



Research article

MORPHOLOGY OF THE MYENTERIC PLEXUS AND NANC TRANSMISSION IN THE RAT COLORECTAL REGION

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*The enteric nervous system exerts local control on the gastrointestinal motility, exocrine and endocrine secretions, ensures proper blood supply, and modulates the immune and inflammatory processes in the gut. The aim of this paper is to give insights into current advances and present knowledge about morphology of the myenteric plexus and the neurochemical coding of adult rat myenteric neurons in the large intestine with a special emphasis on non-adrenergic, non-cholinergic (NANC) transmitters. Major neuroactive substances involved in the NANC transmission include nitric oxide (NO), tachykinins, mostly substance P (SP), and adenosine triphosphate (ATP), co-localized in variable proportions with other neuroactive substances. After applying NADPH-diaphorase enzyme histochemistry and neuronal NO synthase (nNOS) immunohistochemistry, we were able to demonstrate numerous nitrergic somata of myenteric neurons with Dogiel type I morphology. In addition to the observed nitrergic distributional patterns, significant variations were registered in the staining intensity of myenteric structures in the colon and anorectal area. Immunohistochemistry for SP revealed an abundance of intensely stained SP-immunoreactive varicose fibers, ensheathing the immunonegative myenteric neuronal cell bodies in a basket-like manner. ATPergic structures were mostly limited to fiber bundles surrounding unstained myenteric neurons and penetrating the two muscle layers. An important finding was the notable presence of purinergic immunoreactive nerve fiber bundles in the longitudinal muscle layer. In conclusion, our results show that the cytoarchitecture and morphometric properties appeared to mimic their equivalent in the more proximal parts of the rat digestive tract and in other experimental animals. **Biomed Rev 2023; 34: 139-153***

Keywords: colorectal region, myenteric plexus, non-adrenergic, non-cholinergic (NANC), rat

Received 12 November 2023, accepted after revision 29 November 2023.

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INTRODUCTION

One of the most remarkable features of the enteric nervous system (ENS) is the incredibly complex system of connections between nerve cells that have the most diverse morphological and functional affiliations. This structure allows it to not only manage its various functions but also to achieve a remarkable autonomy in terms of regulation by the central nervous system (CNS) that is unlike any other body system. The myenteric (Auerbach) plexus is a network of small myenteric ganglia and their connecting interganglionic strands, located between the circular (CM) and the longitudinal layer (LM) of the muscularis externa. As adrenergic transmission has not been shown to have functional significance in the ENS (1), it is widely accepted over time that there are two major transmitter systems in the ENS: non-adrenergic, non-cholinergic (NANC) transmission. The latter includes a broad range of transmitters, often classified into three major groups: peptides (tachykinins, vasoactive intestinal polypeptide, etc.), purines, e.g. adenosine triphosphate (ATP), and gases, such as nitric oxide (NO).

The structure of the myenteric plexus has been investigated in mammals and some rodents, but systematic morphological and neurochemical studies in the rat are inconsistent. The studies done so far are primarily based on wholemount preparations, which are informative but also often difficult to apply in diagnostic practice. Furthermore, studies in the field of neurochemical affiliation of myenteric neurons in the rat are still insufficient, and comparative studies on differences in the expression of NANC transmitters in the colorectal region are notably lacking.

Therefore, the aim of the present paper is to characterize the myenteric plexus morphology on tissue sections, with a particular focus on the neurochemical features of the NANC transmission in the rat colorectal region. For that purpose, the fundamental morphological characteristics of the myenteric plexus on tissue sections will be examined. Secondly, the presence and distribution of myenteric neurotransmitters in the colorectal region will be addressed, and the results compared with existing data of enteric transmitter systems. Furthermore, a statistical analysis will be performed on the regional differences of transmitter expression in the colorectal region and their potential functional role in the distal interstitial tract will be analyzed.

1.1. Structural organization of the myenteric plexus

Conventional histological techniques were used to study the neuromorphology of the myenteric ganglia on tissue sec-

tions through the intestinal wall. In this regard, three planes of tissue sections were used: cross, longitudinal and oblique (tangential). In the first two types, the plane of the cut passes at a right angle through the muscle sublayers and the interposed myenteric plexus. In tangential sections, it passes relatively parallel to the plane of the muscle and plexus, which had a significant impact on the way in which ganglia and their constituent parts were visualized.

In all parts of the colorectal region, the ganglia showed similar morphological features. They were located strictly in the cleft-like border area between the CM and LM muscle (Fig. 1-3). No muscle fibers were found to be separated from one layer and “added” to the other. The myenteric ganglia had highly variable dimensions. By means of visual estimation, the largest size exceeded 500 µm along their long axis. Their shape was oval to elongated with the long axis oriented parallel to the intestinal wall. Along their vertical axis, they contained between 1 and 5 rows of cells, no more than one of which was a neuron. Inside the ganglia, we found three main structural elements - neuronal bodies, glial elements and fiber bundles. The neuronal number varied and in the larger ganglia reached up to 8.

On the H&E stains, the neuronal cytoplasm was granular and basophilic (Fig. 1 A, B). The nuclei were relatively large, rounded, and hypochromic, often with a distinct nucleolus. The other cell type detected were noticeably smaller in size, corresponding in structure to glial cells. Their nuclei were slightly elongated and heterochromatic. The extracellular space was filled with loosely arranged fibrous tissue. No myelinated fibers were observed therein.

The other non-specific stains used in microscopy also helped in elucidating the morphology of the ganglia and the neurons therein. The Nissl staining with cresylviolet showed that the neuronal cytoplasm was finely granulated by the Nissl bodies (Fig. 1-C). The circular, well-contoured nuclei were unstained, while the nucleoli were positive and notable. The small, elongated nuclei of the enteric glia also stood out well. Only in limited ganglia, a fine reticular network, probably corresponding to bundles of nerve fibers, was discernible. The muscularis externa was generally unstained, except for the muscle cell nuclei, which showed medium staining intensity. In the toluidine blue staining the cytoplasm of the myenteric neurons had a finely granular appearance, stained in native dark blue color (Fig. 1D). The contours of the unstained nucleus, and the nucleolus were clearly distinguishable. In some specimen, an intensely stained structure with a fibrous appearance

