

Synaptic transmission from the submucosal plexus to the myenteric plexus in Guinea-pig ileum

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Abstract Stimulation of the myenteric plexus results in activation of submucosal neurons and dilation of arterioles, one way that motility and secretion can be coupled together. The present study aimed to examine the converse, whether myenteric neurons receive synaptic input from the submucosal plexus (SMP). Intracellular recordings were made from guinea-pig ileal myenteric neurons while the SMP was electrically stimulated. Of the 29 neurons studied (13 S and 16 AH neurons), stimulation of the SMP evoked a synaptic potential in only seven cells, or 24% of neurons. When the SMP was situated oral to the myenteric plexus, 4 of 13 (31%) myenteric neurons had synaptic input. When it was situated circumferential, 2 of 8 (25%) had input, and when the SMP was situated anal 1 of 8 (13%) had input. Overall, 5 of the 13 (38%) S neurons responded with fast excitatory post-synaptic potentials (EPSPs), one of which also showed a slow EPSP, while 2 of the 16 (13%) AH neurons responded with a slow EPSP. This study indicates that the synaptic input from the SMP to myenteric neurons is relatively sparse. Whether this input is less important than the myenteric to submucosal input or simply represents a more selective form of control is unknown.

Keywords electrophysiology, myenteric plexus, submucosal plexus, synaptic transmission, ileum.

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INTRODUCTION

A clear correlation between motility and secretion has been shown in *in vivo* studies in humans,¹ rats,² ferrets³ and guinea pigs,⁴ with a similar correlation of motility and blood flow seen in dogs.⁵ It is generally thought that the myenteric plexus controls motility, whereas the submucosal plexus (SMP) controls secretion and vasodilation.⁶ It is possible that the correlation seen between motility and secretion/blood flow is due to communication between these two plexes.

Anatomical connections from the submucosal to myenteric plexus were described by Kuntz⁷ who showed that axons from the SMP formed synapses with neurons in the myenteric plexus. Since then it has been shown that intrinsic sensory AH/Dogiel type II (AH/DII) neurons containing acetylcholine (ACh) and substance P project from the SMP to the myenteric plexus^{8,9}, as do a class of vasoactive intestinal polypeptide (VIP) containing neurons that do not have branches to the mucosa,⁹ indicating they may be interplexus interneurons. From the myenteric plexus, three types of descending interneurons containing nitric oxide synthase/VIP,^{10,11} somatostatin,¹² and 5-hydroxytryptamine or serotonin (5-HT)¹³ project to the SMP, the latter of which are thought to be the source of serotonergic fast EPSPs in submucosal neurons.¹⁴ In addition, sensory AH/DII neurons containing ACh, substance P¹⁵ and calbindin¹⁶ have been shown to project to the SMP without first traversing multiple myenteric ganglia, indicating they may solely act to communicate with the SMP.

Electrophysiological studies have shown that when the myenteric plexus is removed and the nerve terminals allowed to degenerate, the occurrence of fast EPSPs in all submucosal S neurons is markedly reduced, and the occurrence of slow EPSPs is reduced in VIP neurons.¹⁷ Other experiments utilizing a preparation containing exposed myenteric ganglia in one half and SMP in the other half found that when orally situated myenteric ganglia were stimulated most

submucosal S neurons received synaptic input,¹⁸ and in addition, vasodilation of the blood vessels in the submucosa occurs.¹⁹ However, similar studies have not been done in the reverse. What is known is that mechanical distortion of the mucosa activates myenteric neurons via the SMP.^{8,20}

This study aimed to examine connections running from the SMP to the myenteric plexus and to investigate their nature. A preparation modified from Moore *et al.*¹⁸ was used where the SMP was electrically stimulated and intracellular recordings were made from myenteric neurons. We show that the synaptic input to myenteric neurons from the SMP is relatively sparse.

