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Electrical mapping of the projections of intrinsic primary afferent neurones to the mucosa of the guinea-pig small intestine

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Abstract *The patterns of innervation of the mucosa by axons of individual primary afferent neurones with cell bodies in the myenteric plexus were studied by mapping sites from which electrical stimulation of the mucosa elicited action potentials (APs) in their cell bodies. Segments of guinea-pig ileum were dissected to reveal the myenteric plexus over half of the intestinal circumference, leaving the mucosa intact over the other half. Intracellular recordings were taken from myenteric neurones located within 1 mm of the intact mucosa. Focal electrical stimuli were applied to the mucosa at multiple locations separated by about 1 mm. Neurones that responded had round or oval cell bodies with several long processes (Dogiel type II) and APs that had an inflection on the falling phase (AH-neurones). Responses consisted of single APs or bursts of APs. Maps of the mucosal projections of 30 neurones were generated. The maximum distances from which individual neurones responded were 7 mm circumferential and 2 mm oral or anal to the cell body with a higher proportion of responses from the oral regions. The areas of intact mucosa calculated to be innervated ranged from 1 mm^2 up to ≈15 mm^2 (mean 3.9 mm^2; median 2.5 mm^2). It is estimated that the areas innervated would be two to three times larger under conditions where part of the mucosa is not removed. Some neurones also responded to a chemical or a mechanical stimulus applied to the mucosa within the electrically mapped area. It is concluded that intrinsic primary afferent neurones have overlapping receptive fields with 230–350 neurones innervating the same region of mucosa.*

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INTRODUCTION

Propulsion of food along the intestine requires the coordinated actions of large numbers of neurones within the enteric nervous system.[1,2] References have been recorded These include motor neurones that either excite or inhibit the muscle layers, several populations of interneurones and the recently identified intrinsic primary afferent neurones. The identities of these different populations of neurones in the enteric nervous system of the guinea-pig small intestine have been established in a series of correlated electrophysiological, neuroanatomical and immunohistochemical experiments.[2–5]

The primary afferent neurones that innervate the gut can be placed into two categories: extrinsic neurones with cell bodies in either the sensory ganglia of the vagus or the dorsal root ganglia; and the recently described intrinsic neurones with cell bodies in the myenteric or submucosal plexuses.[6] These latter two populations of intrinsic neurones have large smooth cell bodies and several long, axonal processes (Dogiel type II morphology), and, in quiescent tissue, they fire action potentials (APs) that are followed by prolonged after-hyperpolarizing potentials.[7–10] Hence, they can be termed AH/Dogiel type II neurones.

Dogiel[11] suggested that the type II neurones were sensory because he could trace at least some of their processes to the mucosa, while other processes went to neighbouring ganglia. Recent studies of the AH/Dogiel type II neurones in the myenteric plexus have confirmed this pattern of projections and have shown that all such neurones appear to have at least one axon that projects to the mucosa as well as extensive ramifications within myenteric ganglia and within some submucosal

ganglia.[12–15] The mucosal projections are sensitive to chemical stimulants such as acid, acetate or 5-hydroxytryptamine (5-HT) to which they respond with a burst of APs that then travels back to the cell body.[7,9] It has been estimated on the basis of retrograde tracing studies that each of these neurones sends processes to at least 10 villi, but the areas of mucosa supplied by individual neurones have not been determined.[15]

The aim of this study was to analyse the patterns of projections of AH/Dogiel type II neurones to the mucosa using electrical stimulation to identify all axonal processes arising from a particular neurone and, when possible, to compare this pattern to the receptive fields for chemical or mechanical stimuli applied to the same region.

MATERIALS AND METHODS

Preparation

Guinea-pigs of either sex (160–280 g, Hartley strain, from the University of Melbourne) were stunned by a blow to the head and killed by severing the carotid arteries and spinal cord. A 2–3-cm segment of ileum, 10–30 cm from the ileocecal junction, was placed in oxygenated (95% O_2/5% CO_2) physiological saline of the following composition (mM): NaCl, 117; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2.5; KCl, 4.7; $NaHCO_3$, 25; and glucose, 11. The physiological saline contained nicardipine (3 μM) and scopolamine (1 μM) to minimize muscle movements. The segment was cut open along the line of mesenteric attachment and the mucosa, submucosa and circular muscle were removed over one half of the circumference (Fig. 1), leaving the myenteric plexus exposed; in every case, the same side of the preparation was dissected. The preparation was placed in the base of a recording chamber (volume 2 mL) that was lined with a silastic elastomer, pinned flat, and superfused with physiological saline (35–36 °C) at a flow rate of 4–6 mL min^{-1}.