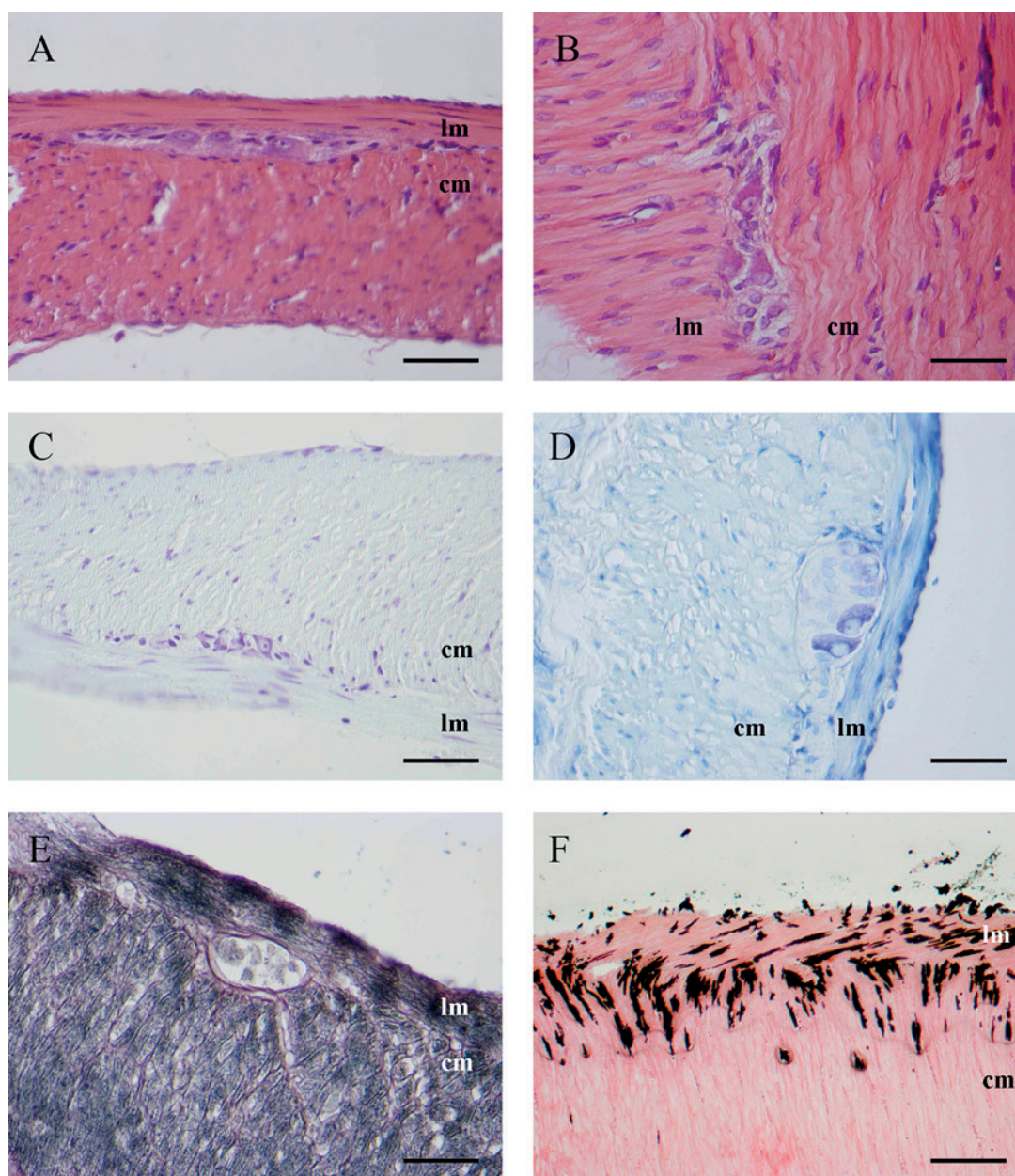


Figure 1. Rat myenteric ganglia visualized by means of conventional staining techniques on tissue sections. Routine H&E staining in the colon (A) and the anorectum (B). Please note the myentric plexus situated between the circular muscle layer (cm) and the longitudinal muscle layer (lm). At a higher magnification in tangentially-sectioned myenteric plexus (B), the cell bodies of myenteric neurons are clearly visible. Nissl (C) and toluidine blue (D) staining reveal presence of stellate-shaped cell bodies. (E) Gomori method of silver nitrate impregnation. Note the thick reticular layer encircling the ganglion. (F) Golgi method of silver nitrate impregnation shows notable presence of fiber bundles in both musce layers. Scale bars: A-E 50 μ m, F 100 μ m.

was observed on the outer surface of the LM, corresponding to the subserous plexus (Fig. 1D).

1.2. Neuronal morphology

It is well established that the largest percentage of guinea-

pig myenteric neurons fall into two morphological groups of neurons, Dogiel type 1 and 2, albeit there are more groups (2). Although oversimplified, the perikarya of neurons with Dogiel type 1 morphology are similar in shape to multipolar neurons, i.e., their contours are stellate (3). Conversely, the bodies of

Dogiel type 2 are more rounded and have smoother contours (4). Similar to the previous group, they also have perikaryal outgrowths but they do not greatly alter the round or oval shape of the body, given the limited magnifications inherent to light microscopy. According to the available data for the guinea pig (5), the myenteric neurons are flattened in the plane that separates the CM and LM and their dendrites are branching predominantly in the same plane. In other words, the plane of both cross and longitudinal sections would pass through the narrow profile of these flattened perikarya. In concur with that, we did not find any significant differences in the neuronal shape in the transverse and longitudinal sections. The huge number of myenteric neurons were slightly elongated, with distinct tapering outgrowths. The other large group included rounded neurons with smooth contours. The distinct similarity with the guinea-pig pattern was already noted. On the tangential sections, the presence of these two cell types became even more apparent. For example, we were able to detect almost entirely stellate neuronal profiles with multiple processes with the histochemical staining for NADPH-d (Fig. 3-A, B). This is consistent with the guinea pig data, where nitrergic neurons are motor neurons and interneurons that are predominantly Dogiel type 1 and 3 morphology (5).

In terms of neuronal size, comparative analysis of cell surface of the myenteric neurons showed some differences both in the different segments of the colorectal region, and in the different orientations of the sections. The Kruskal-Wallis test showed no statistically significant differences in the four sectional planes examined: on the transverse sections $H(3) = 3.422$, $p = 0.3310$; on the longitudinal sections $H(3) = 2.706$, $p = 0.4393$, and on the tangential sections $H(3) = 2.280$, $p = 0.5164$. However, individual comparison of the median values in the individual segments showed larger neuronal size in the colon than in the anorectum (Tables 1), which is valid for all section types. When comparing the values for the same segment but with different section orientation, it was evident that

Table 1. Median values of neuronal surface area along the colorectal region of the rat registered with different section orientation (μm^2).

