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Histochemical Localization of NADPH-Diaphorase Reactive Neurons in the Colorectal Region of the Rat

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The presence and distribution of nitrergic structures have been examined by means of NADPH-d histochemistry in the myenteric plexus, applied to the four main divisions of the rat large intestine. We first identified the exact location of the myenteric ganglia, the distribution of their internodal strands and fiber bundles in the adjacent muscle layers. Many NADPH-d–positive neurons were registered in the myenteric ganglia of all the examined segments and their morphology was categorized as Dogiel-type-1. Only single reactive fibers were found penetrating the longitudinal muscle, whereas in the circular muscle layer the varicose nerve fibers formed prominent bundles, running between the myocytes. We also observed an obvious predominance in the reaction intensity of NADPH-d-positive nerve structures in the recto-anal region, compared to the more proximal gut segments. In conclusion, our results provide histochemical evidence for the presence of nitrergic neurons in the rat colorectal region.

Key words: enteric neurons, myenteric plexus, nitrergic structures, NANC transmission, NADPH-diaphorase

Introduction

The inhibitory motor neurons of the enteric nervous system are an essential executor of a series of colonic reflexes and, therefore, have been a subject of interest for many years. There is now an abundance of evidence for the role this neuronal population plays not only during normal conditions, but also in a variety of disorders, like oesophageal or internal anal sphincter achalasia, hypertrophic pyloric stenosis and Hirschprung's disease [2, 8].

These neurons are the sole substrate of the so-called non-adrenergic, non-cholinergic (NANC) transmission. For many years, endogenous nitric oxide has been appointed as their main neurotransmitter, although a significant amount of evidence has shown extensive coexistence with vasoactive intestinal polypeptide (VIP) and adenosine

triphosphate (ATP) [1, 4, 6, 7, 10]. There is a distinct difference in the proportional distribution of these nitrergic neurons in the two enteric plexuses [2]. While they represent only a few percent of the total neuronal count in the submucous plexus, in the myenteric plexus their proportion can exceed 50%. This is hardly a surprise, since most of them are either inhibitory motor neurons, or inhibitory interneurons, both of which are involved exclusively in the innervation of the intestinal smooth muscle.

In different tissues, nitric oxide (NO) can be produced by all three isoforms, i.e. neuronal, endothelial and inducible, of the enzyme nitric oxide synthase (NOS). Naturally, in the enteric neurons the predominant isoform is neuronal NOS (nNOS). Nevertheless, the other two isoforms are also shown to be present there in small quantities, and, even more, their relative functions may alter during pathological conditions [2, 9].

The nNOS is proven to colocalize with NADPH-diaphorase [11]. Therefore, in this study we aimed to define the presence, distribution and staining intensity of nitrergic structures in the rat myenteric plexus that have been examined by means of NADPH-d histochemistry.

Material and Methods

In our study we used 20 mature (3-month-old) Wistar rats of both sexes with average weight (200-300 g). The entire array of experiments was carried out at the Institute of Neurobiology, Bulgarian Academy of Sciences in accordance with ethical principles of the Medical University of Sofia for the care and use of laboratory animals. Following a routine transcardial perfusion with 4% paraformaldehyde, we collected four colonic segments, each measuring approximately 10 mm, from the four major parts of the rat large intestine – proximal and distal colon, rectum and anal canal. Thereafter, the specimen was cut via a freezing microtome into 30 μm sections and mounted on glass slides, precoated with chrome gelatin. For the detection of NADPH-d activity, we applied the histochemical technique of Scherer-Singler et al. (1983). Briefly, the sections were incubated for 30–60 min at 37◦C in a staining solution consisting of 1mg/ml NADPH, 0.25mg/ml nitroblue tetrazolium (both from Sigma), and 0.3% Triton X-100 dissolved in Tris-buffered saline (TBS), pH 7.4. This mixture was freshly prepared and filtered just prior to use. After incubation, the sections were first rinsed in TBS, followed by washing in distilled water (3×15 min) and coverslipped in an aqueous-based mounting medium, glycerol jelly. For control purposes, sections were treated in the same way with omission of the substrate from the incubation medium. The slides were examined and photographed with Nicon Eclipse 80i (Japan), equipped with image analysis software NIS-Elements Advanced Research (ver. 2.30), and the images processed with Adobe Photoshop CC software.

Results

We first identified the exact location of the myenteric ganglia and the internodal strands connecting them. We observed many NADPH-d–positive neurons and their internodal fibers, located in the myenteric ganglia of all of the examined segments (**Fig. 1**). Those appeared to be monoaxonal, multipolar neurons – the stellate somata had numerous dendrites, oriented in a plane, parallel to the muscle layer. We also registered a definite predominance in the reaction intensity of NADPH-d–positive nerve structures of the recto-anal region (**Fig. 1 c, d**), relative to the colonic one (**Fig. 1 a, b**). Moreover, image analysis revealed that in the rectum the reaction intensity was slightly lower than that in the anal canal.