Electrophysiology of enteric neurones

Voltage recordings were made using 1 M KCl/2% Neurobiotin-filled micropipettes and an Axoclamp 2 A amplifier (Axon Instruments, Foster City, CA, USA) in bridge mode. Signals were digitized at 1–20 kHz, recorded on a personal computer (using Axotape 2.02 from Axon Instruments), and then analysed with Origin 4.1 (MicroCal, Northampton, MA, USA). AH-neurones were identified by their broad APs which had inflections on their falling phases and were generally followed by prolonged after-hyperpolarizing potentials.[16,17] S-neurones were those neurones whose APs lacked these two features and responded with prominent fast excitatory synaptic potentials (EPSPs) upon internodal fibre tract or mucosal stimulation. S-neurones have not been included in the current study.

Electrical stimulation of the mucosa

Bipolar stimulating electrodes were made from two segments of 114-μm-diameter stainless steel wire insulated with a 15-μm-thick layer of Teflon (MedWire, Mt. Vernon, VT, USA). The two segments were twisted together and coated with an epoxy resin to enhance their stability. The tips of the wires were cut flush with the insulation, yielding a flat tip profile consisting of two circular faces of metal separated by about 30 μm of

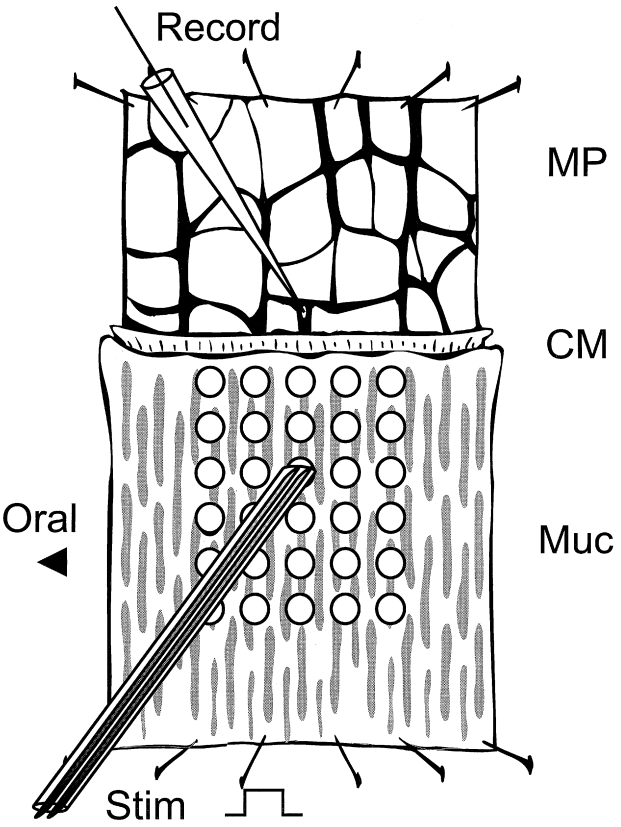

Figure 1 Schematic illustration of the experimental arrangement. A top view of the preparation with mucosa uppermost. The mucosa (Muc), submucosa and circular muscle (CM) have been removed over half the circumference, leaving the myenteric plexus (MP) exposed. The recording electrode (Record) was within 1 mm of the intact mucosa. The stimulating electrode (Stim) was positioned within a search grid (pictured here as an array of open circles). The myenteric plexus and the villi are not drawn to scale; there are ≈2 circumferential rows of myenteric ganglia and 6 circumferential rows of villi per 1 mm of length along the guinea-pig small intestine.

Teflon insulation. These electrodes were used to stimulate several rows of villi at a time. Stimulus pulses from 1 to 15 V (typically 5 V) and a 0.5-msec duration were used (S-48 stimulator, SIU5 stimulus isolation unit, both from Grass, Quincy, MA, USA).

In each preparation, the ability of the electrode to stimulate the nerve fibres effectively was tested by placing the electrode on an interganglionic fibre tract in the myenteric plexus and determining the threshold voltage needed to evoke a nonsynaptic AP. With the electrode on the mucosa, the threshold voltages needed to evoke a nonsynaptic AP were generally a factor of 2 greater than required to stimulate fibres in interganglionic tracts.

The repeatability of the test stimuli was determined by placing the electrode on the mucosa and determining the threshold voltage as above. During 100 test pulses given at 20-sec intervals, the threshold increased by about 10%. In mapping experiments, the threshold was determined at a single position on the mucosa and then the stimulus strength was increased to 150% of this value to reduce errors associated with minor changes in threshold.