	Cross section	Longitudinal Section	Tangential Section
Proximal Colon	236.29	224.76	278.84
Distal Colon	245.90	228.30	273.59
Rectum	216.52	209.29	252.22
Anal Canal	204.75	203.99	261.51

the values are lowest in longitudinal sections, intermediate in cross sections and highest in tangential sections.

Although the comparative tests did not report statistically significant deviations, it is noteworthy that the recorded dimensions of the myenteric neurons still show certain differences. For example, the mean area values of the neurons in the colon were notably higher than those in the anorectum. Such results are analogous to data from previous studies (6, 7). Comparing the data from the same segment but with different section orientation, some basic conclusions regarding the cell shape can also be drawn. Since in all examined segments the values are larger in the cross sections compared to the longitudinal ones, it could be assumed that the myenteric neurons are slightly elongated and that their long profile is perpendicular to the gut axis. Provided that in the tangential sections the values are appreciably higher than in both of the previous groups one can conclude that the neurons are flattened in the myenteric plane. Although tentative, these conclusions are fully supported by literature data (2, 5).

1.3. Myenteric ganglia

Current evidence shows that the myenteric ganglia are unencapsulated formations that are attached directly to the connective tissue permeating the muscle sublayers (8, 9). Moreover, studies involving morphometric measurements in the relaxed and distended intestinal wall have shown that this factor influences morphometric performance, i.e., mechanical forces applied to the intestinal wall are directly transmitted to ganglion cells (6). All this indicates that the myenteric ganglia differ fundamentally from the ganglia of the autonomic nervous system.

The routine staining and the Gomori method of silver nitrate impregnation, commonly used in histological practice for visualization of reticular fibers, aided analysis of the bordering region of myenteric ganglia. On the routinely H&E-stained slides, the thin fibrous layer that separated the two muscle layers continued directly onto the periphery of the ganglia (Fig. 1A, B). Moreover, this structure in no way resembled a well-formed dense fibrous capsule as it is generally seen in the autonomic ganglia. Instead, it had the appearance of a thin layer of loose tissue containing no more than a single row of cells of relatively small size. Their nuclei were small, flattened and heterochromatic and they very likely to be the fibroblast-like cells that have been described in electron microscopic studies (10). Accordingly, the ganglia rather seemed to be devoid of a capsule of their own, and were visually situated among

the loose tissue separating the two muscle layers. Evidence suggests that enteric glia leave extensive portions of neurons and their processes exposed and that nerve cells are found in seemingly direct contact with muscle cells (9), i.e., our findings fully corroborate the data available in the literature.

Despite its popularity and widespread use, we found no evidence for an application of the Gomori silver method in ENS studies. In our hands, this techniques revealed a thin reticular substance in the area between the muscle layers (Fig. 1E). It was in direct contact with the adjacent muscle layers and continued to form an envelope around the individual muscle fibers in both layers. Close to the myenteric ganglia, this layer split and fully encircled the ganglia, resembling a basal lamina. The visual assessment detected that the reticular layer surrounding the ganglia was slightly thickened compared with that in the areas devoid of ganglia. In addition, we observed a significantly thicker-layered structure with a negative staining reaction. These results once more support the thesis of a direct physical relationship between the myenteric ganglia and muscularis externa. Fine bundles of reticular fibers can be regarded as a normal finding in the extracellular matrix of smooth muscle (11). It is evident from the results that these bundles directly merge with the thin layer of analogous tissue surrounding most of the myenteric ganglia. In turn, it extends into the border zone between the muscle layers.

1.4. Nerve structures in the muscularis externa

One of the main functions of the myenteric plexus is the regulation of intestinal motility by means of direct innervation of the surrounding smooth muscle (12). Due to their staining affinity, the techniques used so far were not effective in examining the presence of nerve elements in the muscle layers. A non-specific method which proved to be useful for that purpose was the Golgi method of silver nitrate impregnation, which we performed with eosin counterstaining. The myenteric plexus was visualized in contrasting black color either as a continuous positive layer or as separate cell clusters with uneven borders, located between the two eosinophilic muscle layers (Fig. 1F). Despite the homogeneous staining of all nerve structures, oval thickenings interlinked by more constricted areas were evident in certain locations, most likely corresponding to myenteric ganglia and their interganglionic strands, respectively. A notable finding in all examined tissue specimen was distinct concentration of positive structures in both muscle layers. They had an elongated fibrous nature and were oriented parallel to the course of the surrounding muscle fibers. We did not find a predominance of the

mentioned structures in either of the two sublayers. The sections that seemingly lacked staining in the muscular sublayers proved to contain fine punctate axonal varicosities when observed at a higher magnification. The latter formed discontinuous lines parallel to the muscle fibers. A distinct finding observed in all histological slides was the significant accumulation of argyrophilic structures on the outer surface of the LM. In the more oblique sections, in which the submucosal layer was also included, positive oval structures were also observed in the latter, although in smaller quantity. Such findings were often located at the boundary between the submucosa and the circular muscle. The enzyme histochemical and immunohistochemical methods were also beneficial and they all showed similar histological results. Regardless of their thickness, fiber bundles in the circular muscle are always parallel to smooth muscles, as already described by Heinicke and Kiernan (13).

The innervation of the LM has been the subject of long-standing debates. The classical view is that its motor innervation is most strongly mediated by the so-called tertiary plexus, which is a subdivision of the myenteric plexus (14). It is a fine network of nerve fibers located on the inner surface of the LM. Another widely held belief is that the innervation characteristics of the LM are directly dependent on its thickness (15). In animal species in which its occurrence is shown as an exceedingly thin layer, traversing fiber bundles have not been reported. This effectively means that it is innervated entirely by the tertiary plexus, which is the case in the guinea pig (16), and in humans in the regions between the teniae (17). Conversely, bundles of fibers piercing the muscle are present in species that have a thickened LM. If so, we should not have found nerve structures crossing the LM in the rat. Indeed, in nearly all of the specimens examined, we encountered notable reactive staining on its inner aspect, which is reasonable to assume is due to the tertiary plexus, and no piercing fibers. The only exceptions were the fibers stained with the immunohistochemical (IHC) techniques for ATP and SP. In the former, such fibers were present along the entire colorectal region, whereas in the latter they were found only in the anorectum. We conclude that it is possible that the described findings point to specific peculiarities of the motor innervation accomplished by these transmission systems.