METHODS

Tissue preparation

Guinea pigs of either sex weighing 180–350 g were stunned and killed by severing the carotid arteries and spinal cord in accordance with guidelines of the University of Melbourne Animal Experimentation Ethics Committee. A 3-cm segment of ileum was removed 10–20 cm from the ileocecal junction and placed into physiological salt solution (in mmol L⁻¹: NaCl 118, NaHCO₃ 25, D-Glucose 11, KCl 4.8, CaCl₂ 2.5, MgSO₄ 1.2, NaH₂PO₄ 1.0) containing nicardipine (L-type Ca²⁺ channel blocker, 1.25 μmol L⁻¹) and AF-DX 116 (M₂ antagonist, 300 nmol L⁻¹) to minimize muscle movement, and bubbled with 95% O₂ and 5% CO₂. The ileum was opened along the mesentery and tightly pinned flat in a Sylgard (Compound 184, Dow Corning Corp, Midland, MI, USA) lined Petri dish with the mucosa uppermost that was stripped off with fine forceps. On one half of the preparation only the mucosa was removed leaving the SMP, circular muscle, myenteric plexus and longitudinal muscle (Fig. 1). On the other half of the preparation, the SMP and circular muscle were also removed to leave the myenteric plexus and longitudinal muscle. Care was taken to avoid damaging the connections between the submucosal and myenteric plexus. However, it is likely that the submucosal collaterals originating from the myenteric plexus-to-mucosa projections were damaged during removal of the mucosa. Fig. 1 shows the preparation schematically with the myenteric plexus positioned circumferentially to where the SMP remains. This preparation style was implemented in three configurations: myenteric plexus circumferential to the SMP, myenteric plexus anal to the SMP and myenteric plexus oral to the SMP. The preparation, with approximate dimensions of 1.5 cm by 0.8 cm was then removed from the Petri dish and

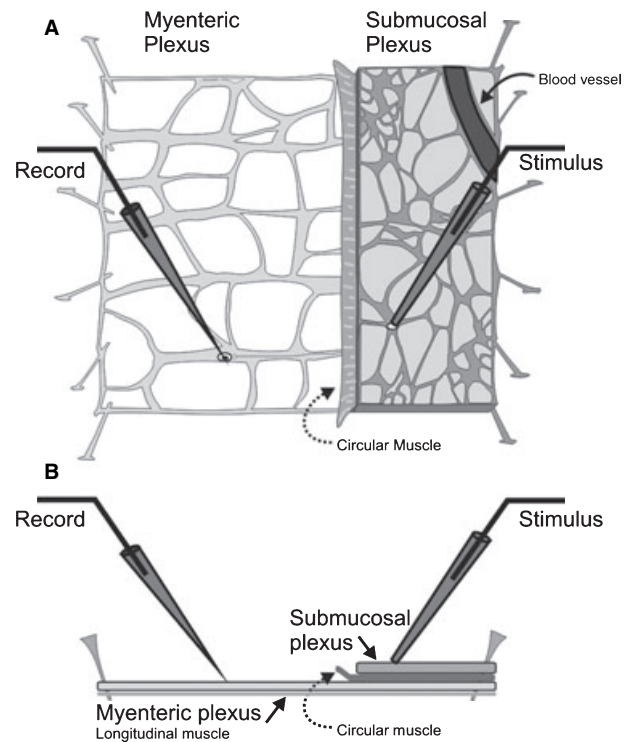


Figure 1 Combined submucosal and myenteric plexus preparation. The mucosa was removed over the whole preparation and then the myenteric plexus was exposed over one half. A preparation with the submucosal plexus located circumferentially with respect to the myenteric plexus is shown. Preparations were also made with the submucosal plexus located oral or anal with respect to the myenteric plexus. (A) Myenteric neurons were impaled and a 7-μm carbon fibre concentric bipolar stimulating electrode placed over the submucosal plexus (with underlying myenteric plexus). A submucosal blood vessel and the edges of the circular muscle are also illustrated. This preparation ensured neuronal connections between submucosal and myenteric neurons were intact for half of the preparation. (B) Side view of this same preparation highlighting the removal of the submucosal plexus and circular muscle from the left side to allow intracellular recordings to be made from myenteric ganglia. The right side shows that the submucosal and myenteric connections are left intact to allow inputs from the submucosal to myenteric plexus to be assessed.

pinned out in an organ bath (volume 1.3 mL) SMP side up. Physiological salt solution was pre-warmed to 36 °C and superfused at a rate of 2–4 mL min⁻¹. The preparation was allowed to equilibrate for 1 h before intracellular recordings began.

Electrophysiology

Myenteric ganglia were visualized at 300× using an inverted microscope (IX-70, Olympus, Tokyo, Japan)

with Hoffman modulation optics. Neurons were impaled with glass microelectrodes containing 1 mol L⁻¹ KCl and 2% biocytin (w/w; tip resistance 100–200 MΩ) and characterized electrophysiologically as either S or AH type²¹ based on the presence of a fast EPSP or a prolonged afterhyperpolarizing potential, respectively. Voltage recordings were made using an Axoclamp 2A amplifier (bridge mode), digitized at 5–20 kHz (Digidata 1200B) and recorded on a personal computer using Axoscope 9 (all from Axon Instruments, Foster City, CA, USA). Once each experiment was complete, the position of the neuron within the preparation was noted for later identification following immunohistochemical processing to identify the biocytin (described in detail in ref. 14).

