Enkephalin Neurons in the Guinea Pig Proximal Colon: An Immunocytochemical Study Using an Antiserum to Methionine-Enkephalin-Arg^{6}-Gly^{7}-Leu^{8}*

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Summary. The distribution and structure of the neurons containing opioid peptide-like immunoreactivity (enkephalin neurons) in the antimesenteric border of the guinea pig proximal colon were immunocytochemically investigated using an antiserum for methionine-enkephalin-Arg^{6}-Gly^{7}-Leu^{8} (R-0171). Whole-mount preparations of the different layers of the intestine perfusion-fixed with Bouin's fluid were immunostained by peroxidase-antiperoxidase techniques. Immunopositive nerve fibers were apparent in the longitudinal muscle layer, myenteric plexus, circular muscle layer and submucosa. Immunopositive perikarya of the ganglionic cells were found in the myenteric plexus. A Golgi-type panoramic view was obtained in the intensely-immuostained enkephalin neurons. Distinct immunoreactivity was shown in the many Dogiel type 1 neurons, characterized by short broad processes (winglets or alulae) and one long axon-like process, as well as a few type 2, characterized by several tapering processes, and type 3 neurons, characterized by dendrite-like processes. Many twig-like processes originated from the free margin of the winglet of the enkephalin neurons (wing-ramuli). A part of them entered the intramuscular fasciculus, while the rest remained inside the ganglion. There were transitional forms between these wing-ramuli and the tapering processes of the type 2 neurons or the dendrite-like processes of the type 3 neurons. The axon-like processes sent out branches (axon-ramuli) along their courses or into the intramuscular fasciculus. At the origin of these axon-ramuli, there was a nodulous or humped swelling of the axon-like process (nodulus or crista). In the myenteric ganglion, the axon-ramuli formed varicose terminals. In the guinea pig proximal colon, many axon-like processes of the enkephalin neurons ran in the oral direction. This polarity of neuronic processes may have a functional significance in the neuronal control of the antiperistalsis.

The occurrence and distribution of various peptide-containing neurons in the enteric nervous system have been extensively studied (FURNESS and COSTA, 1980; FURNESS et al., 1981; KEAST et al., 1984). Investigations of the neurons with opioid peptide-like immunoreactivity (enkephalin neurons) have been performed using sections of the gastrointestinal tissue (POLAK et al., 1977; ALUMETS et al., 1978; LARSSON et al., 1979; SCHULTZBERG et al., 1980; NEANDER et al., 1981; TANGE, 1983, DOMOTO et al., 1984; NIHEI

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and Iwanaga, 1985). These studies revealed the presence of the perikarya, and the processes of the enkephalin neurons in the different layers of the gastrointestinal canal. However, it was impossible to clarify the detailed morphology of individual enkephalin neurons merely through the study of the intestinal sections.

Using whole-mount preparations of the guinea pig small intestine, Furness et al. (1983) investigated the shapes of the enkephalin neurons. From their experimental results, they deduced the destination of the various projections of the enkephalin neurons. Furthermore, Bornstein et al. (1984) injected fluorescent dye, Lucifer Yellow CH, for investigation of the shapes of the enkephalin neurons in the myenteric plexus. They concluded that the myenteric enkephalin neurons are all Dogiel type 1. However, both immunofluorescence and dye-injection methods have an inherent weak point in that repeated and long-term examination of the cytochemical specimens is difficult.

We have recently improved the immunocytochemical method for whole-mount preparations invented by Costa et al. (1980) (Kobayashi et al., 1984). A peroxidase-antiperoxidase method is used in place of the immunofluorescence method. Furthermore, we introduced the perfusion-fixation technique. Thus was obtained immunostained permanent preparations of the guinea pig duodenum, in which we found a Golgi-type panoramic view of the Dogiel type 1, as well as type 2 and 3 enkephalin neurons (Kobayashi et al., 1984). We confirmed the description by Furness et al. (1983), that there were many enkephalin neurons with a single long axon-like process running in an anal direction, which suggested the possibility that these axon-like processes play a role in the neuronal control of the peristalsis.

