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# ELECTROPHYSIOLOGY AND ENKEPHALIN IMMUNOREACTIVITY OF IDENTIFIED MYENTERIC PLEXUS NEURONES OF GUINEA-PIG SMALL INTESTINE

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SUMMARY

1. Intracellular injection of the fluorescent dye, Lucifer Yellow CH, revealed the shapes of neurones in the myenteric plexus of the guinea-pig ileum, and these shapes were correlated with the electrophysiological properties and enkephalin immunoreactivity of the neurones.

2. A total of eighty-three neurones were filled using electrodes containing a 5% solution of the dye. Forty-six cells had many short processes and a single long process (Dogiel type 1) and twenty-four cells had essentially smooth somas and one to eight long processes (Dogiel type II). Thirteen cells could not be put into either group. Enkephalin-like immunoreactivity was detected in twenty-two of the forty-six Dogiel type I cells. Eighteen of these had club-like short processes. No other cells of the eighty-three showed enkephalin-like immunoreactivity.

3. Electrodes filled with a 0·5% solution of Lucifer Yellow in 0·5 M-KCl were used to record from and simultaneously to inject dye into 240 neurones. Eighty-six nerve cells had a slow after-hyperpolarization following the action potential (AH cells) and forty-six nerve cells had no after-hyperpolarization but exhibited a fast excitatory synaptic potential (S cells). The other cells could not be unequivocally identified by their observed electrophysiological characteristics. Almost all S cells (forty-two of forty-six) were Dogiel type I, while eighty-two of the eighty-six AH cells were Dogiel type II.

4. Fifty S cells (eight located geometrically, forty-two by dye injection) and ninety-one AH cells (twenty-six located geometrically, sixty-five by dye injection) were examined for enkephalin immunoreactivity. Fifteen of the S cells were reactive, whereas all of the AH cells were unreactive. It appears that prolonged impalements reduce immunoreactivity so that the proportion of reactive neurones in this series is an underestimate of the true proportion of S cells with enkephalin-like immunoreactivity.

5. The results suggest that a substantial proportion of the S cells in myenteric

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ganglia contain enkephalin immunoreactivity while none of the AH cells do. The enkephalin neurones have a distinctive shape and are all Dogiel type I cells. AH cells are nearly always Dogiel type II.

## INTRODUCTION

When intracellular recordings are made from neurones in the myenteric plexus of the guinea-pig small intestine two classes of neurone can be defined. These are AH cells, in which an action potential is followed by a slow after-hyperpolarization and in which stimulation of the presynaptic nerves nearly always fails to evoke a fast excitatory post-synaptic potential (fast e.p.s.p.), and S cells in which fast e.p.s.p.s can be evoked and which exhibit no slow after-hyperpolarization (Hirst, Holman & Spence, 1974; see also North, 1982). In contrast, at least eight apparently distinct classes of neurone have been identified histochemically in the guinea-pig myenteric plexus (Schultzberg, Hökfelt, Nilsson, Terenius, Rehfeld, Brown, Elde, Goldstein & Said, 1980; Sundler, Häkanson & Leander, 1980; Costa & Furness, 1982). Both the membrane properties and the natures of the synaptic inputs of the different histochemically defined neurones are yet to be determined.

The study described here was undertaken to address some of these problems by correlating the electrophysiological properties of neurones in the myenteric plexus of the guinea-pig ileum with their morphology and with the presence or absence of immunoreactivity for enkephalin. Enkephalin-immunoreactive cells make up a significant proportion of myenteric neurones; estimates of this proportion range from 15 to 24% (Schultzberg *et al.* 1980; Bu'lock, Vaillant, Dockray & Bu'lock, 1982; Furness, Costa & Miller, 1983). The majority of these cells have many short processes and a single long process (Furness *et al.* 1983), a morphology defined as Type I by Dogiel (1899). They also have a well-defined pattern of projections within the wall of the small intestine (Furness *et al.* 1983). Opioid agonists, including enkephalin, exert profound effects on the motility of the guinea-pig small intestine via their interaction with cholinergic motor neurones (Trendelenburg, 1917; Paton 1957; Waterfield, Smokcum, Hughes, Kosterlitz & Henderson, 1977); evidence has been presented that endogenous enkephalin is released by neuronal activity within the myenteric plexus (Puig, Gascon, Gravisco & Musacchio, 1977; Waterfield & Kosterlitz, 1977; Corbett, McKnight & Kosterlitz, 1981).

The shapes of the myenteric neurones were studied by intracellular injection of the fluorescent dye, Lucifer Yellow CH (Stewart, 1978) from micro-electrodes containing solutions of either a mixture of KCl and the dye, thus allowing simultaneous electrophysiological recordings to be made, or the dye alone. The enkephalin immunoreactivity was studied in the same preparations by an indirect immunofluorescence technique.

Brief reports of some of these data have been published elsewhere (Bornstein, Lees, Costa & Furness, 1983; Bornstein, Costa, Furness & Lees, 1983).

## METHODS

Guinea-pigs weighing 200–450 g and of either sex were killed by stunning and were bled out. A 3 cm segment of small intestine was taken from the region 15–30 cm oral to the ileo-caecal junction, flushed clean with warm (37 °C) modified Krebs solution, cut along its mesenteric border and pinned

flat with its mucosal surface uppermost in an organ bath. A number of myenteric ganglia were then cleared of the overlying mucosa, submucosa and circular muscle using methods similar to those of Wood & Mayer (1978).

During the dissection and the subsequent recording period, the preparation was kept in a modified Krebs solution (composition in mM: NaCl, 118; KCl, 4·75; $CaCl_2$, 2·54; $MgSO_4$, 1·2; $NaH_2PO_4$, 1·0; $NaHCO_3$, 25·0; glucose, 11·1; bubbled with 95% $O_2$, 5% $CO_2$). Initial experiments showed that immobilizing ganglia by close pinning as in previous studies (e.g. Nishi & North, 1973; Hirst *et al.* 1974; Hodgkiss & Lees, 1983), could interfere with the subsequent immunohistochemical analysis. Accordingly, in most experiments verapamil (5–15 $\mu$M) and hyoscine (2 $\mu$M) were added to the superfusate to reduce myogenic and neurogenic contractions and the ganglia were left unpinned.