In order to determine whether fibres running exclusively within the submucosal plexus were stimulated by electrodes placed on the mucosa, some tissues were prepared in which the mucosa was removed to reveal the submucosa. In these preparations, the threshold voltages needed to evoke nonsynaptic APs in AH-neurones in the myenteric plexus from the exposed submucosa were 10–20-fold greater than those required for stimulation of internodal fibre tracts in the myenteric plexus of the same preparations. Thus it is unlikely that the stimuli applied to the mucosa would have directly excited axons in the underlying submucosal plexus.

The diameter of the region excited by each electrode was determined by stimulation of interganglionic fibre tracts within the myenteric plexus. Nonsynaptic APs were evoked in AH-neurones from a fibre tract as above, and the threshold stimulus strength was determined with the electrode centred just above the fibre tract. The stimulus strength was increased to ≈200% of this value and the electrode was progressively moved from one side of fibre tract to the other and directly up, away from the fibre tract. The stimulus was observed to fail once the whole of the electrode was moved beyond the region of the fibre tract or when the electrode had been vertically lifted by approximately the same distance. This indicates that the radius for an effective stimulus at these voltages was ≈0.125 mm. Thus the effective stimulus radius at comparable voltages within the mucosa should be similar.

Mapping of projections in the mucosa

To map the projections of a neurone within the mucosa, the stimulating electrode was placed just above, or touching, the mucosa in the middle of a search grid (Fig. 1). After determining whether a nonsynaptic AP could be evoked from this location, the electrode was moved by ≈0.5 or 1 mm to another location on the grid. Thus, each location tested was separated by 2–4 times the effective stimulus diameter from the previous test site. The tip of the electrode was positioned with the aid of an eyepiece graticule and a 4× lens on the microscope. The data generated by this protocol were combined to produce plots on a square grid with locations that were separated by 1 mm. All maps and calculations based upon these data (e.g. calculations of total area innervated) are constrained to this level of precision; this might lead to overestimates of area in some cases. Thus, locations on the mucosa that were shown to respond from one or more locations within a 1-mm^2 area would be counted as a full 1 mm^2.

Not all locations were tested for each neurone, in particular, if the edge of a terminal field was determined by locations that failed to respond, then locations even further from the cell body were not tested systematically. An edge was identified by the presence of two or more adjacent locations that were next to responsive areas but from which responses could not be evoked; edges were identified in all maps, but not all responsive areas could be fully defined by the presence of edges.

Mechanical stimulation of the mucosa

The bipolar stimulating electrode described above was also used to deform small areas of mucosa mechanically. A micromanipulator was used to lower the electrode manually onto the mucosa over a time course of a few hundred milliseconds and was lifted 5–10 sec later. The area deformed was 0.2 mm^2 or greater, the same as the surface area of the face of the electrode. The force generated by the electrode as it was lowered onto the mucosa was estimated by using the micromanipulator to lower the electrode gently onto a microbalance. The force measured was between 2.5 and 7.5 mN. However, when the electrode was pushed down until it bent over upon itself (as a von Frey hair is calibrated) the force generated was greater than 60 mN. This type of force was never generated during an experiment. Because the preparation was pinned onto a silastic elastomer, it is possible that the tissue beneath the deformed mucosa was also distorted. However, the extent to which this occurred could not be quantified.

Solutions used for stimulation of the mucosa

The mucosa was also stimulated with chemicals made up in a buffered saline solution (NaCl, 117 mM; HEPES, 50 mM), and applied by pressure ejection (50–300 msec duration, 10 p.s.i., Picospritzer II, General Valve Corp, USA) from a micropipette (10–20 μm tip diameter) positioned over the intact mucosa.[7] Chemicals were applied at least twice at each site under direct visual control.

Drugs were purchased as follows: TTX from Alomone Labs, Jerusalem, Israel, and all other drugs from Sigma, St. Louis, MO, USA.

Morphological analysis

After electrophysiological testing, neurones were injected with Neurobiotin by passing depolarizing current pulses (0.5 nA, 500 msec pulse duration, 0.5 Hz, 5–10 min train duration) through the recording electrode. Preparations were fixed overnight in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0, cleared in three changes of dimethylsulphoxide, and placed in phosphate-buffered saline. The remaining mucosa and submucosa was removed by dissection and the tissue was reacted with streptavidin coupled to Texas Red to reveal Neurobiotin. Fluorescence images were visualized using a standard upright microscope (Axoplan from Zeiss) or an inverted microscope outfitted for confocal analysis (IMT-2, Olympus; MRC 1000, BioRad).