In the present study, we investigated whole-mount preparations of the antimesenteric border of the proximal colon. This segment of the guinea pig intestine is characterized by the antiperistalsis, i.e. a wave of contraction moving in an oral direction (Elliott and Barclay-Smith, 1904; Hukuhara and Neya, 1968). The immunocytochemical method described in a previous paper (Kobayashi et al., 1984) has been slightly modified. This methodological improvement enabled the visualization of the fine filamentous processes of the enkephalin neurons.

**MATERIALS AND METHODS**

*Anti-opioid peptide serum*

The same anti-methionine-enkephalin-Arg<sup>6</sup>-Gly<sup>7</sup>-Leu<sup>6</sup> (Met-Enk-Arg-Gly-Leu) serum (R-0171) which was used previously (Kobayashi et al., 1983, 1984) was also used in the present study. This antiserum was raised in a mixed-breed female rabbit using Met-Enk-Arg-Gly-Leu (synthesized by Yanaihara et al.) conjugated to ascars protein by the glutaraldehyde method. In radio-immunoassay, this antiserum showed no cross-reaction with α-endorphin, β-endorphin, methionine-enkephalin (Met-Enk), leucine-enkephalin (Leu-Enk), or Met-Enk-Arg-Gly-Leu-related peptides such as H-Arg-Gly-Leu-OH. At a high concentration (more than 2,000 fM), H-Phe-Met-Arg-Gly-Leu-OH was slightly cross-reactive with the antiserum (Kobayashi et al., 1983; Ohse et al., 1984).

*Improved immunocytochemical procedure*

For immunocytochemical localization of Met-Enk-Arg-Gly-Leu, the method described in the previous paper (Kobayashi et al., 1984) was used with slight modifications. Seven Hartley strain guinea pigs of both sexes, 300–490 g in body weight, fed ad libitum, were used in the present study. The animals were anesthetized by an intra-
peritoneal injection of pentobarbital sodium (50 mg/kg b. w.). Heparin (1,000 units/kg b. w.) was intraperitoneally injected to prevent blood coagulation during the preparation of the animal specimens. The anesthetized animals were stunned and bled from severed carotid arteries. Oxygenated Tyrode's solution was perfused through a polyethylene tube inserted into the descending aorta for 3 min at room temperature (40 ml/min) followed by Bouin's fixative for 10 min (50 ml/min). The perfusion-fixed intestinal canal was removed and placed in Bouin's fluid for 2 hrs. Segments of the antimesenteric border of the proximal colon between 4 and 14 cm from the ileocaecal junction were removed and placed in Bouin's fluid. Performing this separation of the intestinal layers in the Bouin's fluid improved the preservation of the enkephalin neurons, as compared with the previous method of separation in a phosphate buffered saline (PBS: 0.01 M, pH 7.2 with 0.14 M NaCl). The proximal colon was separated into layers under the dissection microscope by carefully pulling them apart with watchmaker's forceps. The sheets of the proximal colon thus prepared were immunostained by the peroxidase-antiperoxidase (PAP) method (STERNBERGER, 1979).

Details of the immunocytochemical procedures are summarized as follows:

1) Keep intestinal preparations in PBS with 0.3% Triton X-100 for at least 4 days at 4°C.
2) Wash in PBS for 4-6 hrs at 4°C.
3) Treat with PBS containing 3% H₂O₂ for 7 min at room temperature.
4) Wash, for 10 min, in 5 changes of PBS.
5) Treat with non-immune goat serum diluted in PBS (1/10) for 2 hrs at room temperature.
6) Incubate in a pool (1-2 ml) of the anti-Met-Enk-Arg-Gly-Leu serum diluted in PBS (1/2,000) for 2 days at 4°C.
7) Wash, for 10 min, in 5 changes of PBS.
8) Incubate in goat anti-rabbit IgG serum (Miles, Lot. G283) diluted in PBS (1/200) for 2 hrs at room temperature.
9) Wash, for 10 min, in 5 changes of PBS.
10) Incubate in rabbit peroxidase-antiperoxidase complex (Miles, Lot. S171) diluted in PBS (1/200) for 2 hrs at room temperature.
11) Wash, for 10 min, in 5 changes of PBS.
12) Transfer to 0.05 M Tris-HCl buffer of pH 7.6. Wash, for 10 min, in 5 changes of Tris-HCl buffer.
13) Soak in a freshly prepared solution of 0.02% 3',3'-diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H₂O₂ diluted with Tris-HCl buffer for 30 min to 1 hr at room temperature.
14) Wash, for at least 10 min, in 5 changes of distilled water.
15) If desired, osmication and/or counter-staining with hematoxylin can be performed.
16) Dip in 0.05% gelatin for 1 min at 30°C. Use of concentrated gelatin tends to contaminate the specimen.
17) Place the immunostained layers of the intestinal tissue on a cover glass. Stretch the surface to be observed face down on the cover glass, making sure that the surface does not dry completely. Care should be taken concerning the orientation of the specimen with respect to the long axis of the gut, in order to identify the direction of food flow.
18) Dip in 90% ethanol and then dehydrate in 100% ethanol. Clear in 3 changes
of xylene. Finally, take the preparation fixed on the cover glass, and mount it on a slide glass using Entellan (Merck), in order to obtain a permanent preparation. Care must be taken when turning the cover glass 180° in order to mount on the glass slide.

An Olympus light microscope (Vanox) equipped with a green filter (IF550) was used for examination and photography. Photographs were taken with Kodak Panatomic X-film.

Specificity controls: The following specificity controls were performed in the pieces of the myenteric plexus layer of the proximal colon: 1) phosphate-buffered saline or non-immunized rabbit serum was applied in place of the R-0171 antiserum; 2) an immuno-absorption test was conducted by preincubation of the R-0171 antiserum (1/2,000 in PBS) with Met-Enk-Arg-Gly-Leu (10 or 2.5 μg/ml diluted antiserum), Leu-Enk, Met-Enk or methionine-enkephalin-Arg⁶-Phe⁷ (Met-Enk-Arg-Phe) (10 μg/ml) at 4°C for 24 hrs prior to immunostaining.

RESULTS

Distribution of Met-Enk-Arg-Gly-Leu immunoreactive nerve elements

In the antimesenteric border of the guinea pig proximal colon, Met-Enk-Arg-Gly-Leu immunopositive perikarya of ganglionic cells occurred only in the myenteric plexus. On the other hand, immunostained nerve processes or fibers were found in the longitudinal muscle layer, in the myenteric plexus, circular muscle layer and in the submucous plexus (Fig. 1). There were no immunopositive nerve elements in the mucosa, including the muscularis mucosae.

In the longitudinal muscle layer, the immunopositive nerve fibers formed a loose network. Most of them were thin in caliber (less than 0.3 μm), and possessed small dot-like varicosities. Immunopositive nerve fibers were occasionally seen around the blood vessels (Fig. 1A).

In the myenteric plexus, immunopositive nerve terminals and fibers formed a cluster around both the immunopositive and immunonegative perikarya of ganglionic cells (Fig. 1B, 2). Approximately 30 percent of the total ganglionic cell population of the myenteric ganglia was immunopositive (Fig. 1B). However, the exact percentage of the immunopositive cells was impossible to determine, because the perikaryonic coloration intensity revealed a great range in variation from cell to cell, and because many ganglionic cells showed an ambiguous immunoreaction (Fig. 2). An extremely strong immunostaining was obtained in a few ganglionic cells (vide infra). In the primary fasciculi of the myenteric plexus, there were both immunopositive and immunonegative nerve fibers of various thicknesses (Fig. 1B). The secondary and tertiary fasciculi contained immunopositive nerve fibers (Fig. 1B).

In the circular muscle layer, immunopositive nerve fibers were distributed throughout its thickness, forming an elaborate network (Fig. 1C). However, there was no such extremely-dense network in the guinea pig proximal colon as that seen in the deep myenteric plexus of the duodenum (KOBAYASHI et al., 1984).