After the dissection, the organ bath was placed on the stage of a conventional microscope which was modified so that the preparation could be viewed with fluorescent light epi-illumination or with white or red light transillumination via an interference-contrast condenser (Lees & Gray, 1982). The water-immersion objectives gave final magnifications of 310 or 500. The preparation was continuously superfused at 3 ml/min with the modified Krebs solution pre-heated to give a bath temperature of 33–37 °C.

Three experimental procedures were used:

(1) Random Lucifer Yellow filling of myenteric neurones followed by immunohistochemical localization of enkephalin to correlate the shape of neurones with enkephalin immunoreactivity.

(2) Intracellular recording from myenteric neurones filled with Lucifer Yellow to correlate electrophysiology with the shape of nerve cells.

(3) Intracellular recording from myenteric neurones, either Lucifer Yellow-filled or not, followed by immunohistochemical localization of enkephalin to correlate electrophysiology with enkephalin immunoreactivity.

*Determination of cell shapes*

Micro-electrodes filled with a 5% (w/v) solution of Lucifer Yellow CH in distilled water (resistance 200–500 MΩ) were used to stain randomly selected myenteric neurones with this fluorescent dye. The dye was injected into cells by passing hyperpolarizing current pulses (0·1–2·5 nA, 950 ms duration, at 1 Hz for up to 15 min) through the micro-electrode. In this series of experiments, the criterion for a successful impalement was the appearance in a cell body of the typical yellow fluorescence of the dye. Lucifer Yellow diffuses rapidly through the cytoplasm into which it is injected, so it was possible to determine the morphological characteristics of the stained cell by viewing the Lucifer Yellow fluorescence *in vitro* (Stewart, 1978, 1981). Roughly a quarter of the cells stained were initially impaled in a cell process and partially stained via this process, these cells were usually re-impaled in the soma so that adequate staining of all processes could be ensured. After each cell had been stained, it was sketched and its position noted.

*Electrophysiological recording*

Cells in the myenteric plexus were impaled using methods similar to those described by Nishi & North (1973). Micro-electrode resistance ranged from 40 to 100 MΩ for electrodes filled with 1·0–2·5 M-KCl, and 60–120 MΩ for cells filled with a 0·5% solution of Lucifer Yellow in 0·5 M-KCl.

Nerve fibres within the plexus were stimulated by pairs of silver wire electrodes placed above and below the preparation 3–12 mm from the recording site (transmural stimulation). Stimuli were also applied focally via a glass micro-electrode filled with Krebs solution or saline and having a tip diameter of 8–15 $\mu$m. This electrode was positioned so that it was in contact either with the surface of the ganglion from which recordings were being made or with an interconnecting strand.

The only electrophysiological criterion for a successful impalement was that a cell could be unequivocally classified into one of the two groups, AH or S. Neurones were classified as AH if an action potential was followed by a prolonged hyperpolarization of at least 4 s duration. The hyperpolarization sometimes appeared only after up to 45 min had elapsed following the initial penetration (see also Hodgkiss & Lees, 1978, 1983). Neurones which showed a fast e.p.s.p. in response to either transmural or focal stimulation were classified as S cells. Fast e.p.s.p.s were distinguished from proximal process potentials by the criteria of Nishi & North (1973): the synaptic potentials reach their peak amplitude in 3–8 ms, in contrast to about 1 ms for the proximal process potentials, and they show a 'run down' in amplitude when evoked at stimulus frequencies of 1 Hz or more, as well as an increase in amplitude when the cell is hyperpolarized.

*Geometric localization of cells*

After a cell had been classified electrophysiologically using an electrode containing no dye, the recording electrode was withdrawn. The ganglion was then drawn as accurately as possible and the site of the cell and its relationship to such features as interconnecting strands, capillaries, erythrocytes, and residual circular muscle noted. The position of the ganglion within the preparation and its connexions with neighbouring ganglia were also noted so that the cell could be re-identified after processing for immunohistochemistry.

*Localization of cells after dye injection*

Micro-electrodes filled with 0·5 % Lucifer Yellow in 0·5 M-KCl (resistance 60–120 MΩ) were used to fill the myenteric neurones with dye solution and simultaneously to record from them. More current was required to fill cells with these electrodes than with electrodes containing 5 % Lucifer Yellow, but attempts to use higher concentrations of Lucifer Yellow dissolved in 0·5 M-KCl frequently led to blockages of the micro-electrodes due to precipitation of the dye. Cells were filled by passing a maintained hyperpolarizing current of 0·25–2·0 nA for 10–20 min. In practice, when micro-electrodes filled with the Lucifer Yellow and KCl solution were used, it was found that the electrophysiological properties of cells were markedly improved, provided the cell was subjected to a maintained hyperpolarization soon after impalement; such hyperpolarizing currents were, therefore, applied routinely in this type of experiment. After each impalement the cell was sketched and classified by shape, and its position within the ganglion and in relation to other filled cells was noted.

*Processing for immunohistochemistry*

After most experiments, the preparation was removed from the organ bath, pinned to a piece of clear plastic sheet (1 mm thick) and processed for immunohistochemical localization of enkephalin-like peptides using methods described in detail elsewhere (Costa, Buffa, Furness & Solcia, 1980). In summary, after overnight fixation in formaldehyde and picric acid solution the preparation was dehydrated and rehydrated before being exposed for at least 12 h at room temperature to an anti-enkephalin antibody (antiserum 198B; raised in rabbits) the characteristics of which have already been determined (Miller, Chang, Cooper & Cuatracasas, 1978; Furness *et al.* 1983). After washing, the preparation was exposed for 1 h to a second (anti-rabbit) antibody coupled either to fluorescein isothiocyanate (FITC, Wellcome Diagnostics) or rhodamine (Cappel Laboratories). The preparation was then washed and mounted on a glass slide. Cell bodies immunoreactive for enkephalin-like peptides were identified under a fluorescence microscope. In experiments where Lucifer Yellow was not used the second antibody was that coupled to FITC. When cells had been filled with Lucifer Yellow, a second antibody coupled to rhodamine was used so that the immunofluorescence could be easily distinguished from that of Lucifer Yellow (Reaves & Hayward, 1979). The effect of the immunohistochemical procedures on the Lucifer Yellow fluorescence was monitored at each step and it was found that, although the intensity was reduced, well-filled cells and their processes remained readily identifiable. Once a cell had been identified either using transmitted white light or Lucifer Yellow fluorescence, the filters were switched to show the enkephalin immunoreactivity of the same field and plane of focus.