Statistics

Numbers are given as mean ± standard deviation (SD). A Wilcoxon signed rank test was used to test for significant biases in the patterns of responses; differences were taken to be significant when $P < 0.05$.

RESULTS

Electrical stimulation of the mucosa

The sites on the mucosa from which nonsynaptic APs could be elicited were mapped in a total of 30 AH-neurones. These 30 neurones included 23 neurones whose basic properties, but not the distribution of their mucosal processes, have been reported elsewhere.[7]

Responses evoked in AH-neurones by electrical stimulation of the mucosa appeared to have three distinct patterns, as described before.[7] About half of the neurones in the previous study responded with a nonsynaptic AP that had a short, fixed latency; this has been interpreted as a direct response of the terminal axon to the electrical pulse. Another 25% of the neurones responded with a burst of APs, the first of which had a much longer and variable latency. These responses may be caused indirectly by the electrically evoked release of a substance, or substances, from the mucosa which then acts upon the nerve terminal.[7] The remainder of the neurones exhibited a combination of these two patterns of response. All of the neurones in this study fit into these categories and, in addition, some were shown to exhibit different patterns of response when the test stimulus was applied at different sites upon the mucosa (Fig. 3). If electrical stimulation evoked APs at a particular site, then that site was counted as having an axonal projection from the impaled cell body and was included in the maps.

Examples of the maps generated by electrical stimulation of the mucosa are shown in Fig. 2. In eight maps, the area of mucosa from which APs could be elicited was fully defined by edges where APs could not be elicited from the surrounding regions (e.g. Fig. 2A,C). In the remaining 22 maps, the responsive area was well defined by the presence of edges, but the map contained one or more gaps along its perimeter through which innervation may have continued.

Twenty-five of 30 maps appeared to contain a single, continuous area from which responses could be evoked, while the remainder appeared to contain two responsive areas that were separated by a gap of 1 mm from which responses could not be elicited (e.g. Fig. 2D). Areas separated by gaps larger than 2 mm may have existed, but would not have been detected using the mapping protocol outlined above.

Twenty-five of 30 maps contained two, or more, adjacent 1-mm^2 areas from which responses could be evoked; the remainder of the maps had only a single responsive location. In 28 maps, sites from which responses could be evoked included locations directly circumferential to the impaled cell body (e.g. Fig. 2C). In 14 maps, responses could also be evoked from stimulus sites that lay up to 2 mm oral or anal to the direct circumferential line (e.g. Fig. 2D). The region immediately adjacent to the mucosal edge was only tested in four maps, two of which responded with an AP (e.g. Fig. 2B).

Chemical stimulation of the mucosa

Regions of mucosa from which applied chemicals could evoke APs were tested for 27 of the 30 electrically mapped AH-neurones, of which only 11 responded. For 19 of the 27 neurones, acid saline (pH 3 or pH 5) was used as a stimulant and, in 16 of these cases, neutral acetate was used as an additional stimulant