The submucous plexus consisted of meshworks of nerve fiber bundles and ganglia (Fig. 1D). In the proximal colon, the ganglia appeared to be generally larger in size than those in the duodenum (KOBAYASHI et al., 1984). The tangled lattice of the primary, secondary and tertiary fasciculi was slightly denser than that in the duodenum (KOBAYASHI et al., 1984). The supply of the immunopositive nerve fibers to the sub-
mucous plexus was considerably looser than that of the circular muscle layer. Many nerve fiber bundles were devoid of immunopositive structures (Fig. 1D). In some of the submucous ganglia, there were small numbers of nerve fibers or terminals with immunopositive varicosities. They seemed to make a bouton en passage-type synapse with ganglionic cells (Fig. 1D). No immunopositive perikarya of ganglionic cells were found in the submucous ganglia.

In the submucosa, there was a diffuse network of branching arterioles. Very few nerve fibers in their perivascular plexus showed the positive immunoreactivity (not illustrated).

Shapes of the enkephalin neurons in the myenteric plexus

Nearly complete panoramic views of individual enkephalin neurons were frequently obtained when the immunocoloration of the cell was strong, and not many immunopositive obstacles lay around it. Examples of these panoramic views of the strongly-
immunostained enkephalin neurons are offered in Figure 4 A-Z in a shadow picture.

Shapes of enkephalin neurons in the myenteric plexus corresponded in general to those of the myenteric neurons described by Dogiel (1896, 1899). Thus, for the convenience of description, the strongly-immunopositive enkephalin neurons were temporarily classified into three categories: 1) Dogiel type 1 neurons, characterized by short, broad processes and a single, long axon-like process; 2) Dogiel type 2 neurons, showing a smooth-surfaced perikaryon and several sharp, tapering processes and axon-like process; 3) Dogiel type 3 neurons, with dendrite-like processes branching around other ganglionic cells at one end and a single axon-like process at the opposite end.

There were many strongly-immunopositive neurons which showed various intermediate features among the Dogiel’s three neuron types. Thus, morphological classification of a considerable number of enkephalin neurons was impossible.

**Dogiel type 1**: The majority of the intensely-immunostained enkephalin neurons were classified as being type 1 cells since they had cytoplasmic winglets around the perikaryon and a long axon-like process. As shown in Figure 4 A-Z, the apparent size of the immunopositive neurons differed greatly from cell to cell (compare Fig. 4D and Fig. 4K).

The shapes of 175 type 1 enkephalin neurons were carefully examined. All projected a single axon-like process from the oral, circumferential or anal surface of the perikaryon. Of the 175 axon-like processes, 28 (16.0%) were not traceable beyond the ganglion of origin. Of those which went outside the ganglion, 99 (67.3%) ran orally, 39 (26.5%) ran circumferentially and 9 (6.1%) ran anally. In the type 1 neuron shown in Figure 3, the orally directed axon-like process extends over at least three ganglia. Twenty-four axon-like processes were carefully traced. They measured \(0.59 \pm 0.24\) mm (S. D.) in length (the longest, 1.18 mm; the shortest, 0.20 mm). A knee-like flexure of

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**Fig. 2.** Met-Enk-Arg-Gly-Leu immunopositive nerve elements in a myenteric ganglion. Intensity of the immunostaining varies from cell to cell. Varicose nerve terminals are present in the spaces between ganglionic cells. Nuclei of the ganglionic cells are indicated by *n*.

\(\times 930\)
the axon-like process (genu) was frequently encountered (Fig. 4C, K, O, P, Y). In the enkephalin neurons shown in Figures 4B, E, N, O, T, W, Y, Z and 7 A, B, the axon-like process projected a group of fine filamentous processes (axon-ramuli) of a relatively uniform caliber into the intramuscular fasciculi. At the origin of these ramuli, the axon-like process formed a nodule or humped swelling. Several ramuli were projected along the axon-like process, as shown in Figure 8 A–C. A few of these intrafascicular axon-ramuli were traced to the adjacent ganglion. They entered the space between the ganglionic cells and made a series of strongly-immunopositive boutons. The terminal of the axon-like process was not identified in the present study. However, an intimate connection between the strongly-immunostained swellings of the main trunk of the axon-like process and both immunopositive and immunonegative ganglionic cells was frequently encountered (Fig. 2, 8D).