## RESULTS

### *Morphology of enkephalin immunoreactive cells in relation to a classification of cell shapes*

Enkephalin-immunoreactive nerve cells in the myenteric plexus of the guinea-pig small intestine have a distinctive shape, each has many short (about 20 μm or less), club-like processes and one long process (sometimes with small spines close to the soma (Pl. 1 *A–C*; see also Furness *et al.* 1983).

A total of eighty-six neurones in nine preparations were filled with Lucifer Yellow and eighty-three of these were sufficiently well filled that their morphological features could be studied after fixation and processing for immunohistochemistry. An example of a group of Lucifer Yellow-filled cells in one ganglion is shown in

Pl. 1*D*, and a single cell photographed at a higher magnification can be seen in Pl. 1*E*. Cell shapes ranged from essentially smooth cells with a few long processes to cells with many short processes and covered the range of shapes described Dogiel (1899) and others. Long processes from well-filled cells could often be followed for up to 2 mm within the plexus, sometimes becoming 'beaded' or showing gaps in the fluorescence of what were presumed to be continuous fibres. Short processes were normally clearly defined as shown in Pl. 1*E*, but after fixation poorly filled or damaged cells sometimes showed uneven fluorescence so that some processes appeared as filled regions apparently unconnected with the soma.

Non-neuronal cells were also impaled and filled; these included longitudinal and circular muscle fibres and glial cells. The glial cells could be readily distinguished from neurones as they had very small somata and a profusion of fine branching processes. One filled glial cell is shown in Pl. 1*D* (arrow). When examined with electrodes filled with a Lucifer Yellow and KCl solution, the glial cells had properties very similar to those of glia studied elsewhere in the nervous system (Orkland, Nicholls & Kuffler, 1966; Ransom & Goldring, 1973). The filled nerve cell bodies were divided into morphological groups using criteria based on those of Dogiel (1899), who described three morphological types of neurone in the guinea-pig myenteric plexus: Type I cells which have many short processes and a single long process, Type II cells which have smooth somata and a number of long processes and Type III cells which have several long and several short processes.

From this classification it is clear that the enkephalin immunoreactive cells fall into the Dogiel Type I group (Furness *et al.* 1983). However, the Type I classification includes both cells with short processes that are thin and tapering, and cells whose processes are club-like. In the present study the filled cells were placed in four groups:

(a) Dogiel Type I cells with short club-like processes (e.g. Fig. 1*A*),

(b) Dogiel Type I cells with short, thin and/or tapering, processes (e.g. Fig. 1*B*),

(c) Dogiel Type II cells with smooth somata and a number of long processes (e.g. Fig. 1*C*),

(d) cells which could not be placed in any of the other three categories above; this last group of cells had a wide range of shapes (not illustrated).

Cells were classified independently by each of the authors from photographs similar to that shown in Pl. 1*E* or from drawings. The numbers in each group are shown in Table 1*A*.

Twenty-two cells of those filled with Lucifer Yellow in these experiments were immunoreactive for enkephalin: this is 26·5% of the sample. All the enkephalin-immunoreactive cells were classified as Dogiel Type 1 and all but four of these had short, club-like processes (Pl. 1*F*, *G*). Enkephalin-immunoreactivity was, however, not found in all of the cells with short, club-like processes (Pl. 1*H*, *I*).

*Correlation of electrophysiology with morphology*

Electrodes filled with a 0·5% solution of Lucifer Yellow and KCl were used to fill and record from myenteric neurones at the same time. Cells were usually filled well enough to become classifiable by shape *in vitro* within 10 min of impalement. Many of these nerve cells had very low resting membrane potentials ($< 15$ mV) and fired no action potentials; they were identified as neurones by their shapes. Other, similarly

treated cells were identified as neurones on electrophysiological grounds (e.g. firing of action potentials on impalement, input resistance greater than 20 MΩ, resting membrane potential −35 to −80 mV); their electrophysiological characteristics were similar to those observed in neurones impaled with conventional micro-electrodes and remained stable for up to 3 h. In particular, both fast e.p.s.p.s and slow after-hyperpolarizations were normal in the Lucifer Yellow-filled nerve cells so that these cells could be classified as S or AH (Fig. 2).

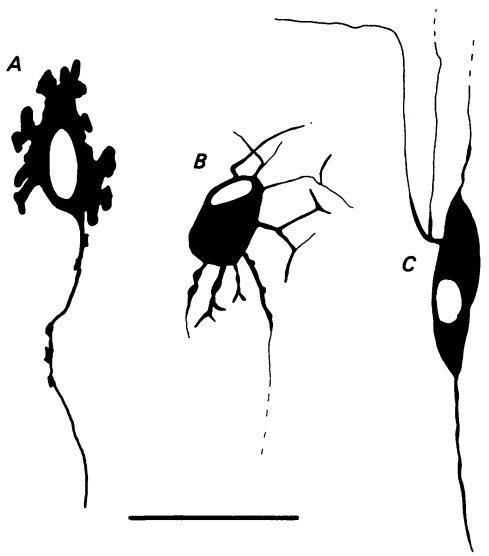


Fig. 1. Examples of scale drawings of the three groups of nerve cells that were classified by their shape. The cells were filled with Lucifer Yellow: *A*, Dogiel Type I cell with club-like processes; *B*, Dogiel Type I cell with thin, tapering processes; *C*, Dogiel Type II cell. No examples of the heterogeneous group of cells with other shapes are shown. Bar 50 μm.