Stimulation of submucosal ganglia

Synaptic potentials were evoked by focal electrical stimulation of submucosal ganglia (up to 1.3 mm away from the impaled neuron). A 7 μm glass coated carbon fibre stimulating electrode was used as it was found to be superior to a 25-μm-insulated tungsten monopolar electrode. Table 1 shows the comparison of stimulus threshold for fast EPSP generation and the current strength at which a maximal fast EPSP was evoked. In addition, the spread of current from the two types of electrodes was tested using the same stimulus strength and moving the electrodes horizontally off the fibre tract until a fast EPSP could no longer be evoked. Carbon fibre electrodes were prepared as in Bertrand *et al.*²² with a 50 μm silver wire (Goodfellow Cambridge Ltd, Huntingdon, UK) wrapped around the outside of the micropipette containing the carbon fibre to act as the ground. A single pulse and/or a train of 10 pulses at 20 or 100 Hz, typically 0.075 mA (0.07–0.09 mA) and 0.5 ms duration was used to stimulate the SMP (Master-8 stimulator, ISO-Flex stimulus

isolation unit, AMPI, Israel). Fast EPSPs were recorded at membrane potentials of -70 to -90 mV and slow EPSPs were recorded at, or close to, resting membrane potential.

To avoid direct electrical stimulation of the myenteric plexus through the preparation, initial control experiments were carried out. When the stimulating electrode was placed on a bare myenteric nerve tract in the myenteric area of the preparation, a stimulus strength of 0.1 mA gave a maximal amplitude fast EPSP, but when the stimulating electrode was placed on the submucosa over the same nerve trunk (determined by focusing through the preparation) no fast EPSP was recorded at this stimulus strength (Table 1, Fig. 2). In addition, when the stimulating electrode was directly over a submucosal ganglion fast EPSPs were recorded in myenteric neurons, but when the stimulating electrode was moved either horizontally or vertically away responses were lost.

When submucosal ganglia were not visible for targeted stimulation (due to the thickness of the preparation) the submucosal area was systematically stimulated at 140 μm intervals in a grid pattern, at 150× magnification, with the aid of 10 × 10 grid eyepiece graticule. Most preparations were stimulated in this manner with an average stimulation area of 1120 μm × 560 μm (as measured with the graticule).

Drugs

The M₂ receptor antagonist AF-DX 116 (Tocris, Australian Laboratory Services, Sydney, Australia) was prepared as a stock solution in dimethyl sulfoxide (DMSO) and kept at room temperature. It was diluted to the final concentration in physiological salt solution before addition to the organ bath by superfusion. The final DMSO concentration in solution was 0.0025%.

Table 1 Properties of the stimulating electrodes and comparison of nerve fibre stimulation

	Stimulus strength for fast EPSP threshold (mA)	Stimulus strength for maximal fast EPSP (mA)	Current/stimulus spread (μm)
CF	0.036 ± 0.009 (<i>n</i> = 9)	0.112 ± 0.025 (<i>n</i> = 9)	160 ± 30 (<i>n</i> = 4)
W	0.300 ± 0.040 (<i>n</i> = 5)	0.520 ± 0.055 (<i>n</i> = 5)	175 ± 50 (<i>n</i> = 2)
MP	0.050 ± 0.035	0.15 ± 0.07	175
SMP	0.15 ± 0.01	0.375 ± 0.11	280

Rows 1 and 2: Fast EPSPs were evoked by stimulating a myenteric fibre tract with a carbon fibre stimulating electrode (CF) or a tungsten wire electrode (W). Rows 3 and 4: The stimulus strength required to evoke a fast EPSP when stimulating through the submucosal plexus and circular muscle to the myenteric plexus (SMP) was 300% of that required for direct myenteric fibre tract stimulation (MP, paired data, *n* = 2).