2. NANC TRANSMISSION IN THE MYENTERIC PLEXUS

The complex regulation of the intestinal muscularis externa involves an excitatory and inhibitory neuronal systems lo-

cated mainly in the myenteric and partly in the submucosal plexus (18, 19). By the 1960s, a notion was established that all components of the autonomic nervous system function on the basis of cholinergic and adrenergic nerve transmission. Over the past nearly six decades, new transmitter systems, diverse in chemical structure and mechanisms of action, have been described. They are all united by the NANC transmission system. Because of the large number of transmitters inherent to this system, they are classified either on the basis of their chemical nature or on the basis of the functional affiliation of the neurons that express them. Based on the first approach, three large groups of transmitters are introduced: peptides, purines and NO, characterizing three corresponding groups of neurons: peptidergic, purinergic and nitrergic.

2.1. Peptidergic transmission in the myenteric plexus

Substance P is the first member of tachykinins, which due to their significant distribution among myenteric neurons, are one of the most intensively studied neuropeptide families in the gut. The vast majority of the cholinergic enteric neurons utilize SP as a cotransmitter (2). The only exceptions to this rule are the descending interneurons and the intestinofugal neurons. These exceptions, however, account for no more than 10% of all myenteric neurons. The role of the SP in the execution of enteric motor reflexes has been the subject of a number of studies in different animal models and in different segments of the gastrointestinal tract (GIT). For example, SP-containing myenteric neurons in the rat small intestine have been proved to mediate intrinsic motor and secretomotor reflexes induced by physiological and pathophysiological stimuli (20, 21).

More specifically, SP-mediated transmission has been confirmed to be involved in three neuronal regulatory systems. In the first, SP is a cotransmitter of excitatory myenteric motor neurons, along with acetylcholine (22, 23). However, the functional significance of these two transmitters, as measured by their ability to induce intestinal muscle shortening, is not uniform. Blocking cholinergic transmission has been shown to result in more severe dysfunction than blocking tachykininergic transmission, i.e. SP has a secondary role in the excitatory motor system (24). The second system includes the ascending interneurons, in which SP is a cotransmitter along with acetylcholine and calcitonin (25). The functional significance of these neurons is in the execution of propulsive intestinal reflexes. The third system is associated with the afferent limb of sensory reflexes and includes the intrinsic primary afferent neurons (IPANs), the primary sensory neurons of the ENS.

In these, SP is a cotransmitter, along with acetylcholine and calbindin (2, 25, 26).

The focus of research to date, regardless of the animal model used, has been on the small intestine. The results of our study suggest that SP-ergic transmission is an essential component of ENS also in the more distal compartments of the GIT. Indeed, we observed numerous clusters of SP-immunostained material consistent with myenteric ganglia. At a higher magnification, we noted abundant SP-immunopositive fibers forming characteristic perisomatic varicosities. In the colon, we did not detect the presence of any SP-containing neuronal bodies (Fig 2A). However, immunostained nerve fibers ran in a basket-like manner around some immunonegative spaces that visibly corresponded in shape and size to neuronal bodies. Furthermore, the fibers were so contrasting that they allowed cell contours to be defined. Although these were found throughout the ganglia, the most distinct varicosities were those in direct contact with cell bodies. The interganglionic strands located between the two muscle layers were also readily apparent because of their marked SP-immunoreactivity and the presence of varicosities. The circular muscle also displayed immunopositive nerve fibers oriented parallel to the muscle fibers. The staining intensity of immunopositive fibers was significantly weaker than that in the interganglionic strands, and they possessed less varicosities. Similar if not identical findings were observed in the anorectum, despite the use of a different chromogen system (DAB) (Fig. 2B). Such negative perikaryal staining has been described in previous studies (27, 28). On the other hand, positive staining has been observed in other experiments (29, 30). One possible explanation for this is the use of colchicine to block the fast axonal transport of neurons in the latter studies.

The present results allow us to speculate about the morphological affiliation of the myenteric neurons with immunoreactive varicose fibers, as well as those with which they contact. However, it should be noted that our results are not inconsistent with recent publications demonstrating that similar SP-immunopositive varicose fibers make synapses on the cell surface of the body of neurons expressing neurokinin 1 receptors (a major cellular receptor for tachykinins) that are at the same time SP-negative (31). The same has been reported in other studies demonstrating that myenteric SP-immunoreactive neurons morphologically belong to the described Dogiel type 1 (motoneurons and interneurons) and type 2 IPANs (32).

Our findings are also quite similar to experiments on the guinea pig colon (33). Moreover, the presence of SP-immunoreactive fiber bundles in the CM is consistent with studies