In addition to the axon-like process, the type 1 neurons had numerous short, broad processes (ramulous winglets or alulae). They were usually projected from the inner surface of the type 1 neurons; thus, practically no winglets were demonstrated on the surface of the enkephalin neurons where they were at the ganglionic surface (Fig. 5A). A few winglets were seen on the initial portion of the axon-like process (Fig. 5A, B). Here, a fimbria-like row of winglets sometimes occurred (Fig. 5C). There were many immunopositive nerve terminal varicosities on the winglets (Fig. 5C, 6 Inset a).

The winglets of the type 1 neurons were ramulous. Many twig-like processes (wing-ramuli) originated from the free margin of the winglet (Fig. 5A, 6A). A group of several ramuli emanating from one winglet entered into the circular muscle layer and joined in the elaborate network of immunopositive and immunonegative nerve fibers (Fig. 4B, T, Z). However, a large proportion of these wing-ramuli ran inside the ganglia and fasciculi. The terminals of the wing-ramuli were not found in the present study.
Dogiel type 2: Immunopositive Dogiel type 2 neurons were the smallest in number of the three enkephalin neuron types.

In addition to the single axon-like process, the type 2 neurons possessed more than one tapering processes. On their very initial portion, there were one or more occasional winglets which were indistinguishable from those on the type 1 neurons (Fig. 6B). Some of these winglets projected a few thin ramuli, whose destination was not clear.

The tapering processes of the type 2 neurons radiated from the perikaryon. These were thinner than the axon-like process, but considerably thicker than the ramuli which originated from the free margin of the winglet. Usually, they could not be traced beyond the ganglion of origin.

Dogiel type 3: The type 3 neurons, regularly possessed, as did the type 1 neurons, a small number of ramulous winglets on their perikaryon. The single axon-like process possessed a few noduli or crista from which a few axon-ramuli emanated in a group (Fig. 4O, 7B).
Fig. 5. A–C. Type 1 enkephalin neurons of the guinea pig proximal colon. A. Camera lucida drawing of this cell is shown in Figure 4R. Winglets (*) with ramuli (thin arrows) and the axon-like process are clearly seen. The immunopositive fiber indicated by the thick arrow belongs to another neuron. There are no winglets on the surface of the cell where it is at the ganglionic surface (arrowheads). ×1,300. B. Camera lucida drawing of this cell appears in Figure 4F. The vertically running line indicated by f is an intramuscular fasciculus. ×1,100. C. Camera lucida drawing of this cell is shown in Figure 4A. A fimbria consisting of ramulous winglets (*) decorates the initial portion of the axon-like process. An axon-ramulus originates from the nodulus indicated by the arrow. The black dot labeled s represents a nerve terminal varicosity which makes a synapse with this enkephalin neuron. ×1,000
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Fig. 6. A and B. Ramulous winglets and the long-type tapering processes of the enkephalin neurons. A. Enkephalin neuron of an unknown type whose camera lucida drawing is shown in Figure 4V. The axon-like process was broken during specimen preparation. A higher magnification of the winglet indicated by * is shown in the Inset b. Several wing-ramuli emanate from this winglet. Met-Enk-Arg-Gly-Leu immunopositive nerve varicosities make synapses with this winglet as shown in Inset a. The tone of each picture was altered in order to show the different structures. ×1,400, Inset a: ×1,200, Inset b: ×2,500. B. Type 2 enkephalin neuron whose camera lucida drawing appears in Figure 4J. Arrows indicate the characteristic tapering processes. ×1,200
One of the most distinguishable characteristics of the Dogiel type 3 neurons was the dendrite-like process. This ran in the narrow space between ganglionic cells. However, the destination of these dendrite-like processes was not determined (Fig. 7B). It was frequently difficult to distinguish between the dendrite-like processes and the wing-ramuli.

**Unclassified enkephalin neurons:** Enkephalin neurons showed a wide range of shapes;
many of them could not be classified into any one of the three neuron categories. Examples are as follows:

1) The enkephalin neurons shown in Figures 4C, D possess smooth surfaced perikaryon and a single axon-like process. They possess neither the ramulous winglets characteristic to the type 1 and 3 neurons, nor long, tapering processes, characteristic to the type 2 neurons.