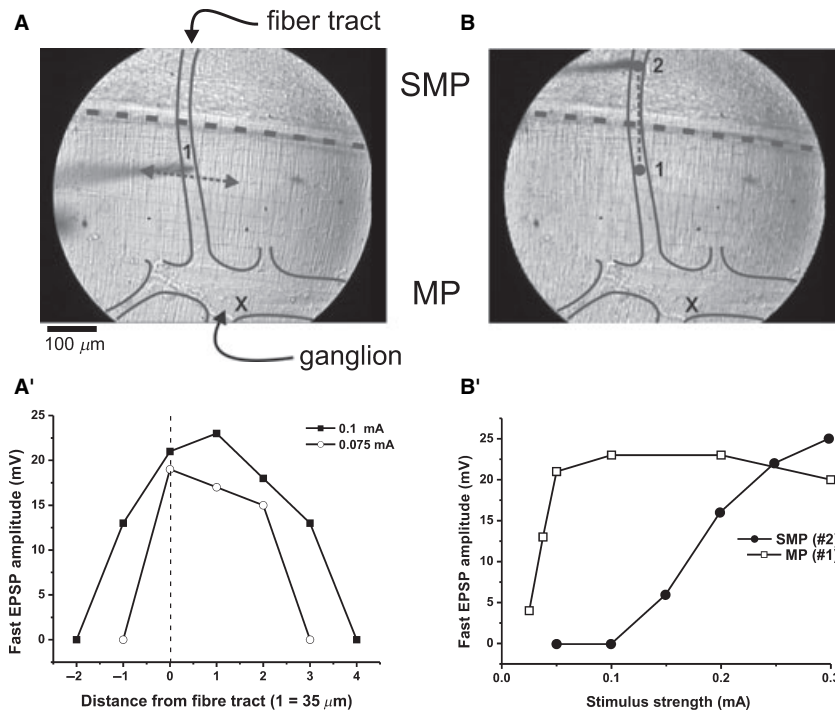


Figure 2 Effective stimulus intensities using a 7- μm carbon fibre electrode to electrically stimulate the submucosal and myenteric plexes. [A,B] Photomicrographs of a single preparation with submucosal plexus (SMP) at the top (oral) and myenteric plexus (MP) at the bottom (anal). A myenteric ganglion is outlined in the lower half and the location of the impaled neuron is marked with an 'X'. The stimulating electrode is on the left. (A) The stimulating electrode was placed on the myenteric fibre tract (marked position '1') and fast EPSPs were evoked in 5 neurons. The stimulating electrode was then moved horizontally off the fibre tract (arrow) to determine current spread. (B) The stimulating electrode was moved from the fibre tract in the MP (position 1) to the area of intact SMP with the myenteric fibre tract running underneath (position #2) and fast EPSPs were evoked to test the strength required to stimulate the MP through the SMP. (A',B') Plots showing the resulting fast EPSP amplitudes from altering stimulus strength and electrode position. (A') Fast EPSP amplitude is on the Y-axis and stimulation position relative to the fibre tract is on the X-axis. For a fixed stimulus strength (0.075 mA, open circle, or 0.1 mA, filled square) with the electrode approaching from the left, the largest amplitude fast EPSP could be evoked on the fibre tract or to the right (+1). For a stimulus strength of 0.075 mA, this equates to an effective stimulus area of approximately 70 μm diameter ($n = 4$). (B') Fast EPSP amplitude is on the Y-axis and stimulus strength is on the X-axis. For a fixed position, either on the fibre track in the MP (open squares) or over the fibre track as it runs under the SMP (closed circles), the stimulus strength was increased and the fast EPSP amplitude recorded. Maximal fast EPSP amplitude is obtained with the electrode directly on the MP fibre tract with a strength that does not give a fast EPSP when it is over the SMP ($n = 2$).

Morphological analysis

Preparations were processed to reveal the presence of biocytin. Briefly, preparations were fixed in Zamboni's fixative (2% formaldehyde plus 0.2% picric acid in 0.1 mol L⁻¹ phosphate buffer, pH 7.0) at 4 °C for between 1 and 24 h, cleared of fixative and permeabilized using DMSO (3 \times 10 min washes), and then washed in phosphate-buffered saline (3 \times 10 min washes). The preparations were incubated in a humidifier at room temperature with streptavidin-Texas Red (1 : 200 or 1 : 400; Amersham Biosciences, GE Healthcare, Rydalmere, NSW, Australia) for 2–2.5 h to reveal the biocytin-containing neurons. After washing, the preparations were mounted in buffered glycerol (pH 8.7) for viewing under a fluorescence microscope (Axioskop, Carl

Zeiss, Oberkochen, Germany) using the appropriate filter cube for Texas Red fluorescence. Images were acquired at 12 bits using a CCD camera and saved as 16 bit greyscale TIFFs using Spot Advanced software (both from Diagnostic Instruments Incorporated, Sterling Heights, MI, USA).

Measurements

Data were analyzed using Axoscope 9. Fast and slow EPSP amplitude, time-to-peak, latency and duration (at 90% return to baseline for slow EPSPs) were measured. The average of 2–6 repetitions was obtained and used for comparisons between neurons; one 'n' refers to the average response from a single neuron. In this study, 52 animals were used. Unless otherwise stated, numbers given are mean \pm SEM.

RESULTS

A total of 70 neurons from 52 preparations were included in this study. Of these, 22 neurons were used for experiments that characterized the carbon fibre stimulating electrode (Methods), 19 neurons were used for experiments investigating synaptic transmission in the presence of a M_2 receptor antagonist to minimize muscle movement and 29 neurons were used in investigating synaptic connections from submucosal to myenteric neurons.