A total of 240 neurones were filled with Lucifer Yellow using the mixed Lucifer Yellow/KCl electrodes. Of these, eighty-six were identified as being AH cells and forty-six were found to be S cells. The remaining 108 cells were not unequivocally identified electrophysiologically, as S or AH, although they were neurones. Each of the electrophysologically identified cells was examined by fluorescence microscopy *in vitro* and put into one of the four morphological categories (Table 1 B). Some cells became swollen during the impalement (see Nishi & North, 1973) but many of these cells returned to their normal shape when the electrode was removed. In nearly every case the cells could nevertheless be classified by shape.

The great majority of S cells (forty-two of forty-six) were Dogiel Type I cells; twenty-eight of these had short, club-like processes. One S cell was classified as Dogiel Type II and three could not be classified as Type I or II. The range of shapes of S cells is illustrated in Fig. 3. The short processes of S cells ranged from fine, branching, varicose fibres (3*J*), to broad, branching processes (3*H*), and short, club-like almost mushroom-like processes (3*A–D* and *G*). Similarly, the shapes of the S cell somata varied from round (3*F*) to cigar shaped (cell 3*I*, which was 5 times longer than it was wide). The sizes of the somata also showed wide variation (e.g. 3*B* and *E*).

Almost all of the AH cells (eighty-two of eighty-six) were Dogiel Type II cells. The remaining four had neither Type I nor Type II morphology in that they had a number of short processes but more than one long process. None of the AH cells had the Dogiel Type I morphology. Fig. 4 shows examples of the range of shapes of AH cells and illustrates the relative paucity of short processes of these cells (compare with Fig. 3). These drawings also illustrate another common property of the AH cells in that their long processes often branch close to the cell body (e.g. cells *A*, *B*, *D*, *E* and *H*; see also Hodgkiss & Lees, 1983).

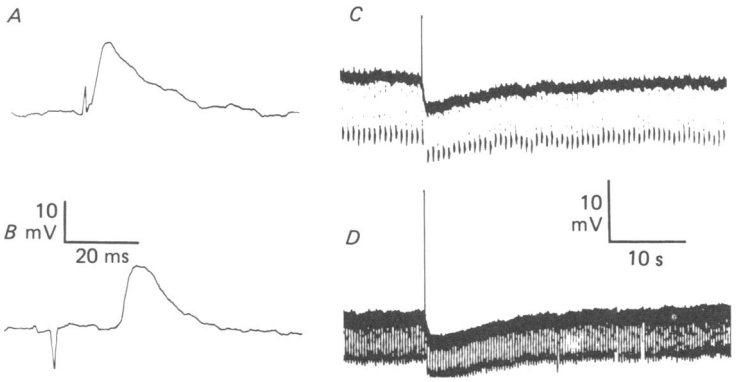


Fig. 2. Fast e.p.s.p.s (*A*, *B*) and slow after-hyperpolarizations (*C*, *D*) recorded in different cells with electrodes filled with either 2·5 M-KCl (*A*, *C*) or 0·5 % Lucifer Yellow in 0·5 M-KCl solution (*B*, *D*). The e.p.s.p. in *A* was evoked by focal stimulation of an interconnecting strand and was recorded with a KCl electrode, while the e.p.s.p. in *B* was evoked by transmural stimulation 12 mm oral to the cell impaled and was recorded with a mixed Lucifer Yellow and KCl electrode. *C* shows a slow after-hyperpolarization following an action potential, recorded in an AH cell with a KCl electrode. The action potential was attenuated by the pen recorder. *D*, a similar slow after-hyperpolarization recorded with a mixed electrode. The records shown in *C* and *D* were obtained while constant sized hyperpolarizing current pulses were being passed through the recording electrodes so that the increased conductance associated with the slow after-hyperpolarization could be demonstrated. Records shown in *A* and *B* were retouched.

The sizes of S cells and AH cells were compared by measuring the long and short axes (excluding processes) of each of the cells that had been drawn to scale. These dimensions were compared statistically using a non-parametric test (Mann–Whitney rank-sum test, pp. 130–131 in Snedecor & Cochran, 1967). On average, AH cells were significantly larger than S cells. The mean length of AH cells (fifteen cells) was $43 \pm 8$ $\mu$m (S.D.) and that of S cells (thirty cells) was $33 \pm 11$ $\mu$m ($P < 0{\cdot}01$); the mean width of AH cells was $20 \pm 4$ $\mu$m and that of S cells was $15 \pm 5$ $\mu$m ($P < 0{\cdot}005$). AH cells were also more uniform in size than S cells as is shown by the relevant coefficients of variation: for AH cells these were 0·20 (length) and 0·18 (width) but for S cells the equivalent values were 0·33 and 0·32, respectively.

*Correlation of electrophysiology with enkephalin immunoreactivity*

The enkephalin immunoreactivity of a total of 141 electrophysiologically identified cells was examined (Table 1C). No enkephalin-like immunoreactivity was detected

TABLE 1

| | Type I (club-like) | Type I (other) | Type I (total) | Type II | Other shapes | Total |
|---|---|---|---|---|---|---|
| **A. Enkephalin-like immunoreactivity in Lucifer Yellow-filled cells of different shapes** | | | | | | |
| No. of cells filled | 25 | 21 | 46 | 24 | 13 | 83 |
| No. of immunoreactive cells | 18 | 4 | 22 | 0 | 0 | 22 |
| Immunoreactive cells (%) | 72 | 19 | 48 | 0 | 0 | 26·5* |
| **B. Shapes of electrophysiologically identified cells** | | | | | | |
| S | 28 | 14 | 42 | 1 | 3 | 46 |
| AH | 0 | 0 | 0 | 82 | 4 | 86 |
| Total number of cells | 28 | 14 | 42 | 83 | 7 | 132 |

