CYTOCHEMICAL DETECTION OF NADH-DIAPHORASE POSITIVE NERVE CELLS IN THE ALIMENTARY CANAL OF FROG

R. GÁBRIEL[1], K. HALASY[1] and M. CSOKNYA[2]

[1]Department of Zoology, Attila József University
H—6701 Szeged, P.O.B. 659, Hungary
[2]Department of Zoology, Janus Pannonius University
Pécs, Hungary

Abstract

Authors studied the nerve cells of the myenteric plexus of the frog alimentary canal using the NBT/NADH-diaphorase technique, as well as silver impregnation and transmission electron microscope. It was concluded that the nerve cells form groups of ganglia in the stomach; on the contrary they show dispersed localization in the small intestine, while both ganglionic and solitary neuronal arrangements are detectable in the transitional duodenal section. The size of the nerve cells varies through the length of the intestinal canal (50—200 μm²). The myenteric plexus is situated in the connective tissue of the subserosa, in this way differing from the higher Vertebrates plexuses. The neuronal density ranges from 600 to 1100 cells/cm² throughout the lenght of the alimentary canal. The organization of the cellular elements shows a transition between the innervation by dispersed nerve cells and the enteric nervous system of ganglionic arrangement.

Key words: frog intestine, myenteric plexus, NBT/NADH diaphorase, "whole mount", cell count

Introduction

The structural organization of the myenteric plexus has been studied by several authors at various sections of the alimentary canal (FILOGAMO and VIGULIANI, 1954; GABELLA, 1971; IRWIN, 1931; LEAMING and CAUNA, 1961; OKHUBO, 1936a and b). The studies firstly deal with the innervation of the mammal intestine, and in this relation also give account on cell count data referring to area unit. Studies of this kind have been launched by the NADH-tetrasolium reductase technique, introduced by GABELLA, (1969). This technique electively stains the nerve cells with good reproducibility, and contrary to the classical staining procedures, demonstrates practically every nerve cell. In the possession of this specific and highly effective technique, it seemed obvious to study the myenteric plexus of the frog *(Rana esculenta L.)* enteric nervous system, since literary data are rather scant in respect to its organization — particularly in regard to the upper sections of the intestinal canal (GUNN, 1951). The aim of our studies was to examine the myenteric plexus of the frog intestine from morphological aspect, and within this, by means of the detection

and counting of the NADH-diaphorase positive nerve cells, we wished to obtain new (comparative) data on the innervation of the studied intestine sections.

Materials and methods

Mature male and female adult frogs *(Rana esculenta* L.) were used in our studies. The method of GABELLA (1969) was used for the histochemical reaction. The animals were decapitated and the distal end of the intestinal canal was tied down, then the intestine was filled up with Krebs-solution. After filling up, the other end of the intestine was also tied down, then the whole intestinal tract was placed into 0.3% Triton-solution, followed by washing for 2x5 minutes. Then the filled up intestine was placed into incubating solution, the composition of which was the following: 2.5 mg of NBT dissolved in 5 ml of tridistilled water, 5 ml of 0.1 M phosphate buffer (pH 7.3), 10 mg of NADH dissolved in 10 ml of tridistilled water. The intestine was kept in the incubating solution till the appearance of the dark blue colour reaction (40—60 min). The fixation of the samples in 10% neutral formaline lasted for at least 12 hours. Using safety razor blade, about 1 cm slices were cut from the intestine, perpendicularly to the longitudinal axis, and "whole mount" specimens were prepared from the slices. During the preparation of the specimens it turned out that the complete muscle layer (tunica muscularis) could be removed without damage to the nerve plexus.

The method of BIELSCHOWSKY-GROS-CAUNA was used for the silver impregnation (LEAMING and CAUNA, 1961).

For the purpose of electronmicroscopic studies, transcardial perfusion was performed with fixative containing 1% glutaraldehyde and 2% paraformaldehyde. 0.2 M Na-cacodylate buffer was used for the preparation of the fixative. Following 2 hours of immersion post-fixation, the material was washed in 0.2 M phosphate buffer containing, 7.5% saccharose. 2% buffered osmium was used for osmification, then the material was dehydrated in ascending alcohol series. During dehydration "block contrasting" was performed in the solution of 75% alcohol saturated with uranylacetate. Using propylene-oxide intermedium, the samples were embedded in Durcupan. The ultrathin sections were also contrasted with lead citrate according to REYNOLDS. The electronmicroscopic figure was prepared by Tesla BS 540 electronmicroscope.

Results

The light microscopic studies of the specimens prepared by the NADH-diaphorase cytochemical reaction showed the morphology of the myenteric plexus to be strongly variable throughout the length of the intestinal canal. Using the present method to study the esophagus could not be accomplished since the proximal end of the intestine could only be tied down on the account of this section of the intestine.