Synaptic potentials in the presence of a M_2 receptor antagonist

Most recent experiments investigating synaptic transmission in the myenteric plexus have been performed in the presence of the non-specific muscarinic antagonist hyoscine (scopolamine) to minimize muscle movement while recording from neurons. The nature of transmission from the SMP to the myenteric plexus is unknown, but muscarinic slow EPSPs could contribute given that submucosal AH neurons projecting to the myenteric plexus contain choline acetyltransferase. Muscarinic slow EPSPs are mediated by M_1 receptors.²³ It is known that M_2 receptors are located on the smooth muscle^{23–26} and so this study used an M_2 receptor antagonist, AF-DX 116 (300 nM), to reduce muscle movement. Before the experiments characterising the inputs from the SMP were carried out, it was thus decided to do a basic characterization of synaptic potentials in myenteric neurons in the presence of the M_2 receptor antagonist. Of the 19 myenteric neurons examined (eight S-type and 11 AH-type), myenteric fibre tract stimulation elicited a fast EPSP in seven neurons, a slow EPSP in 12, an IPSP in four, and five neurons had antidromic action potentials only. This distribution is similar to previously published studies. The range of synaptic potentials found in myenteric neurons could, thus, be evoked in the presence of the M_2 antagonist.

Synaptic potentials in myenteric neurons evoked by stimulation of the submucosal plexus

A total of 29 myenteric neurons, 13 S and 16 AH, from 21 preparations were successfully characterized and had any inputs from the SMP mapped. Of these, seven preparations had the SMP located circumferentially, nine had the SMP oral and five had the SMP located anally. The average resting membrane potential of S neurons was -53 ± 3 mV and for AH neurons -67 ± 2 mV. Interestingly, a further three S neurons

had oscillating membrane potentials (not included in the average above) that were regular in frequency and amplitude and ranged between 10 and 20 mV in amplitude, and 160 and 290 s between peaks (0.006 Hz and 0.003 Hz, respectively). All S neurons were identified by the presence of a fast EPSP in response to myenteric fibre tract stimulation. When submucosal ganglia were not visible to stimulate (as was usually the case due to the thickness of the preparation), the submucosal area was systematically mapped by stimulating at 140 μ m intervals in a grid pattern. Trains of stimuli were also used to maximize the chance of evoking transmitter release from the submucosal neurons. An average of 28 positions (range: 5–72, area: 140 μ m \times 700 μ m to 1120 μ m \times 1260 μ m) was tested in any one myenteric neuron for a response to stimulation of the SMP. Of the 13 S neurons, five responded with a synaptic potential, and of the 16 AH neurons, two responded with a synaptic potential (Fig. 4). Overall, most myenteric neurons did not respond to stimulation of the SMP. No types of synaptic potentials were recorded that have not previously been reported. Because of the low number of neurons with synaptic input, statistical analyses were not performed.

Submucosal stimulation oral to the myenteric plexus

Recordings were made from seven AH and six S neurons when the stimulus site in the SMP was situated oral to the myenteric plexus. Of the AH neurons, two responded with a slow EPSP. The first neuron responded to stimulation in an area of approximately 210 μ m \times 140 μ m, the centre of which was approximately 0.8 mm oral to the impaled neuron. Morphological analysis revealed that this neuron had a large cell body with a long anally projecting axon and extensive varicose fibres within its ganglion. The second neuron responded erratically from an area approximately 70 μ m \times 70 μ m, the centre located approximately 0.77 mm oral to the impaled neuron. The erratic nature of the response would indicate the neuronal pathway between the site of stimulation and the impaled neuron may have been polysynaptic. This neuron had a typical Dogiel type II morphology. The slow EPSPs had a control amplitude of 9 ± 1 mV (latency: 4.5 ± 0.5 s, time to peak: 13 ± 4 s, duration: 58 ± 18 s, $n = 2$). Of the five AH neurons that did not respond to submucosal stimulation, morphological analysis showed two were Dogiel type II and one was dendritic Dogiel type II. The other two neurons were not recovered.