C. Enkephalin-like immunoreactivity of electrophysiologically identified cells

| | Geometrically located | Lucifer Yellow-filled: Type I (club like) | Type I (other) | Type II | Other shapes | Total |
|---|---|---|---|---|---|---|
| S cells | | | | | | |
| No. of cells | 8 | 26 | 13 | 1 | 2 | 50 |
| No. of immunoreactive cells | 2 | 12 | 1 | 0 | 0 | 15 |
| Immunoreactive cells (%) | 25 | 46 | 8 | 0 | 0 | 30 |
| AH cells | | | | | | |
| No. of cells | 26 | 0 | 0 | 61 | 4 | 91 |
| No. of immunoreactive cells | 0 | 0 | 0 | 0 | 0 | 0 |
| Immunoreactive cells (%) | 0 | — | — | 0 | 0 | 0 |
| Total number of cells | 34 | 26 | 13 | 62 | 6 | 141 |
| Immunoreactive cells (%) | 6 | 46 | 8 | 0 | 0 | 10·6* |

* Significantly different $P < 0{\cdot}005$, $\chi^2$ test 1 degree of freedom.

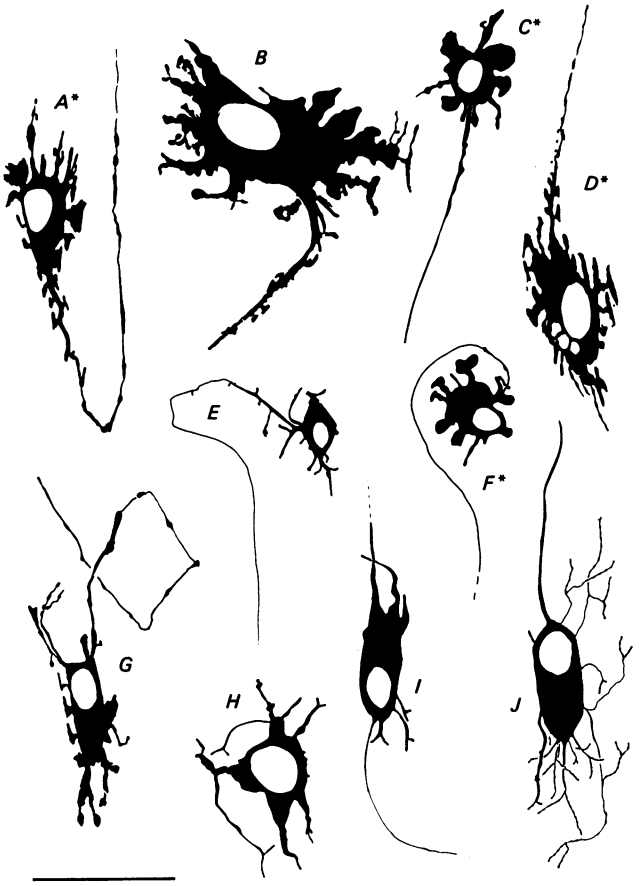


Fig. 3. Scale drawings showing the range of shapes of Lucifer Yellow-filled nerve cells identified electrophysiologically as S cells. All these cells with the exception of cell *I* were classified as being Dogiel Type I cells as they had only a single long process and a number of short processes. Cell *I* was classified as a Dogiel Type II cell on the basis of a careful examination of its processes under the microscope. Cells *A–D*, *F* and *G* had club-like processes. Cells *E*, *H* and *J* had thin or tapering short processes. Cells with asterisks showed enkephalin immunoreactivity (cell *B* was not examined for enkephalin-like immunoreactivity). Note the wide range of cell sizes. Bar 50 $\mu$m.

in any of the ninety-one AH cells. In contrast, fifteen out of fifty S cells showed immunoreactivity for enkephalin. An example of the immunofluorescence and the electrophysiological record of a Lucifer Yellow-filled S cell is shown in Pl. 2.

Thirteen of the Lucifer Yellow-filled S cells showing enkephalin immunoreactivity were sufficiently well filled for their shapes to be classified and all thirteen were Dogiel Type I cells with twelve having short, club-like processes and one having fine branching processes. Examples of the shapes of these cells are shown in Fig. 3 where cells marked with an asterisk (cells *A*, *C*, *D* and *F*) were immunoreactive for enkephalin. Measurement of the dimensions of these immunoreactive S cells indicated that they could not be distinguished from the other S cells on the basis of their length or breadth.

The immunofluorescence of impaled cells was generally less intense that that of

immunoreactive cells which had not been impaled. The apparent loss of immunoreactivity was greater following a prolonged impalement than after a brief one so that those cells most intensely stained with Lucifer Yellow had the faintest enkephalin immunofluorescence.

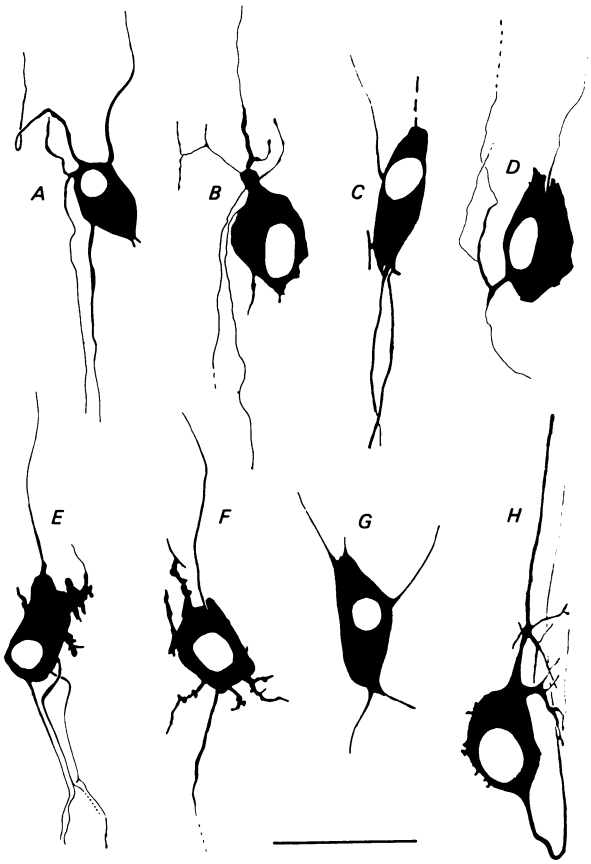


Fig. 4. Scale drawing of Lucifer Yellow-filled nerve cells, identified electrophysiologically as AH cells. Cells *A–E*, *G* and *H* were classified as Dogiel Type II, they had generally smooth somata and more than one long process. Cell *F* was not classified as either Type I or Type II. None of these cells showed enkephalin-like immunoreactivity. Bar 50 $\mu$m.