The nerve cells form groups of ganglia at the area of the stomach (Fig. 1), with 3—10 neurons occupied in each ganglion. The average distance between the ganglia is about 100 μm, but distances of only 30—40 μm were also found between some neighbouring ganglia. The ganglia of the stomach are generally polygonal. The section of the duodenum close to the stomach assumes an interesting appearance from the viewpoint of neuronal arrangement. Solitary neurons are frequent here, with the simultaneous occurrence of ganglional arrangement (Fig. 2). The size of the cells shows great variation. The cell profile ranges from 50 to 200 μm^2 in case of both

the stomach and the proximal end of the duodenum. In the stomach, about 50% of the cells are large: more than 150 μm^2, one third are small (50—75 μm^2), while the remaining parts show moderate profile. From the processes of the nerve cells, generally only onė can be followed till a greater distance from the cell body (Fig. 3a). The histochemical reaction causes well detectable granulation in the plasma of the nerve cells, and it can be observed well close to the cell body, in the initials of the processes (Fig. 3b).

The ganglionic arrangement of the nerve cells disappears completely at the distal part of the duodenum as well as in the small intestine: the cells are arranged sporadically without showing even distribution (Fig. 4). Here the localization of neurons from each other is at distances of 25—75 μm.

In the environs of the blood vessels entering from the mesenterium, the neuronal density of the gut is higher then on the antimesenterical side. The profile of the neurons is small (50—75 μm^2). A few larger neurons are also occassionally observable at the distal end of the duodenum and in the small intestine, but these are consequently found among the entering of mesenterial vessels (Fig. 5a). The size of these neurons surpasses that of the large neurons of the 200—250 μm^2 profile. These large neurons are multipolar (Fig. 5b). The neurons were found in the sections of duodenum and small intestine, resp., had one or two processes (Fig. 6).

The neurons were counted per intestinal section, and the results obtained are given in Table 1. The number of neurons per area unit differs according to various intestinal sections. The lowest amount was found at the initial sections of the stomach (619 cells/cm^2), while an increase was manifested at the pylorus section (948. cells/cm^2).

The duodenum cell count showed slight decrease compared to the pylorus (772 cells/cm^2), but the rest of the small intestine parts was found to be more abundant in nerve cells: the number of cells was found to be the highest here from the studied sections (about 1000 cells/cm^2). At the same time, no significant difference was manifested in respect to cell number between the proximal and distal ends of the small intestine, as observable in the column diagram (Fig. 7).

The results of the NADH-diaphorase procedure serving for the detection of the neurons evidence the majority of the neurons are localized on the outer surface of the tunica muscularis. This arrangement has also been verified by the results of the silver impregnation and electron microscopic studies. The silver impregnation light microscope figure (Fig. 8b) shows that the myenteric plexus is situated on the outer surface of the muscle layer, and this is supported by the figure prepared from the specimen labelled by the diaphorase technique (Fig. 8a). On the electron microscopic picture (Fig. 9) this arrangement is even clearer: the edge of the smooth muscle layer; the centrally located neuron and the neuropile in the direct neighbourhood; furthermore, the wide connective tissue layer of the consecutive subserosa, mainly composed of collagen fibers are all well recognizable in the left upper corner of the Figure.

Discussion

It is evident from both literary data (GABELLA, 1969; 1971; 1979) and our study results that the histochemical detection of NADH-diaphorase activity is suitable for the selective demonstration of the nerve cells, for the determination of neuron number, as well as for studies on the cellular arrangement in the frog intestinal canal.

The localization of the myenteric plexus is somewhat different from that is usual in the case of mammal species. The cellular elements are situated on the outer side of the tunica muscularis, while in the case of higher Vertebrates the usual local-ization is between the circular and longitudinal layers of the muscle sheat. The cause of the deviation might be the weak development of the longitudinal muscle layer, since this layer stands of only 1—4 cell rows in frogs, thus the axons necessary for innervation can easily penetrate through it. Our observations regarding the shape of the diaphorase-positive nerve cells is in correlation with the data of GUNN, (1951) obtained by the silver impregnation method, according to which both small and large cells are found in the small intestine; furthermore, the localization of the large cells rarely detectable at this site is also thought by this author to be in the surroundings of the large veins entering from the mesenterium. There have been no data so far concerning to the arrangement of the nerve cells in the frog stomach. Our studies have proved on the one hand the ganglional arrangement, on the other hand that the small and large neurons in the stomach ganglia are found simultaneously beside each other.

DOGIEL (1896, 1899) classified the vegetative neurons on the basis of their processes. Nowadays, however, it seems more expedient to take the cell size into consideration too, according to the suggestion of LEAMING and CAUNA, (1961) as well as GUNN, (1959, 1968). Based on newer studies, a third basic cell type can also be found in the myenteric plexus of the chicken intestinal tract (CSOKNYA and BENEDECZKY, under publication).

