received fast nicotinic excitatory synaptic inputs (Sharkey et al., 1998).

Conclusion

The present study has demonstrated that recordings from colonic nerves contain action potentials from enteric viscerofugal neurons. A means to identify these neurons has been developed, based on localised DMPP responses and subsequent localisation by dye filling.