## DISCUSSION

### *Shapes of enteric nerve cells*

Dogiel (1896, 1899) classified enteric nerve cells according to their morphology: Type I were cells with several short processes and one long process, Type II cells had generally smooth surfaces and several long processes and Type III cells showed several long and several short processes. In the myenteric plexus of the guinea-pig small intestine we can identify Type I and Type II cells but the remaining cells (about 15%) are quite variable in shape and we have grouped them separately. Amongst the Type I cells we can distinguish two subgroups, Type I cells with club-like short processes and Type I cells with filamentous or tapering short processes.

This distinction of the two subgroups of Dogiel's Type I classification is significant in that most enkephalin-immunoreactive nerve cells have shapes of the first subgroup (see below).

*Estimation of proportions of nerve cell populations*

The present results give various estimates of the proportions of nerve cells that are electrophysiologically AH or S, of cells that have Type I or Type II configurations, and of fractions of these groups that show enkephalin-like immunoreactivity. Table 1 shows that when cells were briefly impaled, simply to fill them with dye, 55% exhibited Type I morphology and 29% Type II. In contrast, of the neurones that were adequately classified electrophysiologically, and were also dye-filled, 32% were Type I and 63% were Type II cells. Type I neurones have smaller and more convoluted cell bodies than do Type II neurones and it is perhaps not surprising that a lower proportion of Type I cells was obtained when it was necessary to maintain impalements. We regard the estimate that about 55% of neurones are Type I and 30% Type II as the more reliable.

A previous study indicated that about 24% of all myenteric neurones are immunoreactive for enkephalins (Furness *et al.* 1983). This is consistent with the proportion of randomly filled reactive cells found in the present work (26·5%). As all enkephalin cells are Type I cells, these data taken together suggest that about 50% of Type I cells are enkephalin positive; of the subgroup with club-like processes, about three-quarters react for enkephalin. Nevertheless, only one-third of electrophysiologically characterized Type I cells showed enkephalin-like immunoreactivity. This observation, and unquantified observations of intensity of staining, suggest that prolonged impalements of neurones reduce their enkephalin-like immunoreactivity. The morphological class of Type I cells and the electrophysiological class S are almost identical, so the results imply that about 50% of S cells contain an enkephalin-like compound.

*Relation of nerve cell morphology, chemistry and function*

Dogiel (1899) considered that the shapes of enteric neurones were an indication of function and proposed that Type I cells are motor and Type II cells are sensory. While the present work cannot substantiate the proposed correlation made by Dogiel, it does indicate an association of shape with functional features. The electrophysiological identification of cells as the AH type was almost always correlated with their having Dogiel Type II morphology; and S cells, electrophysiologically defined, were most often of Type I morphology. Essentially the same correlations of shape and electrophysiology were made in other studies (Hodgkiss & Lees, 1978, 1980, 1983; Erde, Gershon & Wood, 1980). Moreover, all cells with enkephalin-like immunoreactivity had the Dogiel Type I morphology and none had Type II morphology. Enkephalin immunoreactivity was associated with a proportion of S cells, but never with AH cells. These observations support Dogiel's idea that the shapes and functions of enteric neurones are related. The Dogiel Type I neurones which contain enkephalins receive a cholinergic input from other enteric neurones. Action potentials initiated synaptically in the cell body may be expected to invade processes which project to the circular muscle layer; they might also invade enkephalin-positive processes which project orally to other myenteric ganglia and are thought to arise from these cells

(Furness *et al.* 1983). Further experiments to correlate nerve cell shape and neurochemistry with physiological, pharmacological and ultrastructural properties will predictably play an important role in understanding the modes of operation of the enteric nervous system (Furness & Costa, 1980).

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## REFERENCES

BORNSTEIN, J. C., COSTA, M., FURNESS, J. B. & LEES, G. M. (1983). Correlation of the electrophysiology, morphology and enkephalin-immunoreactivity of neurones in guinea-pig myenteric plexus. *J. Physiol.* **341**, 59*P*.

BORNSTEIN, J. C., LEES, G. M., COSTA, M. & FURNESS, J. B. (1983). Morphology of intracellularly-stained guinea-pig myenteric neurones correlated with enkephalin immunoreactivity. *Neurosci. Lett.* **11** suppl., S31.

BU'LOCK, A. J., VAILLANT, C., DOCKRAY, G. J. & BU'LOCK, J. D. (1982). A rational approach to the fixation of peptidergic nerve bodies in the gut using parabenzoquinone. *Histochemistry* **74**, 49–55.

CORBETT, A. D., MCKNIGHT, A. T. & KOSTERLITZ, H. W. (1981). Tetrathyl-ammonium facilitates the stimulation-evoked loss of the enkephalins from the myenteric plexus of guinea-pig ileum. *Proc. R. Soc.* B **213**, 171–182.

COSTA, M., BUFFA, R., FURNESS, J. B. & SOLCIA, E. (1980). Immunohistochemical localization of polypeptides in peripheral autonomic nerves using whole mount preparations. *Histochemistry* **65**, 157–165.

COSTA, M. & FURNESS, J. B. (1982). Neuronal peptides in the intestine. *Br. med. Bull.* **38**, 247–252.

DOGIEL, A. S. (1896). Zwei Arten sympathischer Nervenzellen. *Anat. Anz.* **11**, 679–687.