2) The large enkephalin neuron shown in Figure 4H appears to be type 2. Careful examination confirmed the continuity of the perikaryon and the tapering processes. However, there is a possibility that this profile of the enkephalin neuron is composed of overlapping multiple cellular elements.

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**Fig. 8.** A–D. Branchings and varicosities of the axon-like process of enkephalin neurons. A and B. Branches of axon-like process running in the primary fasciculus of the myenteric plexus, which sends out twigs (axon-ramuli) at the noduli indicated by the arrows. \( \times 1,500 \). C. Main trunk of an axon-like process running in the primary fasciculus of the myenteric plexus. At the nodulus indicated by the arrow, this axon-like process projects at least two ramuli \( (r) \). \( \times 2,100 \). D. Terminal varicosities of the axon-ramuli in the myenteric ganglion. Synapse formation between the intensely immunostained varicosities and a weekly-immunostained enkephalin neuron is seen in the center of this micrograph \( (s) \). \( \times 1,100 \)
3) The enkephalin neuron shown in Figure 4I resembles the type 1 neuron. However, it is devoid of the axon-like process.

4) The perikaryon of the enkephalin neuron shown in Figure 4L is of a Y shape. Each of the three projected processes is of a different thickness.

**Nerve terminals in the myenteric and submucous plexus**

Strongly-immunopositive nerve terminals were disseminated in both the myenteric and submucous plexus (Fig. 2, 8D). In the myenteric plexus, they were in close contact with the immunopositive as well as immunonegative perikarya of ganglionic cells. However, in the submucous plexus, there were only immunonegative cells (Fig. 1D). Several immunopositive nerve terminals were connected into a row by a thin, weakly-immunopositive nerve fiber (Fig. 8D). The overlapping of the immunopositive varicosities and the winglets of the enkephalin neurons were frequently seen in the myenteric plexus (Fig. 5C, 6A, 8D). There was no network formation of the beaded nerve fibers in either the myenteric or submucous ganglia.

**DISCUSSION**

The present study of the guinea pig proximal colon revealed that the myenteric ganglia contained a large number of perikarya of ganglionic cells which had an opioid peptide-like immunoreactivity. Immunopositive nerve fibers and terminals were demonstrated in the longitudinal muscle layer, myenteric plexus, circular muscle layer, and in the submucous plexus. The perivascular plexus contained a limited number of immunopositive nerve fibers. The results of the specificity control experiments showed that the immunoreaction was specific to Met-Enk-Arg-Gly-Leu. Therefore, it is probable that the enkephalin neurons of the guinea pig proximal colon, like those of the duodenum (KOBAYASHI et al., 1984), contained this opioid octapeptide, which is characteristic only to the preproenkephalin A molecule among the three known opioid peptide precursors (KAKIDANI et al., 1982).

The shape of the enkephalin neurons deduced from the present investigation is illustrated in Figure 9. The features of their processes are summarized as follows:

1) The axon-like process rapidly tapers, projecting groups of ramuli into the intramuscular fasciculus. These axon-ramuli also run in the internodal fasciculus and ganglion. There is a nodulus or crista at the origin of the axon-ramuli. In the myenteric ganglion, both the main trunk and branches of the axon-like process frequently make a series of bead-like varicosities. The main trunk of the axon-like process may enter the submucous ganglia after penetrating through the circular muscle layer. However, such a phenomenon could not be ascertained in the present study. The true terminals of the axon-like process are unknown.

2) The ramulous winglets or alulae are regarded as a site of synapse formation, because many nerve terminal varicosities lie on them. Groups of fine filamentous processes emanate from the free margin of the winglet. These wing-ramuli enter the intramuscular fasciculus, or remain within the ganglion. However, their terminals remain to be elucidated.