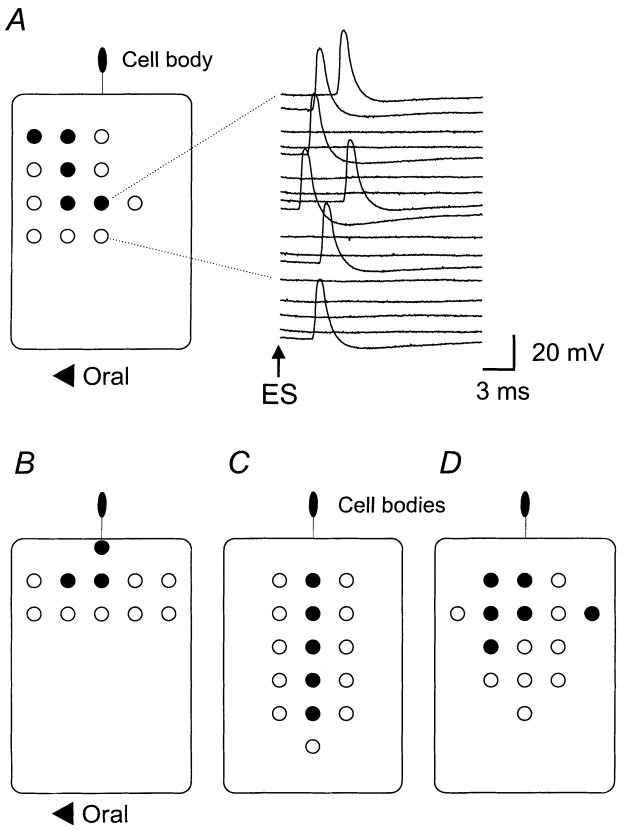

Figure 2 Representative maps of the projections of myenteric AH-neurones. A. On the left is a map from a single AH-neurone. The filled circles indicate locations from which APs were elicited in response to electrical stimulation of the mucosa; open circles indicate locations that were not responsive. The filled oval above the map indicates the approximate position of the cell body. On the right are representative voltage records that were used to generate the map showing either a single AP or not in response to electrical stimulation. A single electrical stimulus (ES) was applied at the time indicated by the arrow. Each trace is from a different location on the mucosa; the dotted lines indicate the locations for two of those traces. B–D. Three representative maps, using the same conventions as above, of the responsive sites of three AH-neurones.

(Table 1). One of the 27 neurones was tested with neutral acetate alone and another was tested with acid and 5-HT. The remaining seven neurones were tested with 5-HT as the sole stimulant. Where tested, responses to chemical stimuli were found within the electrically mapped area.

About 25% of neurones tested responded to one or more chemical stimuli applied to the electrically mapped terminal field (Table 1). Of six neurones that were tested with both acid and neutral acetate, four responded to acetate only, one responded to acid and one to both.

Table 1 Numbers of AH-neurones that responded to chemical or mechanical stimulation of the mucosa with a burst of APs

Stimulant	Responsive neurones	Total neurones tested
Acid	4	19
Base	0	1
Acetate	5	17
5-HT	3	8
Chemical	11	27
Mechanical	7	30
All	15	30

$n = 30$ AH-neurones. Chemical stimulants were made up in buffered saline at the following concentrations: acid (pH 3–5), base (pH 9–11), acetate (100 mM, pH 7), 5-HT (3–10 μM, pH 7). *Chemical* is a sum of all chemical stimulants, while *All* is the sum for both chemical and mechanical stimuli. Mechanical stimuli were applied by gently lowering a stimulating electrode onto the mucosa as described in the *Methods*.

Mechanical stimulation of the mucosa

Mechanical stimulation of the mucosa in at least one location was examined for all 30 neurones whose projections were electrically mapped. The electrode was gently lowered down upon the mucosa in a region in which electrical stimulation had been successful. In seven of 30 neurones, such stimuli elicited a burst of APs which occurred at the beginning of the stimulus and in one neurone, the burst continued for several seconds at a low (< 1 Hz) frequency. These responses were often not seen when the mechanical stimulus was repeated at the same site. Four of these seven neurones also responded to electrical stimulation of the same location with a burst of APs; the remainder responded with a single, directly activated AP.

Chemical and mechanical stimuli outside the electrically mapped regions, when attempted, were not successful in evoking responses in the impaled neurones.

Comparison of mechanical and chemical stimulation

All seven neurones which responded to mechanical stimulation were also tested with a chemical stimulus applied to the same region; only three responded. One responded to both mechanical stimulation and neutral acetate, but was unresponsive to acid. For the other two responsive neurones, only a single chemical stimulant was tested; one responded to acid, and the other responded to 5-HT.

Comparison of electrical, mechanical and chemical maps

In five neurones, the areas responsive to chemical (three neurones) or mechanical (two neurones) stimuli were directly compared with the electrically defined maps (e.g. Fig. 3). The three neurones that responded to chemical stimuli did so from areas which were 25%, 33% and 71% (mean of 43%) of the electrically defined map. The two neurones responsive to mechanical stimulation did so from areas which were 17% and 33% of the areas of the electrically defined maps.

Extent of innervation

The maps generated by electrical stimulation of the mucosa from individual neurones were pooled to form a composite map (Fig. 4). This composite illustrates the maximal extents of mucosal innervation by individual neurones and the proportion of neurones responding at that location *vs* the total number tested. The proportions of responses fall from a high of 75% (21 responses from 28 neurones) on the midline, 2–3 mm circumferential from the impaled neurone, to 30–40% for positions a further 1 mm circumferential, and 1 mm oral to the midline. Positions further circumferential, or displaced longitudinally, were less likely to respond. The proportions of responses from these regions were from 8 to 27%. There was a higher proportion of successful responses near the impaled neurone (between 1 and 2 mm) *vs* away from it (between 4 and 7 mm; Wilcoxon signed rank test, $P < 0.05$; excluding responses from 3 mm). Similarly, there was a higher proportion of successful responses on the oral (between 1 and 2 mm) *vs* the anal (between 1 and 2 mm) side of the midline (Wilcoxon signed rank test, $P < 0.05$; excluding the midline).