DOGIEL, A. S. (1899). Ueber den Bau der Ganglien in den Geflechten des Darmes und der Gallenblase des Menschen und der Säugerthiere. *Arch. Anat. Physiol. Anat. Abt.* 130–158.

ERDE, S. M., GERSHON, M. D. & WOOD, J. D. (1980). Morphology of electrophysiologically identified types of myenteric neuron: intracellular recording and injection of lucifer Yellow in the guinea-pig small bowel. *Neurosci. Abstr.* **6**, 274.

FURNESS, J. B. & COSTA, M. (1980). Types of nerves in the enteric nervous system. *Neuroscience* **5**, 1–20.

FURNESS, J. B., COSTA, M. & MILLER, R. J. (1983). Distribution and projections of nerves with enkephalin-like immunoreactivity in the guinea-pig small intestine. *Neuroscience* **8**, 653–664.

HIRST, G. D. S., HOLMAN, M. E. & SPENCE, I. (1974). Two types of neurones in the myenteric plexus of the guinea-pig duodenum. *J. Physiol.* **236**, 303–326.

HODGKISS, J. P. & LEES, G. M. (1978). Correlated electrophysiological and morphological characteristics of myenteric plexus neurones. *J. Physiol.* **285**, 19–20*P*.

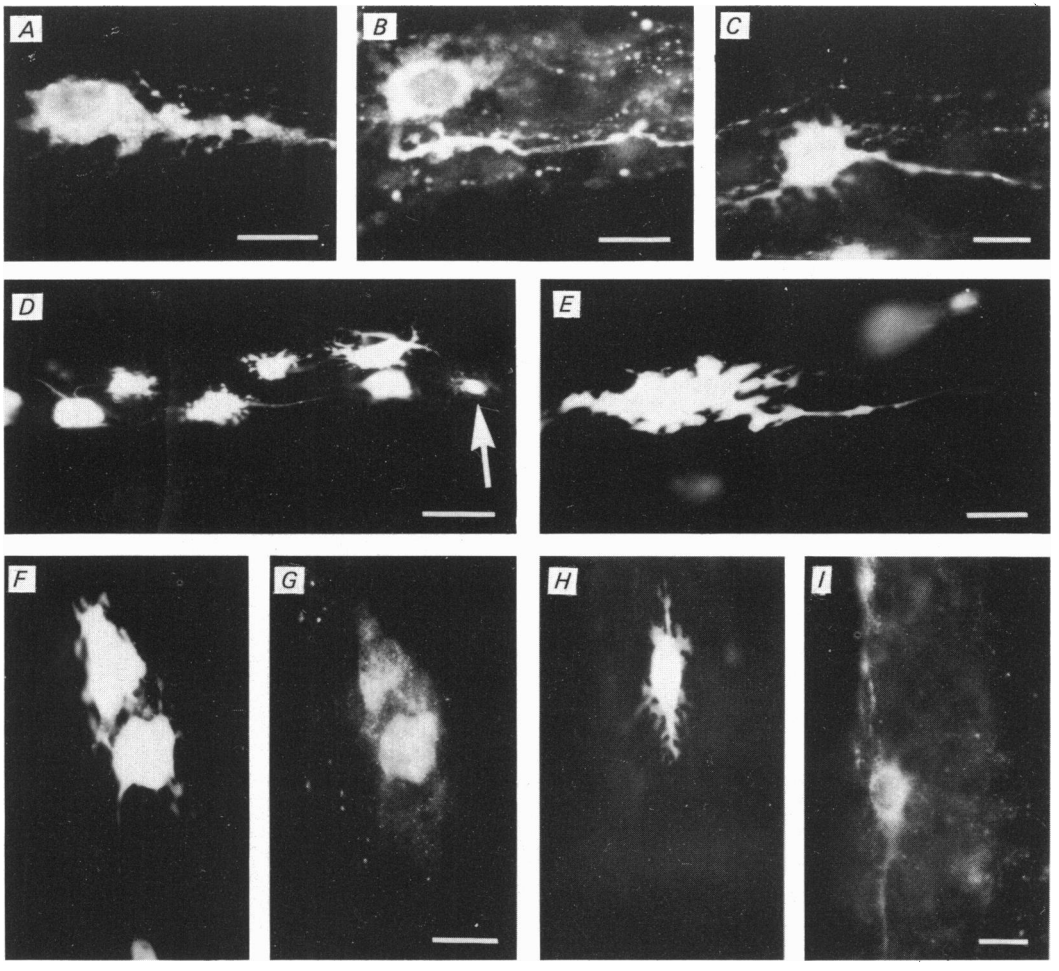
HODGKISS, J. P. & LEES, G. M. (1980). Morphological features of guinea-pig myenteric plexus neurones. In *Gastrointestinal Motility*, ed. CHRISTIENSEN, J., pp. 111–117. New York: Raven Press.

HODGKISS, J. P. & LEES, G. M. (1983). Morphological studies of electrophysiologically-identified myenteric plexus neurons of the guinea-pig ileum. *Neuroscience* **8**, 593–608.

LEES, G. M. & GRAY, M. J. (1982). A method for simultaneous visualisation and electrophysiological recording of enteric neurons with intracellular fluorescent markers. *Scand. Jnl Gastroenterol.* **17**, suppl. 17, *Autonomic Nerves of the Gut*, 169–170.

MILLER, R. J., CHANG, K. J., COOPER, B. & CUATRECASAS, P. (1978). Radioimmunoassay and characterization of enkephalins in rat tissues. *J. biol. Chem.* **253**, 531–538.