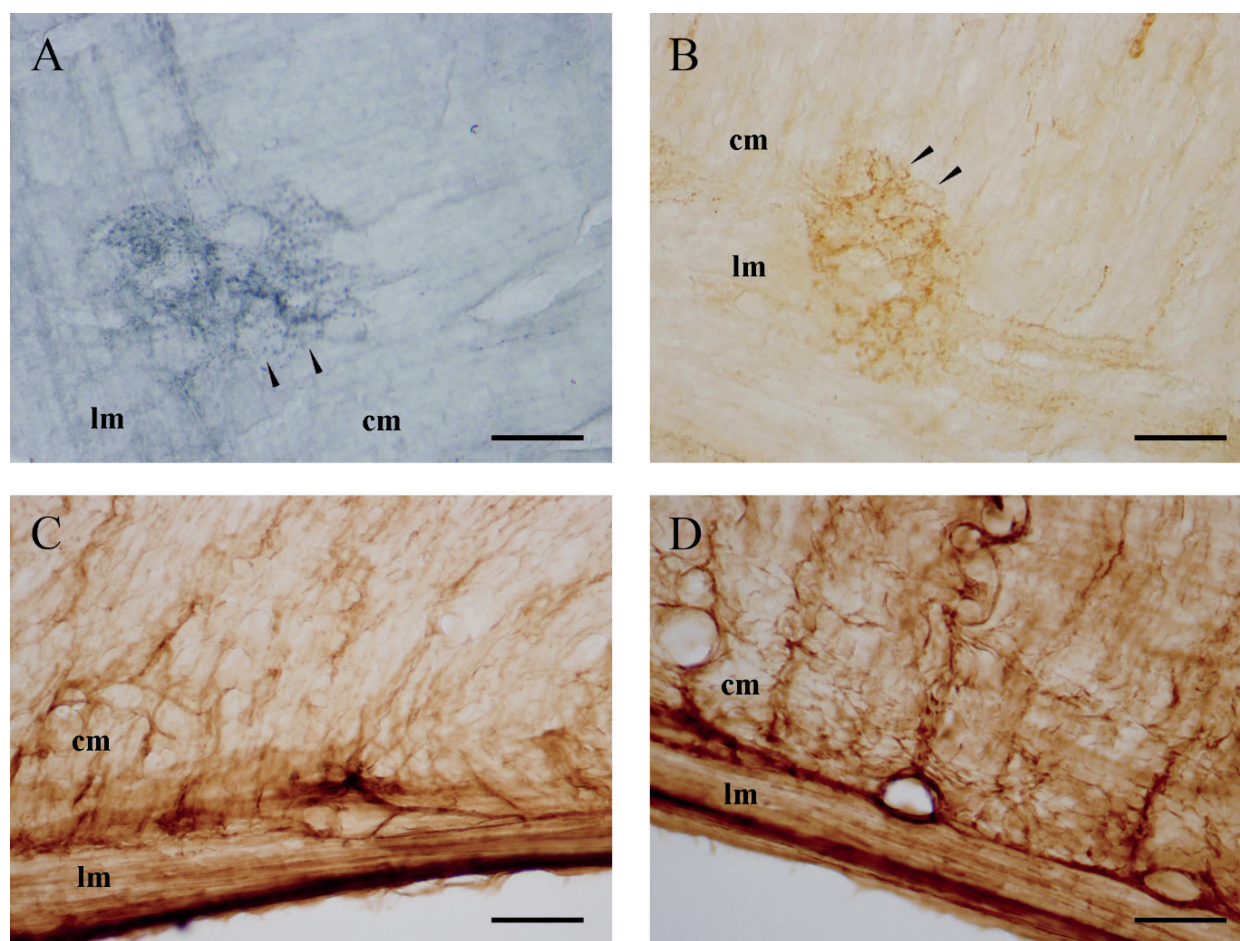


Figure 2. Myenteric immunoreactive nerve structures in the rat colon and anorectum. (A, B) SP-immunoreactive structures in the colon (A) and anorectal (B) region. Note the SP-positive beaded fibers that surround the immunonegative ganglionic perikarya in a basket-like manner (arrowheads). (C, D) ATP-reactive nerve structures in the colon (C) and anorectum (D). Note that the majority of ATP-positive structures are fiber bundles between or traversing the muscle layers. lm – longitudinal muscle; cm – circular muscle. Scale bars: 50 μ m.

done in a number of other animal models (12, 32). However, the data in the LM are quite diverse. For example, there is a complete absence of immunoreactive fibers in this layer in the pig large intestine (34) whereas such finding not only has been described in the rat and guinea pig but also evidence about their effect on excitatory motor transmission has been provided (35). In this work, we registered presence of nerve structures in the LM only in the anorectum.

2.2. Purinergic transmission in the myenteric plexus

There is experimental evidence that purinergic transmission plays a role in the relaxation of gastrointestinal muscle (36). In fact, a complete colocalization of ATP and NO has been described in inhibitory motoneurons of the rat colon, whereas

in the anorectal region this is only true for a fraction of purinergic neurons (37). Although Furness (38) has presented evidence for the involvement of purinergic transmission in descending myenteric interneurons, ATP still remains the major representative of the NANC transmitters associated with muscle relaxation, along with NO and the vasoactive intestinal polypeptide.

The purinergic structures observed in this study were mainly fiber bundles located in the periphery of the myenteric ganglia, in the interganglionic connections, and in the two muscular layers (Fig. 1C, D). Due to the presence of immunoreactive nerve fibers running along the periphery of the ganglia, the latter were distinctly outlined and separated from the surrounding tissues, though mostly appeared optically empty (Fig. 2D). At

certain locations within the ganglia, the immunostained fibers formed punctate structures with the appearance of varicosities that surrounded optically empty fields corresponding in shape and size to immunonegative neuronal bodies. Immunopositive neurons was extremely rare. In addition to the core and periphery of ganglia, we observed immunoreactive nerve fibers in the form of interganglionic strands on all preparations and also such entering the CM. Their course was parallel to the direction of the muscle fibers. A remarkable finding in these immunohistochemical reactions was the palpable presence of fiber bundles also in the LM, which was not observed in all other immunohistochemical reactions reported here. Significant staining was also observed in the subserosal layer (Fig. 2C, D). Moreover, in the CM, the latter contained noticeably thicker fibrous bundles in the vicinity of the myenteric plexus, which towards the submucosal layer seemed to progressively thin out. These findings suggest an intrinsic origin of these fibers in the rat from the myenteric plexus.

2.3. Nitrgenic transmission in the myenteric plexus

There are three generally accepted isoforms of the enzyme nitric oxide synthase generating the mediator NO (39). The neuronal isoform (nNOS) shows colocalization in the corresponding neurons with the NADPH-diaphorase (NADPH-d) (40, 41). For this reason, we assume that the results obtained in our study with the enzyme histochemistry for NADPH-d and the immunohistochemical reaction for nNOS refer to the same neuronal population. The literature data have unequivocally confirmed the presence of nitrgenic neurons in the ENS and in particular in the myenteric plexus of the guinea pig (42), dog (43), pig (44), and other animal species. They are thought to be a major link in descending inhibitory motor reflexes, as nNOS is localized in inhibitory neurons of both muscle layers, in descending interneurons, and in intestinofugal neurons (38). There is also data on their distribution in other compartments of the rat GIT such as the duodenum (45).

We observed an obvious presence of NADPH-d-positive structures, predominantly in tangential sections (Fig. 3A, B). In the myenteric ganglia, the number of NADPH-d-positive neurons ranged from 3-4 in the smaller ganglia to 15-16 in the larger ones. Their cytoplasm appeared granular, and the staining intensity was markedly weaker in the colon, compared to the anorectum. The colonic neurons showed weak to medium staining intensity (Fig 3A). The weakly stained ones had cytoplasmic granules in an amount insufficient to surely delineate the body contours. In neurons showing moderate

staining, the granules allowed rough contouring of the cell shape, with a negative nucleus. Numerous ganglia displayed diffusely scattered poorly pigmented material, which made the outlines of the occasional negative bodies distinguishable. In the same incubation environment, the staining of a considerable proportion of the neurons in the anorectal region may readily be defined as strong (Fig. 3B). The proportion of neurons with weak, medium or negative staining was notably less. In the intensely-stained neurons, the cytoplasmic granules were so numerous and densely labeled that the cytoplasm appeared homogeneously stained. Since those were tangential cuts, the neuronal bodies were sectioned in an optimal manner relative to their three-dimensional shape. This, in turn, would allow inferences to be made about their morphological affiliation. Inside the ganglia, fiber bundles of different direction were observed, forming a distinct network, whereas in the region of the interganglionic strands they had a markedly parallel course (Fig. 3B). In addition, positive fiber bundles were also registered in the surrounding muscle. Just as with the neurons, all fibers showed strikingly more intense staining in the anorectal region, largely due to the present axonal varicosities.