Of the six S neurons studied, two responded with fast EPSPs. The first S cell responded from an area approximately $140\ \mu\text{m} \times 70\ \mu\text{m}$, the centre of which was located approximately 0.91 mm from the impaled neuron. The fast EPSP had an amplitude of 15 mV (latency: 6 ms, time to peak: 9 ms). Morphological analysis revealed the axon of the neuron had been severed during dissection, indicating it projected out of the myenteric plexus. The second S cell responded erratically from only one stimulus position located approximately 1.05 mm from the impaled neuron. The response to every stimulus was different with variable fast EPSP amplitudes and pattern of response. Once again this suggests a polysynaptic pathway between the stimulus and recording sites. A single stimulus did not always give a fast EPSP. When a train of stimuli (10 pulses, 20 Hz) were delivered the latency ranged between 30 ms and 180 ms with the number of fast EPSPs ranging from 1 to approximately 11. The fast EPSPs barely outlasted the stimulus train. This neuron had a mix of lamellar and filamentous dendrites. The four other neurons that did not respond to stimulation could not be recovered for morphological analysis.

To ensure the nine neurons (five AH and four S) that did not respond to stimulation actually had no response, a large stimulation area was tested. An average of 32 spots were tested, corresponding to an area approximately $980\ \mu\text{m} \times 840\ \mu\text{m}$.

Submucosal stimulation circumferential to the myenteric plexus

When the SMP was situated circumferential to the myenteric plexus three AH neurons and five S neurons were studied. None of the AH neurons responded to stimulation. Two of these AH neurons had Dogiel type II morphology, and one had a long anally projecting axon. Of the five S neurons, one responded with a fast EPSP, and one with both a fast and slow EPSP. The neuron that responded with only a fast EPSP was located 1.05 mm circumferential to the centre of the effective stimulus area which was $140\ \mu\text{m} \times 70\ \mu\text{m}$. The fast EPSP had an amplitude of 11 mV (latency: 11 ms, time to peak: 9 ms). Morphological analysis of this neuron showed filamentous dendrites. The second neuron that responded to circumferential stimulation had both a fast and slow EPSP that could be evoked from one position located 1.33 mm circumferential to the impaled neuron. The slow EPSP had an amplitude of 4 mV (latency: 400 ms, time to peak: 1.8 s, duration: 28 s). Morphological analysis showed this neuron was an anally projecting motor neuron. Of the three S neurons that did not respond, morphology showed one

was a circular muscle motor neuron, whereas the other two were not recovered.

The six neurons (three AH and three S) that did not respond to stimulation had an average of 30 spots tested, an approximate area of $980\ \mu\text{m} \times 700\ \mu\text{m}$.

Submucosal stimulation anal to the myenteric plexus

When the SMP was situated anal to the myenteric plexus six AH neurons and two S neurons were recorded from. None of the AH neurons responded to stimulation or were recovered for morphological analysis. Of the two S neurons, one responded with fast EPSPs (Fig. 3) that could be evoked from a region approximately $140\ \mu\text{m} \times 280\ \mu\text{m}$, the centre of which was located approximately 0.42 mm anal to the impaled neuron. Each location evoking a response was tested with single trials of three different stimuli (single pulse, train of 10 pulses at 20 Hz and train of 10 pulses at 100 Hz) before the impalement was lost. When fast EPSPs evoked from two positions were compared their latencies were found to be 8 ms and 13 ms. The average number of fast EPSPs from the two positions in response to a stimulus train were 28 (10 pulses, 20 Hz; duration of stimulus 450 ms, duration of response 510 ms) and 18 (10 pulses, 100 Hz; duration of stimulus 100 ms, duration of response 275 ms). Morphological analysis revealed this neuron was an anally projecting motor neuron. The other S neuron did not respond to stimulation and had Dogiel I morphology with an orally projecting axon.

Of the seven neurons (six AH and one S) that did not respond to stimulation, an average number of 37 spots were tested corresponding to an approximate area of $980\ \mu\text{m} \times 840\ \mu\text{m}$.

DISCUSSION

This study has shown that input from the SMP to the myenteric plexus appears to be sparse. Neurons of the SMP receive many inputs from the myenteric plexus,^{17,18} but the present study indicates that the reverse may not be true. Of the 29 neurons studied, 13 S and 16 AH, stimulation of the SMP gave a response on only seven occasions, or 24% of neurons. When the SMP was situated orally, 4 of 13 (31%) neurons had synaptic input; when circumferential, 2 of 8 (25%) had input; and when anal, 1 of 8 (13%) had input. In terms of neuronal type, 5 of 13 (38%) S neurons and 2 of 16 (13%) AH neurons responded.