Area of innervation

The maps generated by electrical stimulation of the mucosa from individual neurones were used to calculate the average area of innervation. Totals for individual maps were based upon each location tested encompassing a full 1 mm^2. Thus, for a map with five locations from which APs could be evoked (e.g. Fig. 2A), the total area is 5 mm^2. The total areas for all 30 maps ranged from the minimum of 1 mm^2 for maps that responded from only one location, up to 15 mm^2 in a map that responded from 15 locations. The average area of the maps was 3.9 ± 3.1 mm^2 (mean ± SD, $n = 30$) and the median was 2.5 mm^2.

Morphology of cell bodies

All 30 AH-neurones whose projections were mapped electrically were filled with Neurobiotin and were recovered following histochemical processing. All were Dogiel type II neurones with large smooth cell bodies that were round or oval and had several long, axonal processes which ran circumferentially in either direction. In each case, at least one of the long processes could be traced to the position of the mucosa, although the ramifications of these processes within the mucosa could not be determined. Thirteen of the 30 AH-neurones were sufficiently well filled with Neurobiotin to allow more detailed morphologies to be determined.

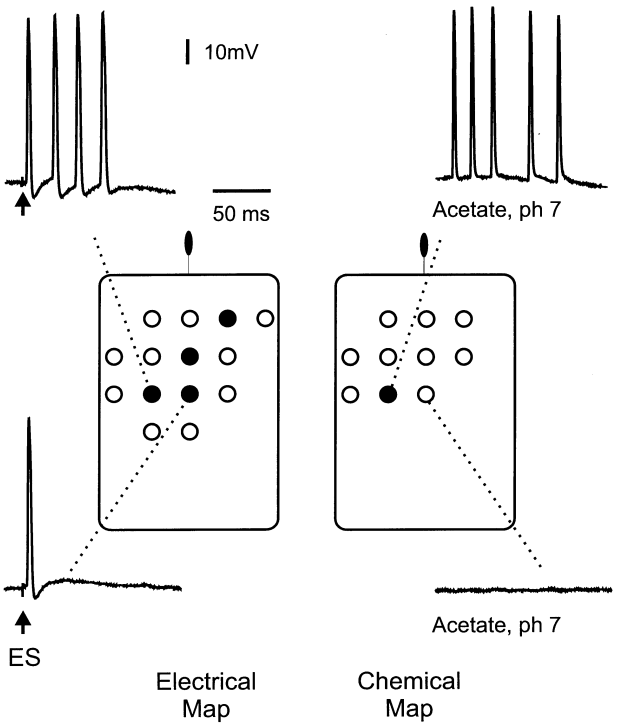

Figure 3 Comparison between electrically and chemically generated maps. Records taken from a single myenteric AH-neurone. Records at left: representative traces taken from two locations in response to electrical stimulation at points indicated by the lines. A single electrical stimulus (ES) was applied at the arrow. The lower left region was the only location to respond with more than one AP for a single electrical pulse (latency 3 msec, average frequency 45 Hz, record inset). Records at right: responses to neutral acetate from the same two locations whose responses to electrical stimulation are shown. A neutral acetate solution (100 mM) was applied ≈700 msec before the start of the trace (latency 735 msec, average frequency 42 Hz). The region which responded to the chemical stimulus also responded with the greatest number of APs to electrical stimulation.