The IHC for nNOS demonstrated the presence of cellular aggregations with intense positive staining that unambiguously represented myenteric ganglia (Fig. 3C, D). In certain places, highly immunoreactive neuronal bodies stood out in them. Their shape varied, but polygonal perikarya, with several distinct processes, were frequently observed. The nuclei were negative and slightly elongated. Negative perikarya with distinguishable contours were also a common finding. This was due to the fact that the areas unoccupied by neuronal bodies were homogeneously pigmented or had a fibrillary appearance, with medium intensity. Immunoreactive interganglionic strands were not detected. All specimens showed punctate or slightly elongated accumulations of pigment in the CM, most likely resulting from the presence of transversely or obliquely cut bundles of immunopositive fibers. Such findings were not found in the LM.

Analyzing the results of our study, we can unequivocally confirm the presence of a stable population of nitrgenic neurons in all parts of the rat colorectal region. Their shape was identical to what we know as Dogiel type 1 neurons. This is in agreement with previous findings (46). The ganglia showed intense reactivity with the two methodologies used. However, the neuron-free spaces in the enzyme histochemistry showed a relatively weak reaction. This made the nitrgenic neurons easily accessible for

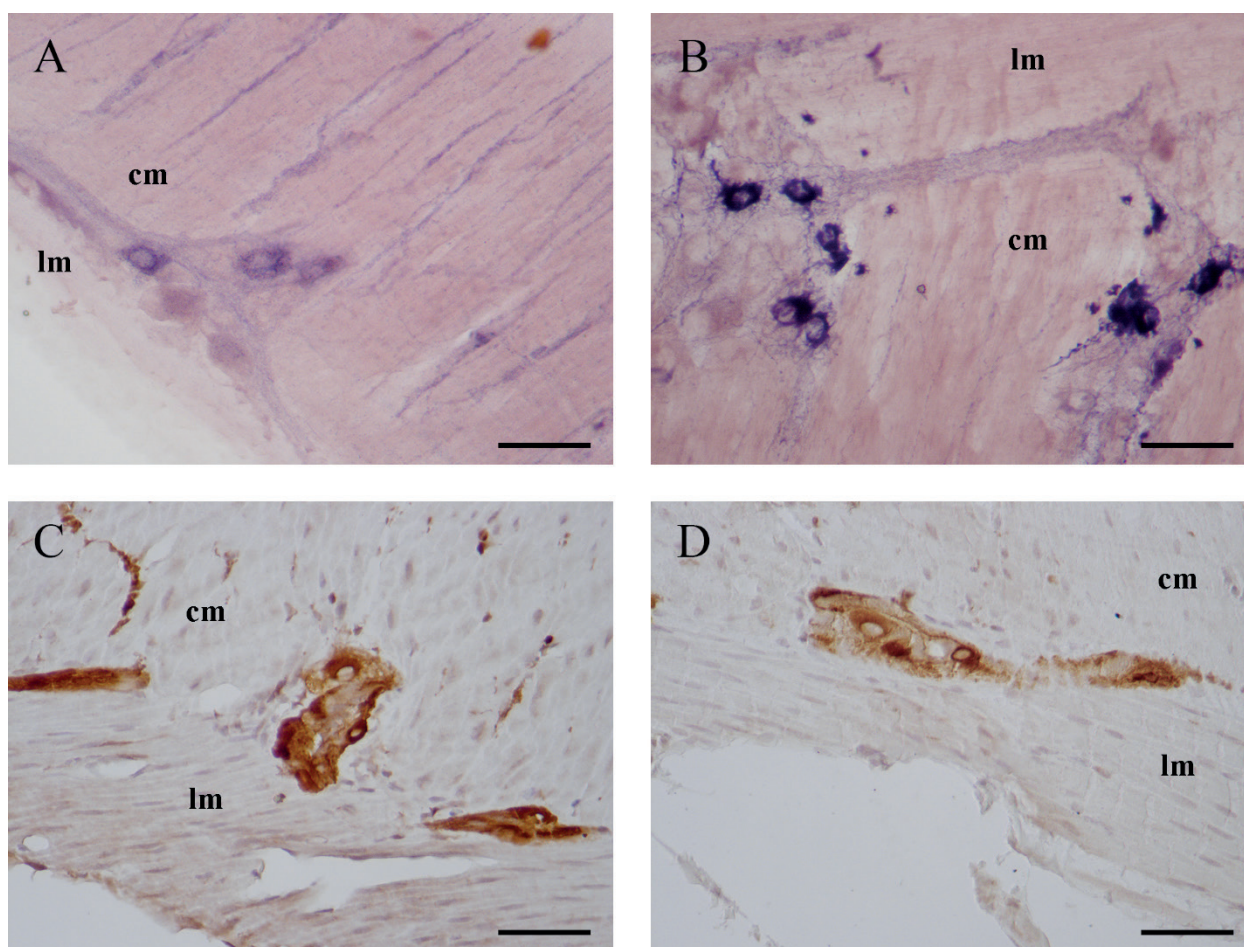


Figure 3. Histochemical localization of NADPH-d-positive structures in the colon (**A**) and the anorectum (**B**). Note that the staining intensity of the neuronal bodies is notably higher in the anorectum and the predominant morphology is analogous with Dogiel type I. (**C, D**) Immunohistochemistry for nNOS in the colon (**C**) and anorectum (**D**). In addition to the positive reaction in the myenteric neurons, note the significant staining of the intraganglionic spaces surrounding them. lm – longitudinal muscle; cm – circular muscle. Scale bars: 50 μ m.

qualitative analysis. In the IHC reactions, the staining of the neuron-free spaces was weaker compared with that of neurons, but was appreciably stronger than that in the histochemical reactions. This made morphological analysis of nitrergic neurons in immunohistochemical examination more difficult.