We are confident that the small number of responses to submucosal stimulation is representative. For

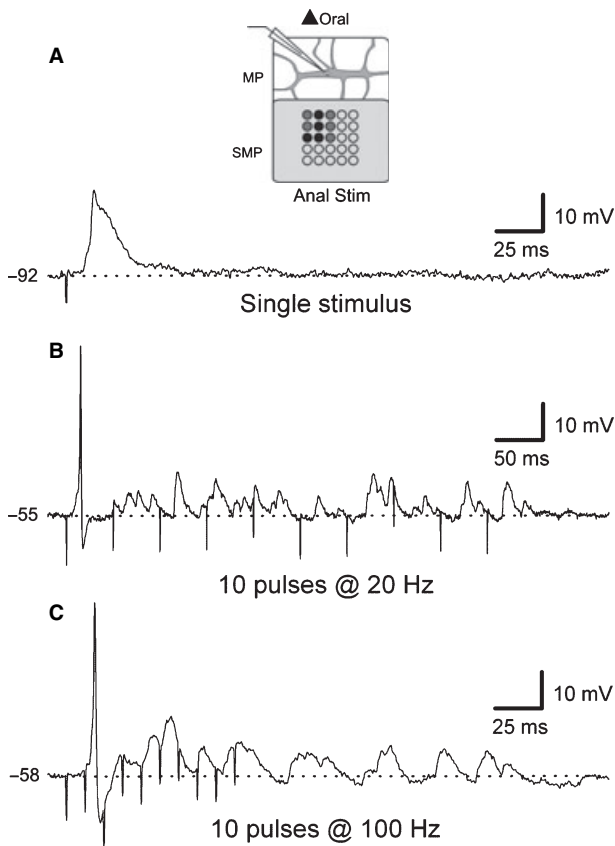


Figure 3 Burst of poorly entrained fast EPSPs in an S myenteric neuron evoked by electrical stimulation of the submucosal plexus. (A–C) Voltage traces from a single S neuron from the myenteric plexus (the dotted line is the resting/holding membrane potential). (A) A fast EPSP was evoked by a single pulse applied (at the downward deflection) to intact SMP (anally situated – see inset). (B–C) Trains of 10 stimuli applied at either 20 or 100 Hz (at the downward deflections) evoked a burst of fast EPSPs that did not follow the stimulus and outlasted it. Inset: The relative positions of the impaled myenteric neuron (top) and the intact SMP (bottom). The black dots were positions that yielded a fast EPSP while the dark grey dots were positions that did not; the light grey dots were untested (each dot is approximately 140 μm diameter). Note: the myenteric ganglion has been drawn for illustration purposes only.

example, the accidental stimulation of myenteric ganglia was unlikely to have contributed. To support this, for every S neuron recorded from, a myenteric fibre tract was first stimulated. This ensured both that the impaled neuron had synaptic input, and that the stimulus strength used was just adequate to evoke a synaptic potential (generally 2× threshold, <0.1 mA). This in turn acted to minimize current spread by the stimulus. Stimulating electrode controls showed that the stimulus strength used was not strong enough to penetrate through the circular muscle to directly stimulate the

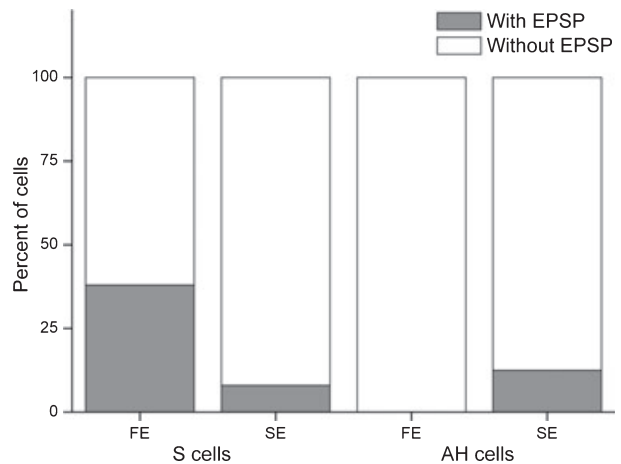


Figure 4 Histogram showing the proportions of fast and slow synaptic transmission in S and AH myenteric neurons when stimulated from the submucosal plexus. Summary data from 13 S neurons and 16 AH neurons from the myenteric plexus (MP). Single pulses and trains of electrical stimuli were applied to the submucosal plexus (SMP) approximately 1 mm from the impaled neurons which were situated anally, orally or circumferentially to the intact submucosa. Each neuron was stimulated from five or more positions with a strength shown to selectively stimulate the SMP and not the underlying MP (Fig. 2). The open bar indicates the percentage of neurons that did not respond, while the filled bar represents the percentage that did respond. All S neurons were shown to have a fast EPSP when the myenteric plexus was stimulated. FE, fast excitatory post synaptic potential; SE, slow excitatory post synaptic potential.

myenteric plexus. In fact, the finding that not every cell responded to submucosal stimulation indicates that the myenteric plexus was unlikely to have been directly stimulated. In addition, systematically testing the stimulus across the surface of the submucosa would be expected to stimulate submucosal fibre tracts and ganglia. This could enhance the probability of evoking a response in the impaled myenteric neuron.