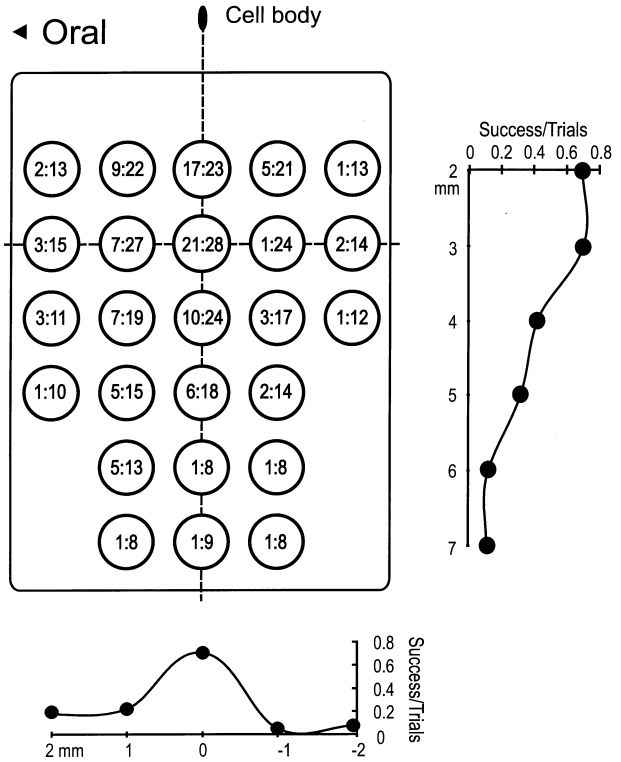

Figure 4 A composite map of the extent of mucosal innervation by the endings of 30 AH-neurones. A composite map in the same orientation as in Fig. 1; oral is to the left and the average position of the cell body is indicated by the filled oval above the map. Each circle represents a region of mucosa that was tested. Within each circle, the proportion of responsive *vs* total tested neurones is given, i.e. 17:23 indicates that 17 of 23 neurones responded from this location. A response was defined as the generation of one or more APs due to electrical stimulation of that region of mucosa. The greatest probability of obtaining a response occurred directly circumferential to the impaled neurone, within the first 3–4 mm. At the bottom and right side, the proportions of neurones yielding responses are plotted for an oral to anal axis and for the axis directly circumferential to the cell bodies (axes indicated by dotted lines).

None of these neurones had a significant projection anally nor did any have five or more fine dendrites.[12]

DISCUSSION

The results provide electrophysiological confirmation of previous morphological studies which indicated that AH/Dogiel type II neurones project locally to supply axons to the mucosa.[13,15,18] Although individual neurones differed in the extents of mucosa they supplied, the data obtained in this study can be used to estimate the probability of a single neurone projecting a particular distance to a site within the mucosa and the average area of innervation for the population of intrinsic primary afferent neurones.

These analyses show that about 10% of the intrinsic primary afferent neurones project to the mucosa up to 7 mm circumferential from their cell bodies. The relaxed circumference of the small intestine is from 15 to 20 mm in the guinea-pig and it is probable that the neurones project in both directions around the intestinal circumference. Thus, some neurones may supply axons to the mucosa around most of intestinal circumference, although clearly most neurones innervate the mucosa to a smaller extent. There was a pronounced oral bias in the longitudinal projections to the mucosa. This is consistent with the results of retrograde tracing studies that found that the neurones project predominantly circumferentially with a tendency for those projections to terminate on the oral side.[15]

Where a response was evoked in the impaled neurone by application of a mechanical or chemical stimulus to the mucosa, this came from only a part of the total field that was electrically mapped. One reason for this could be that not all terminals were sensitive to, or were reached by, the chemical or mechanical stimuli used, while focal electrical stimulation would activate all terminals.

The sensitivity of the terminals to physiological stimuli might depend on either their properties or the properties of the epithelium (see below). The neurones tested in this study appeared to discriminate between stimuli. For instance, most neurones that responded to neutral acetate did not respond to acid and just over half of the neurones responsive to mechanical stimuli were not excited by chemicals.

It is likely that the late bursts of APs in response to electrical stimulation of the mucosa were due to stimulation of other structures within the mucosa that subsequently excited the nerve terminals. The mucosa contains several structures that might release excitatory substances onto the nerve terminals; the terminals of other intrinsic primary afferent neurones, the terminals of extrinsic primary afferent neurones, enterochromaffin or other enteroendocrine cells, and mast cells. A comprehensive list of the substances that act upon the terminals of the intrinsic primary afferent neurones is not known; however, 5-HT, which is released from enterochromaffin cells, is an effective stimulant in up to 50% of the neurones investigated.[7] The late burst of APs (indirect response, Fig. 2) seen in some neurones after electrical stimulation has the characteristics that might be expected of a response produced by release of 5-HT or another chemical intermediate in the sensory transduction pathway.[7] It is

not clear whether the involvement of an intermediate compound would have any affect upon the sizes of the electrically mapped fields in relation to the area stimulated physiologically.