2.4. Myenteric nerve fibers

Quite visible on examination of the histological slides was the presence of varicosities along the course of the various fiber systems. Definitely the ones with the largest size and highest density were the SP-ergic fibers. In other transmitter systems the varicosities were less abundant, and in others they were hardly detectable. The presence of immunoreactive varicosities along the course of SP-ergic fibers has been reported in

previous studies (47, 48). They also show that SP injection leads to changes in the dendrites of neurons expressing the neurokinin-1 receptor, which is believed to be the main cellular receptor for SP. Receptor internalization after a single (49) or continuous (50) application of SP was found to lead to the formation of multiple varicosities along the course of these neurons. It has been suggested that the formation of varicosities may have consequences for neuronal function as it alters the geometry of the dendrites (51). Therefore, there are theories that varicosities may be a means of reduction of nerve fiber conductivity and isolating perikarya from excitatory or inhibitory influences (52). On the basis of the methodologies applied, we cannot infer on the functional significance of these varicosities, but we believe that a thorough study of

the possible influence of these structures on enteric motility is necessary.

Less abundant, but definitely present, were the varicosities along the course of the purinergic and nitrergic fibers. The reasons for the presence of varicosities in ATP-containing nerve fibres may be related to the accumulation of ATP in large amounts in synaptic vesicles (53). Based on a significant research experience, ATP has been identified as the major purine neurotransmitter in the gut (12, 18, 54). However, there is evidence that other purine derivatives, such as nicotinamide adenine dinucleotide phosphate (NADP+), also play these roles, including in the ENS (55, 56). Indeed, we found that varicose nerve fibers were a well-established finding in the histochemical reaction for NADPH-d but not in the immunohistochemistry for nNOS, though these reactions should identify the same neuronal population. It is therefore possible that the higher reactivity of the varicosities in question is explained by the presence of NADP+ in inhibitory motoneurons. Despite scientific advances in the field of NANC transmission, many gaps have been found in the relations between purinergic and nitrergic transmission and their mechanisms to date. Therefore, our results could be a good starting point for future research in this direction.

3. REGIONAL DIFFERENCES IN THE NANC TRANSMITTER EXPRESSION IN THE COLORECTAL REGION

Histochemical and immunohistochemical techniques remain a major tool for the study and analysis of neuronal transmission in all parts of the somatic and autonomic nervous system, including the ENS. They provide comprehensive information on both the quantitative and qualitative characteristics of different neuronal systems, including the examined in this study neurotransmitters. It is evident, both in extant literature and in our data that NANC neurons are a constant and well-developed cell population in the mammalian ENS of the healthy gut. However, this is not the case in a number of gastrointestinal pathological conditions, in which significant abnormalities in the quantity or activity of particular neuronal groups can be observed. Fair examples are Hirschsprung's aganglionosis (57), hypertrophic pyloric stenosis (58), internal anal sphincter achalasia (59), and some others, in which significant deviations in the characteristics of nitrergic transmission are observed. This suggests that failure of specific transmitter systems may be directly connected to the pathophysiology of these socially relevant diseases. Hence the need to accumulate as much as

possible data on the neurochemically distinct neuronal groups.

In order to summarize the information we gathered; our expression-related findings are listed in Table 2.

Table 2. Summary of the obtained results, presented as a semiquantitative assessment of the expression of neuroactive substances in the myenteric plexus and the apposed muscle layers.

Neuroactive Substance	Positive Reaction in Neuronal Bodies	Positive Interganglionic Fibers	Fibers in CM	Fibers in LM
SP	-	+	+/-	-
ATP	-	+/-	+	+
NADPH-d	+	+	+	-
nNOS	+	+/-	+	-

Note: The intensity of immunoreaction was subjectively coded: a negative reaction with -, a positive reaction with +. When a positive reaction is registered only in isolated regions, it is marked with +/-.

The statistical data were analyzed using the Kruskal-Wallis test, which is a non-parametric alternative to single-factor analysis of variance for comparing three or more groups with different sample sizes. Comparative analysis of the immunoreactivity of myenteric neurons showed statistically significant differences alongside the rat colon in the majority of the staining methods performed. Regarding immunoreactivity to SP, the Kruskal-Wallis test showed statistically significant differences in the medians of the groups studied: proximal colon, Mdn = 38.312, distal colon, Mdn = 19.201, rectum, Mdn = 19.156, and anal canal, Mdn = 51.015; $H(3) = 17.28$, $p = 0.0006$. Dunn's test showed significant differences in SP-immunopositive nerve fiber staining intensity between proximal and distal colon ($p < 0.05$), proximal colon and rectum ($p < 0.05$), distal colon and anal canal ($p < 0.05$), and rectum and anal canal, $p < 0.01$ (Fig. 4A). Assessment of immunoreactivity for ATP with the Kruskal-Wallis test showed no statistically significant differences in medians among the four groups studied, $H(3) = 5.495$, $p = 0.1389$ (Fig. 4B). For the staining intensity of NADPH-d-positive structures, the medians of the proximal colon (Mdn = 152.737), distal colon (Mdn = 132.959), rectum (Mdn = 105.331), and anal canal (Mdn = 72.640) showed statistically significant differences, $H(3) = 105.239$, $p < 0.0001$. Dunn's test revealed differences in neuronal staining intensity between the proximal colon and rectum ($p < 0.001$), proximal colon and anal canal ($p < 0.001$), distal colon and rectum ($p < 0.05$), distal colon and anal canal

($p < 0.001$), and rectum and anal canal, $p < 0.01$ (Fig. 4C). For nNOS-immunopositive structures, the Kruskal-Wallis test showed no statistically significant differences in nNOS expression in the four groups examined, $H(3) = 2.455$, $p = 0.4835$ (Fig. 4-D).