Gunn also had made an attempt to differentiate the small and large neurons from the viewpoint of transmitter content, however, she could not achieve un-ambiguous results (GUNN, 1968). Newer studies (VINCENT et al. 1983a and b, 1986) have shown that the comparison of the results of the diaphorase reaction with those of the acetylcholine-esterase reaction can successfully be applied in the central nervous system. Performing the reactions separately, the neurons giving both histo-chemical reactions could be identified on consecutive sections. Supporting the result of the acetylcholine-esterase reaction with choline-acetyltransferase reaction, VINCENT et al. (1983a) came to the conclusion that the NADPH-diaphorase posit-ivity of certain central nervous system structures assumes cholinergic transmission. In their other studies, however, these authors could not unambiguously support this statement (VINCENT et al. 1983b). In a recent publication, VINCENT et al. (1986) have been successful in proving the coexistence of the cholinergic-peptidergic system in respect to the ascending reticular cholinergic systems. In our opinion, both the cholinergic and adrenergic neurons of the intestinal myenteric plexus became

stained during the course of our experiments. This statement is supported by the fact, that in case of consecutive diaphorase- and acetylcholinesterase reaction performed on the same "whole mount" preparation, such cells are found that show only diaphorase-activity (GÁBRIEL, unpublished data).

We could not find any literary data on nerve cell count concerning the frog intestine. Our data show 2—7 times less cells per intestinal section than the summarizing data of GABELLA, (1979) related to mammal species. There are no data, however, on the tissue volume innervated by certain ganglia or cells. Calculations in this respect are rather difficult since the muscle layer is not of the same thickness even throughout the same intestinal section. On the other hand, distending with Krebs-solution produces the elongation of the intestinal muscle cells. Therefore, these do not show their original volume during the course of measurement, and preliminary measurements are not possible owing to the filling up the whole intestine.

Our present results suggest that the development of the myenteric plexus of the frog intestine shows an intermediary state phylogenically at the various sections of the intestine: the myenteric plexus of the stomach is ganglionic, the small intestine contains sporadically arranged nerve cells. In case of chicken as well as mammals, it is known (DOGIEL, 1896, 1899) that the whole myenteric plexus shows ganglial arrangement. The alimentary canal of the developing chicken and mammal, however, does not show clearly this kind of arrangement, since the cells still migrate and there is also an increase in their absolute number with further development (GABELLA, 1971).

Our results obtained with silver impregnation and transmission electron microscope have unambiguously supported our assumptions regarding the localization of the plexus, according to which the nerve plexus is not localized at the "usual" place between the two muscle layers, but rather outside this, in the subserosa.

The morphological and functional significance of this characteristic localization is to be clarified by further studies.

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Table 1. Results of the cell count

Section of the intestine	cell count (total)	area (mm^2)	cells/unit (nu./cm^2)	average
stomach cardia	524[a]	72	728	
	523[b]	104	503	619
	634[c]	101	627	
stomach pylorus	241[a]	21	1100	
	445[b]	48	927	948
	393[c]	48	818	
duodenum	363[a]	43	844	
	462[b]	66	700	772
small intestine proximal	367[a]	45	814	
	379[b]	35	1083	966
	210[c]	21	1000	
small intestine distal	351[a]	36	975	
	124[b]	12	1033	1004

The labellings a, b, c mean the cell count data of the total amount of specimens prepared from three different experimental animals.

Legend to figures

Fig. 1.   The majority of the nerve cells in the stomach form groups of ganglia (arrow.) 200 x

Fig. 2.   Both ganglional and solitary neurons are observable in the transitional section of the stomach-
duodenum. 200 x
G: ganglion, S: solitary cell

Fig. 3a.   In general, the cells possess well detectable processes. 400 x

Fig. 3b.   Strong granulation can mainly be observed in the neuronal processes (arrow.) 800 x

Fig. 4.   The neurons of the small intestine do not form groups of ganglia and do not show even distri-
bution (arrows: cells). 200 x

Fig. 5a.   Large neurons can be seen (arrows) in the surroundings of the vessels entering from the
mesenterium. 200 x

Fig. 5b.   Several processes are recognizable (arrows) projecting from the large cells. 800 x

Fig. 6.   The small neurons mostly possess 1 — 2 processes at the area of the small intestine.
The processes are indicated by arrows. 200 x

Fig. 7.   On the column diagram the neuron density of the intestinal canal can be seen according to
the various sections of the intestine.

Fig. 8a.   The plexus is localized outside the muscle layer: smc: smooth muscle cells, n: neuron. 200 x

Fig. 8b.   The plexus (indicated by arrows) is situated at the outer edge of the muscle layer, beneath the
connective tissue limiting the intestine from outside. 200 x

Fig. 9.   Detail of the myenteric plexus. In the upper left corner of the Figure the detail of a smooth muscle
cell (smc) is observable, with a neuron (n) in the neighbourhood, close to which a neuropil (np)
can be seen. The thick layer of the subserosa, packed with the collagen fibres (co), is situated near
the outer surface of the intestine. 25 000 x