It may be that not all terminals of the AH/Dogiel type II neurones are sensory. Most of these neurones contain choline acetyltransferase (ChAT), the synthetic enzyme for acetylcholine (ACh), and many also contain a tachykinin.[4,19,20] As both ACh and the tachykinins are potent secretogogues in the mucosa, it is possible that some of the projections of these neurones have a motor function rather than, or in addition to, a sensory one.[21–23]

The total area innervated by a single AH/Dogiel type II neurone in the intact intestine is almost certainly larger than the average field measured in this study. Injection of biocytin into individual AH/Dogiel type II neurones has shown that, on average, these neurones have symmetrical myenteric projections around the circumference of the intestine and immunohistochemical studies have shown that the axons in the mucosa probably arise from cell bodies in myenteric ganglia.[12,13,24] Thus, the removal of half the mucosa, that was required in the present study to allow intracellular recording from myenteric neurones, would have disrupted many of the mucosal projections of impaled neurones. Indeed, as the impaled neurones lay up to 1 mm from the intact mucosa, the actual area of mucosa innervated by a single AH/Dogiel type II neurone could be estimated to be 2–3 times the 3.9 mm^2 measured, i.e. 8–12 mm^2. When corrected to compensate for the fact that the tissue had been stretched taut in the presence of nicardipine to prevent smooth muscle contraction, this estimate corresponds to 5.5–8.5 mm^2 in the undistended gut.[13] The number of neurones whose projections would overlap with that of any individual neurone can be deduced from the number of Dogiel type II nerve cells in a 5.5–8.5 mm^2 area of undistended intestine. As there are 42 Dogiel type II neurones per mm^2 of undistended intestine,[13] at least 230–350 Dogiel type II neurones would have projections to the mucosa that overlap with those of any one AH/Dogiel type II neurone (this estimate does not include axons from cell bodies outside the 5.5–8.5 mm^2 area). This massive overlap of potential receptive fields is consistent with the data of Song *et al.*[18] who estimated using retrograde tracing methods that a single villus received axons from about 65 Dogiel type II neurones. Song *et al.*[18] also found that, although most Dogiel type II neurones supplied villi locally, they could project up to 7 mm circumferentially and 2 mm longitudinally. This observation, too, is consistent with the present study. However, Song *et al.*[18] estimated that each Dogiel type II neurone could only innervate about 10 villi. By contrast, in this study we calculate that, with 10 villi per mm^2 and an area of 5.5–8.5 mm^2 for the terminal fields, 55–85 villi would be innervated.[13] This estimate assumes that the AH/Dogiel type II neurones innervate every villus within their terminal fields. One reason for the discrepancy in estimates may be that the intrinsic primary afferent neruons only innervate a subset of the villi within a region, i.e. the innervation pattern of the villi within a potential receptive field would be discontinuous. This is consistent with the observation in the present study that some AH/Dogiel type II neurones innervate discontinuous regions of mucosa.

The substantial convergence of Dogiel type II neurones onto single villi and the wide extent of the mucosal projections of the intrinsic primary afferent neurones indicate that the receptive fields of many intrinsic primary afferent neurones overlap. Thus, the terminals of numerous primary afferent neurones would be expected to be excited by a stimulus applied to a specific villus. It is unlikely that any physiological stimulus would be confined to a single villus, and thus the number of neurones activated could be several hundreds. Overlap is also seen in the terminal fields of the intrinsic primary afferent neurones within the myenteric plexus. Each myenteric neurone may receive inputs from 10 or more intrinsic primary afferent neurones and each of these neurones may contact more than 20 other myenteric neurones.[12,14,25,26]

The activity of intrinsic primary afferent neurones can evoke slow EPSPs in other neurones of the same type.[7,27] Thus, activation of one or more intrinsic primary afferent neurones by a stimulus applied to a single villus would be expected to increase the excitability of many other intrinsic primary afferent neurones in the same region. This would tend to couple the activity of these neurones within a particular region. Coupled activity may be important for the functioning of the sensory neurones as they transmit to interneurones and motor neurones via slow EPSPs and via subthreshold fast EPSPs.[26,27] For mucosal stimuli to result in the generation of APs in motor neurones and interneurones may require either, or both, high-frequency firing in the intrinsic primary afferent neurones in order to evoke a slow EPSP or substantial convergence of inputs onto the second-order neurones causing summation of fast EPSPs.

In conclusion, this study indicates that individual intrinsic primary afferent neurones send axons to areas of mucosa that, although large in comparison to the circumference of the gut, would be small compared to that occupied by a natural stimulus. Analysis of the

patterns of projections indicates that neurones may project up to half way around the circumference of the intestine and more than 2 mm orally along the intestine to supply the mucosa. Taken together with the results of earlier studies, these data indicate that individual intrinsic sensory neurones may never act alone because their receptive fields and synaptic outputs appear to overlap substantially.

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