Comparisons of the reaction density of specific neurons in a given segment of the GIT have been presented by many authors (60-62). However, we did not find data on the average staining intensity of NANC neurons along the course of the rat colorectal region, and therefore find our results valuable for a comparative topographic analysis of transmitter expression in the gut. When applying enzyme histochemistry, it is important to consider the details of tissue processing. The first empirical histochemical study of NADPH-d activity in neurons was that of Scherer-Singler *et al* (63), who experimented with different tissue incubation conditions. They appointed 30 min as the optimal incubation time for the brain. Kuo *et al* (64) reported

that increasing the incubation time in the CNS did not really increase the number of positive neurons, but did increase their staining intensity. In other words, arbitrary changes in the parameters of the methodology would not be relevant for the quantitative analysis of neuronal expression, but would have a major impact on the qualitative results. For this reason, the uniformity of the methodology is of paramount importance for adequate comparative analysis. Hence, for our studies, we strictly followed the exact values suggested by Scherer-Singler (63), i.e. 30 min incubation time. From data available in the literature, variations in expression levels, cell size and shape suggest the existence of functional subcategories of nitrergic neurons. The largest of these is that of inhibitory motor neurons, inducing relaxation of enteric muscle (65). The remainder of these are very likely to perform functions of inhibitory interneurons and may even be involved in sensory reception from the CM (66). Cracco and Filogamo (67) reported that

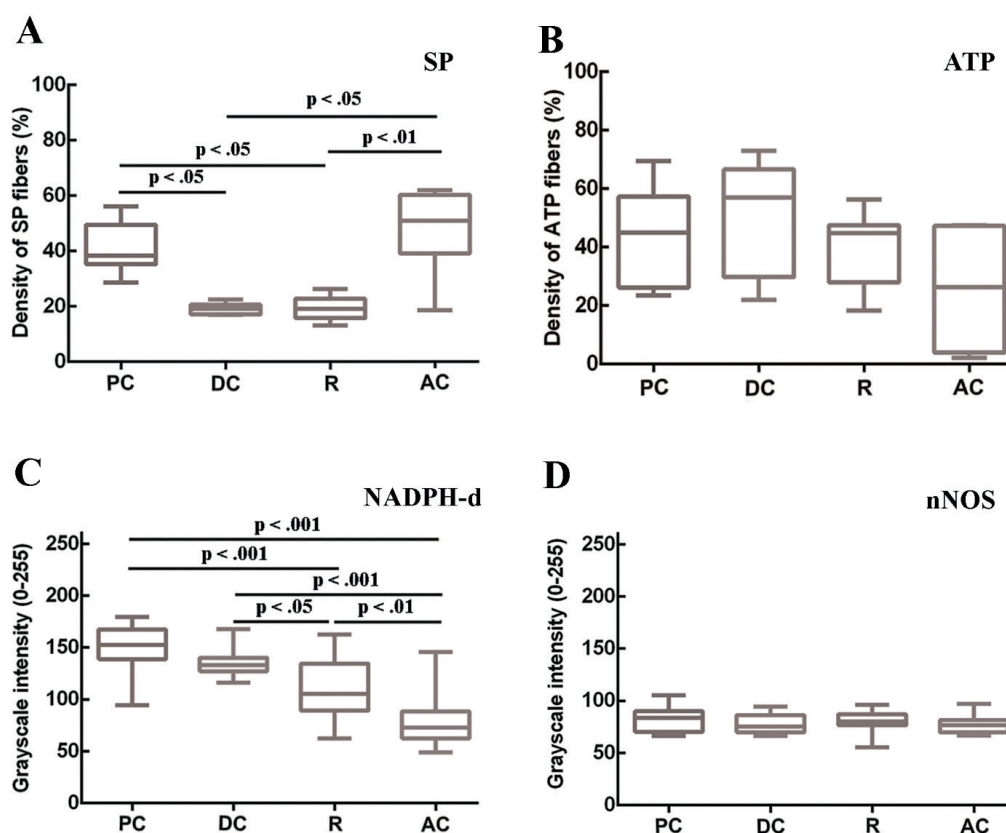


Figure 4. Box plot diagrams showing statistical comparison of density of SP-positive (A) and ATP-positive (B) nerve fibers, and staining intensity for NADPH-d-positive (C) and nNOS-positive (D) somata in the colorectal region of the rat. The data are presented as box and whisker plots, where the line within the box represents the median and the boxes represent the second and third quartiles (25%–75%). The data were compared by Kruskal-Wallis test followed by Dunn's pairwise multiple comparison procedure. PC – proximal colon, DC – distal colon, R – rectum, AC – anal canal.

differences in staining density might be related to differences in the quantity of NADPH-d, differences of enzyme activity, or to local specifics of isoenzyme proportions. Although hypothetical, according to Cserni (66), stronger expression is most likely to be present in inhibitory nitroergic motor neurons and weaker expression is intrinsic to the other functional subsets listed. It is possible that these relations could also be valid for the expression of ATP, which is a NO cotransmitter in the same populations of neurons (37).

Our data related to nitroergic transmission indicate an apparent discrepancy. Comparative analysis of the results of the histochemical reactions for NADPH-d shows statistical differences along the course of the colorectal region. Specifically, staining intensity was significantly higher in the anorectum, being the highest in the anal canal and lowest in the proximal colon. We consider it quite likely that specific mechanisms of motor reflex regulation in these areas determine this. However, comparative analysis of the immunohistochemical data for nNOS did not indicate the presence of significant differences in expression. Several putative explanations are possible. The first is that these two types of reactions are at least partially positive for different populations of cells, i.e. there is no complete colocalization between NADPH-d and nNOS. We find this possibility unlikely because of the undisputable evidence for the opposite (40, 41). We also believe that, although statistically possible, there was no technical error that would have led to false positive or negative staining. The other, and in our opinion the most likely possibility, relates to the fact that the specimens with histochemical reactions numerically outnumbered those for immunohistochemistry. Consequently, the statistical population analyzed was appreciably larger for the NADPH-d reaction, which is inevitably a prerequisite for a more accurate study. Similarly, there is some risk that the data obtained for nNOS are not fully representative of the entire population. ATP expression results also showed no statistically significant differences along the course of the colorectal region. Because of the considerably larger set of histological specimen that we were able to prepare for this study, we do not consider it probable that the statistical analysis provided misleading data.

CONCLUSION

The general morphology, cytoarchitecture and morphometric properties of the myenteric plexus in the rat colorectal region appeared to mimic its equivalent in the more proximal parts of the rat GIT and in other experimental animals. We can

confirm that working with tissue sections provides relatively limited but reliable morphological information regarding the structure of the myenteric plexus and its constituent neurons. In the rat, this plexus has a reticular structure consisting of interganglionic strands and ganglia, which do not have a well-developed connective tissue capsule. The constituent myenteric neurons correspond in morphological features to their counterparts described in other animal species and with usage of alternative methods. Based on a thorough literature review and our own results we confirm that myenteric neurons in the distal intestinal tract of the rat utilize a wide range of neurotransmitters, including classical neurotransmitters such as acetylcholine and NANC, also the neuropeptide SP, the purine mediator ATP, and the gaseous signaling molecule NO.

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