NISHI, S. & NORTH, R. A. (1973). Intracellular recording from the myenteric plexus of the guinea-pig ileum. *J. Physiol.* **231**, 471–491.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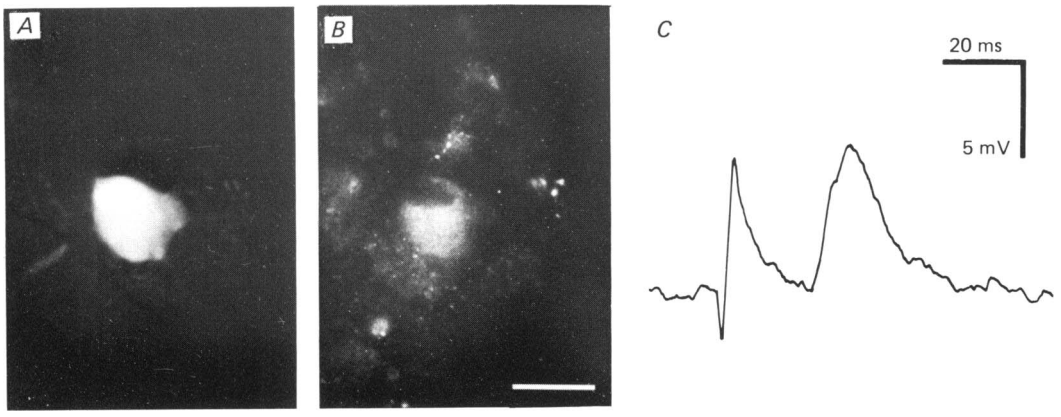
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NORTH, R. A. (1982). Electrophysiology of the enteric nervous system. *Neuroscience* **7**, 315–325.

ORKLAND, R. K., NICHOLLS, J. G. & KUFFLER, S. W. (1966). Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. *J. Neurophysiol.* **29**, 788–806.

PATON, W. D. M. (1957). The action of morphine and related substances on contraction and on acetylcholine output of coaxially stimulated guinea-pig ileum. *Br. J. Pharmac.* **11**, 119–127.

PUIG, M. M., GASCON, P., GRAVISCO, G. L. & MUSACCHIO, J. M. (1977). Endogenous opiate receptor ligand: electrically-induced release in guinea-pig ileum. *Science, N.Y.* **195**, 419–420.

RANSOM, B. R. & GOLDRING, S. (1973). Ionic determinants of membrane potential of cells presumed to be glia in cerebral cortex of cat. *J. Neurophysiol.* **36**, 855–868.

REAVES JR, T. A. & HAYWARD, J. N. (1979). Intracellular dye-marked enkephalin neurons in the magnocellular preoptic nucleus of the goldfish hypothalamus. *Proc. natn. Acad. Sci. U.S.A.* **76**, 6009–6011.

SCHULTZBERG, M., HÖKFELT, T., NILSSON, G., TERENIUS, L., REHFELD, J., BROWN, M., ELDE, R., GOLDSTEIN, M. & SAID, S. I. (1980). Distribution of peptide and catecholamine neurons in the gastrointestinal tract of rat and guineå-pig: immunohistochemical studies with antisera to substance P, VIP, enkephalins, somatostatin, gastrin, neurotensin and dopamine $\beta$-hydroxylase. *Neuroscience* **5**, 689–744.

SNEDECOR, G. W. & COCHRAN, W. G. (1967). *Statistical Methods*, 6th edn. Ames, Iowa: Iowa State University Press.

STEWART, W. W. (1978). Functional connections between cells as revealed by dye-coupling with a highly fluorescent naphthalimide tracer. *Cell* **14**, 741–759.

STEWART, W. W. (1981). Lucifer dyes – highly fluorescent dyes for biological tracing. *Nature, Lond.* **292**, 17–21.

SUNDLER, F., HÅKANSON, R. & LEANDER, S. (1980). Peptidergic nervous systems in the gut. *Clinics in Gastroenterology* **9**, 517–543.

TRENDELENBURG, P. (1917). Physiologische und pharmakologische Versuche über die Dünndarmperistaltik. *Arch. exp. Path. Pharmak.* **81**, 55–129.

WATERFIELD, A. A. & KOSTERLITZ, H. W. (1977). Stereospecific increase by narcotic antagonists of evoked acetylcholine output in guinea-pig ileum. *Life Sci.* **16**, 1787–1792.

WATERFIELD, A. A., SMOKCUM, R. W. J., HUGHES, J., KOSTERLITZ, H. W. & HENDERSON, G. (1977). *In vitro* pharmacology of the opioid peptides, enkephalins and endorphins. *Eur. J. Pharmacol.* **43**, 107–116.

WOOD, J. D. & MAYER, C. J. (1978). Intracellular study of electrical activity of Auerbach's plexus in guinea-pig small intestine. *Pflügers Arch.* **374**, 265–275.

## EXPLANATION OF PLATES

### PLATE 1

*A–C*, examples of enkephalin-immunoreactive nerve cells in the guinea-pig myenteric plexus. Bar 20 $\mu$m. *D* and *E*, myenteric nerve cells filled with 5% Lucifer Yellow. Note that both Dogiel Type I and Dogiel Type II cells are shown in *D* together with filled glial cell (arrow). The micrograph in *E* shows a Dogiel Type I cell with club-like processes. Bar in *D*, 50 $\mu$m; *E*, 20 $\mu$m. *F* and *G*, two Dogiel Type I Lucifer Yellow-filled cells (*F*) and, in *G*, the same cells showing rhodamine labelled enkephalin immunoreactivity. Bar 20 $\mu$m applies to both *F* and *G*. *H* and *I*, micrographs of the same field. The Dogiel Type I cell in *H* is filled with Lucifer Yellow but does not show enkephalin immunoreactivity while a non-filled cell in the same field does show enkephalin immunoreactivity (*I*) Bar 20 $\mu$m applies to both *H* and *I*.

### PLATE 2

*A–C*, example of an electrophysiologically identified S cell which belongs to the Dogiel Type I group and shows enkephalin immunoreactivity. The neurone was filled with 0·5% Lucifer Yellow (*A*), a fast e.p.s.p., evoked by transmural stimulation applied 8 mm oral to the cell, was recorded via the same Lucifer Yellow/KCl micro-electrode (*C*), and the enkephalin immunoreactivity was demonstrated with rhodamine fluorescent label (*B*). Bar in *A* and *B* 20 $\mu$m.