3) The sharp tapering processes of the type 2 neurons vary in thickness. The winglets regularly adhere to their initial portion. It is frequently difficult to differentiate between the thinner tapering processes of the type 2 neuron and the thicker wing-ramuli of the type 1 and 3 neurons. The thicker tapering processes of the type
2 neuron are sometimes indistinguishable in morphology from the axon-like processes of the smaller type 1 and 3 neurons.

4) The dendrite-like process of the type 3 neurons enters into the space between the ganglionic cells, however its terminals remain unknown. There are various transitional forms between the dendrite-like processes of the type 3 neurons and the wing-ramuli of all the three neuron types; in particular, the distinction between the dendrite-like process of the type 3 neurons and the sharp tapering process of the type 2 neurons is uncertain.

The use of reliable histological techniques following strict criteria is essential for the interpretation of the shapes of the enteric neurons. DOGIEL (1896, 1899) used methylene blue staining for his well-known investigations. FURNESS et al. (1983) performed their experiments using an immunofluorescence method, and deduced the shapes of enkephalin neurons. BORNSTEIN et al. (1984) studied the shapes of myenteric plexus neurons by an intracellular injection of fluorescent dye and correlated these shapes with the enkephalin immunoreactivity of the neurons. However, none of the previous authors visualized such frequent connections between the wing- and axon-ramuli and the intramuscular fasciculi as noted in the present study.

FURNESS et al. (1983) and BORNSTEIN et al. (1984) stated that all the enkephalin neurons seem similar in morphology and are type 1 according to Dogiel's classification. However, in the present study, we have demonstrated that not only the type 1 but also types 2 and 3 neurons possess an opioid peptide-like immunoreactivity. One possibility may be that the discrepancy between past investigations and the present investigation is due to a difference in the interpretation of Dogiel's criteria. DOGIEL (1896, 1899) did not illustrate the fine filamentous processes originating from both axon-like processes and winglets of the enteric neurons. As revealed in the present study, there are transitional forms between the wing-ramuli of all three classified neuron types and the tapering processes of the type 2 neurons or the dendrite-like processes of the type 3 neurons. We propose that the criteria of Dogiel's three neuron types are not absolutely specific to each neuron type, and that the reexamination of the morphological criteria themselves is needed.

DOGIEL (1896, 1899) proposed that type 1 neurons are motor in function, whereas the type 2 neurons are sensory. However, the question of the functional differentiation of the three types of the enkephalin neurons needs further consideration and investigation. It is the authors' opinion that Dogiel's simple correlation between morphology and physiology cannot be applied to the analysis of the unknown functions of the different morphological types of enkephalin neurons.

BORNSTEIN et al. (1984) reported that the S neurons, which electrophysiologically produced fast excitatory synaptic potentials, were Dogiel type 1 in the shape and contained opioid peptides. They further reported that the AH neurons, which exhibited a slow after-hyperpolarization following the action potential, were Dogiel type 2 and showed no opioid peptide-like immunoreactivity. However, it must be pointed out that the electrophysiologists removed muscular tissues when they performed their intracellular recording of the myenteric neurons. Thus, they could have destroyed most, if not all, of the axon- and wing-ramuli of the enkephalin neurons together with the muscular tissues. The results of the present study suggest that caution must be taken in interpretation of the electrophysiological data of the enkephalin neurons, as the removal of those intramuscular axon- and wing-ramuli may change the electrical properties of the enkephalin neurons.

In the guinea pig proximal colon, a large proportion of the long axon-like processes
of the type 1 enkephalin neurons ran in the oral direction. This observation differs from that in the small intestine where most of these axon-like processes were anally directed (Furness et al., 1983; Kobayashi et al., 1984). In their study on the serotonin neurons in the guinea pig small intestine, Furness and Costa (1982) suggested that the anally directed axon-like processes play a role in the neuronal control of the peristaltic movement. A similar assumption may be applicable to the enkephalin neurons (Kobayashi et al., 1984). Thus, the occurrence of the orally-directed axon-like processes
in the present material may be correlated with the antiperistalsis which regularly takes place in the guinea pig proximal colon (Elliott and Barclay-Smith, 1904; Hukuhara and Neya, 1968). Physiological studies are required to establish the functional significance of the orally directed axon-like processes of the enkephalin neurons.

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