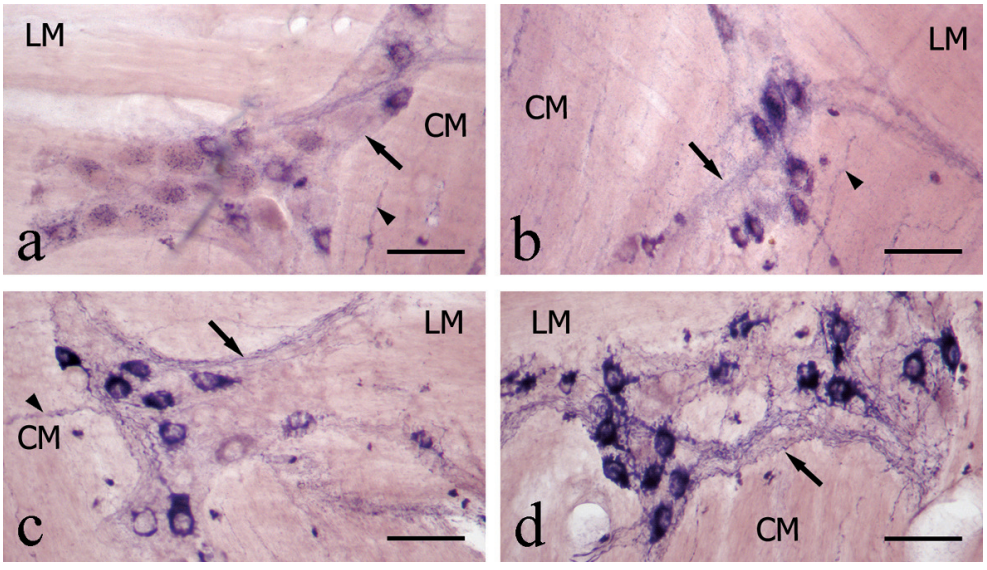

Fig. 1. Histochemical demonstration of NADPH-d activity in neuronal somata and fibers in the myenteric plexus of the colo-rectal region. Note the typical shape of the classical motor Dogiel-type-1 neurons. LM – longitudinal muscle; CM – circular muscle; arrows – internodal strands; arrowheads – fiber bundles in the circular muscle layer. Scale bar: 50 µm. (a) Proximal colon; (b) Distal colon; (c) Rectum; (d) Anal canal.

Analysis of the fiber system revealed only single positive fibers in close contact with the longitudinal muscle, but rarely penetrating it (**Fig. 2**). On the other hand, large fiber bundles, with extensive varicosities, were found traversing the circular muscle. Their orientation was perfectly aligned with the longitudinal axis of the myocytes.

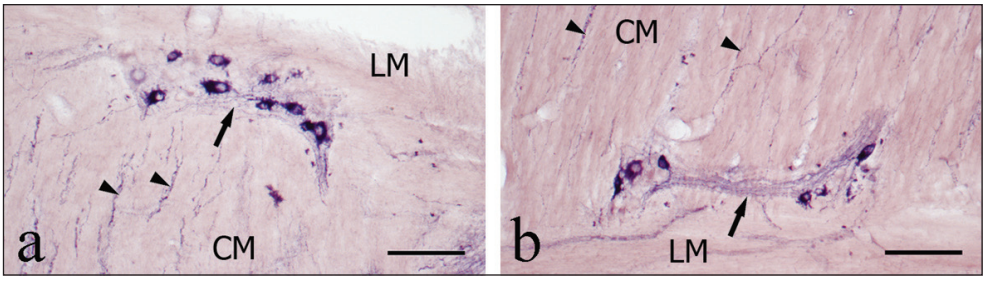

Fig. 2. Distribution of NADPH-d-positive neuronal perikarya and fibers in the myenteric plexus and the surrounding muscle layers. LM – longitudinal muscle; CM – circular muscle; arrows – internodal strands; arrowheads – fiber bundles in the circular muscle layer. Scale bar: 100 µm. (a) Rectum; (b) Anal canal.

Discussion

The aim of this study was to evaluate the morphology of inhibitory nitrergic neurons in the rat colonic myenteric plexus and to compare the results to previous findings. Virtually all of the registered NADPH-d-positive neurons appeared to possess the characteristic Dogiel-type-1 morphology. According to classical descriptions those neurons are

monoaxonal and have flattened stellate somata and numerous lamellar dendrites placed in the plane of the myenteric plexus itself [3]. Such studies have also indicated that those neurons serve as motor neurons and, to a lesser extent, as interneurons. Thorough research in the area has shown that the reported cases of nitrergic positive neurons expressing the features of Dogiel type 2 morphology (multiaxonal neurons with oval and seemingly smooth perikarya) is only due to insufficient magnification [5]. Therefore, the present study fully supports previous findings regarding the morphology of the nitrergic neurons.

Our results are also consistent with another aspect of past research – the perception that colonic myenteric nitrergic neurons exclusively send anal projections, which are responsible mainly for the inhibition of the circular muscle [2, 4]. Moreover, in animal species that possess only a thin layer of longitudinal muscle (such as the rat), the latter receives its innervation solely from the so-called tertiary myenteric plexus (a fine network of thin fiber bundles). In accordance with those postulates, we registered only a scant amount of poorly stained fibers in the longitudinal muscle, whereas in the circular layer fibers showed intense staining reaction and formed distinctive bundles with abundance of varicosities.

Conclusions

We provide histochemical evidence for the presence of abundant NADPH-d-positive neuronal structures in the rat colo-rectal region. Moreover, our results fully support the typical morphology and distribution of nitrergic neurons in the rat large intestine as previously described.

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