The sparseness of the input to myenteric neurons was surprising given how heavily the SMP is innervated by the myenteric plexus.¹⁷ One idea to explain this finding is that the pathways from the SMP to the myenteric plexus run predominately vertically between the muscle and neuronal layers and not horizontally along the layers. Thus, recording 1 mm away horizontally from the stimulus means that there are no strong direct monosynaptic connections between the stimulated neuron and recording neuron. Song *et al.*⁹ showed that when submucosal neurons were retrogradely labelled from the myenteric plexus using DiI, approximately 66% of cell bodies were found within 1 mm oral or anal of the DiI application site, and 22% of cell bodies were found within 1 mm circumferential. In the present

study, the median distance between stimulation and recording sites (binned from all directions and measured using a calibrated eyepiece graticule) was between 0.42 mm and 1.33 mm. This indicates that our recordings were not likely to be out of range of a direct connection. Enteric neurons form long chains with each other and it is thus possible that in the present study, multiple synapses are involved between the stimulated SMP and the myenteric neurons. In a monosynaptic pathway, a train of stimuli should result in one fast EPSP for one stimulus pulse with later fast EPSPs being reduced in amplitude (i.e. rundown²⁷). In a polysynaptic pathway, the temporal information is lost and there is no longer one fast EPSP for every stimulus pulse. Further, it becomes difficult to determine the properties of the inputs, such as the extent of rundown. The poor following of fast EPSPs seen in the neurons in this study (e.g. Fig. 3) suggests a polysynaptic pathway. Morphological analysis showed that two of the myenteric neurons receiving input were muscle motor neurons. It is presumed that they received input from other myenteric neurons first, and not directly from submucosal neurons.

It is clear that some myenteric S neurons are innervated by submucosal neurons; however, it is unclear whether myenteric AH neurons are, as only two of 16 showed synaptic input. AH neurons have extensive projections circumferentially and a projection to the mucosa which sends out a collateral in the SMP.¹⁶ It has been shown that the processes of myenteric AH neurons all conduct action potentials back to the cell body.²⁸ Thus, electrical stimulation of the SMP could have activated these collaterals, initiating an antidromic action potential in the myenteric AH neuron and resulting in an EPSP in another myenteric neuron without any submucosal neuronal input. Our data argue against this, however, as no antidromic action potentials were seen in the myenteric AH neurons. It could be that the submucosal collaterals of these mucosal projections were damaged by removal of the mucosa during the dissection of the preparation. Given that myenteric AH neurons were not antidromically activated by stimulation of the SMP, it is likely that the few slow EPSPs recorded were evoked from a submucosal neuron. It would appear that myenteric AH neurons are innervated by submucosal neurons, but that this innervation is less even than that of the myenteric S neurons.

Immunohistochemical studies can give us an insight into the neurotransmitters responsible for the synaptic potentials recorded. Multiple myenteric neuron types project to the SMP but only a minority of submucosal neurons project to the myenteric plexus – ACh/substance P containing neurons (sensory neurons)

which comprise 11% of submucosal neurons, and a small percentage of VIP containing (in general thought of as vasodilator/secretomotor) neurons.⁹ It would be expected that transmission from the SMP would be via one of these three transmitters (ACh, substance P and VIP). Myenteric neurons are also known to have EPSPs/depolarizations evoked by these transmitters.^{29–31} In addition to these three substances, it is possible that ATP could also be released from the submucosal VIP neurons as purinergic EPSPs have been found in the SMP.^{14,32,33}

If input from the SMP to the myenteric plexus is sparse, then what would the physiological relevance of such input be? The simple conclusion is that this input is less important for the coordination of reflexes than the input from the myenteric plexus to the SMP. Alternatively, we could speculate that submucosal input represents a more selective form of control that might act on a single reflex circuit under relatively specific circumstances. For example, deformation of the mucosa enhances myenteric reflex responses to distension.³⁴ If submucosal sensory neurons are mucosal mechanoreceptors as has been suggested,^{8,35} then the direct input from these neurons into the myenteric motility reflex pathways might represent the kind of highly specific input sparse connections could be involved in. At the other extreme, relatively few inputs from the SMP might accomplish broad but non-specific modulation of the myenteric plexus, similar to the actions of the central nervous system (for review, see ref. 36).

In summary, myenteric neurons appear to receive only sparse/infrequent input from the SMP. The design of any future studies to investigate the pharmacology of this pathway will need to take this into account. The correlation seen between the motility and secretion/blood flow may be due to communication between these two plexes; however, the specific role of submucosal input to the myenteric plexus remains to be determined.

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