

MORPHOLOGICAL EVIDENCE FOR AGE-RELATED
CHANGES IN THE NADPH-d ACTIVITY OF MYENTERIC
NEURONS IN THE RAT RECTO-ANAL REGION

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(Submitted on May 10, 2010)

Abstract

Age-related changes in the nitric oxide-producing neurons in the rat recto-anal region were studied with reduced nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry and morphometry. In 15-day-old rats the myenteric plexus of the distal rectum and anal canal, situated in the space between the longitudinal and circular muscle layers, was exposed by NADPH-d positive perikarya. In the anal canal the positive myenteric neurons were more intensively stained than in the rectum. The mean optical density (OD) of positive neurons in the rectum was 115.8 ± 2.4 vs. 89.5 ± 1.6 in the anal canal. Single nerve fibres and small nerve bundles in the circular muscle layer of the anal canal were frequently seen and were more intensively stained than rectal fibres. In 3-month-old rats a larger number of NADPH-d-positive nerve fibres and an increase in their staining intensity were found. The staining intensity of the perikarya in the anal canal was higher than in the rectum (the OD 70.9 ± 1.1 vs. 55.5 ± 1.5). In the circular muscle layer thicker nerve

This study was supported by a grant from the Medical University of Varna and the Institute of Neurobiology, Bulgarian Academy of Sciences (project No 3/2007).

strands were observed. In 26-month-old rats the staining intensity of NADPH-d-positive perikarya in the myenteric plexus was weaker than in the 3-month-old rats. In the rectum their OD was lower than in the anal canal (93.8 ± 2.05 vs. 78.9 ± 1.7). The nerve strands were sparse and less intensely stained than in 3-month-old rats. Our results are consistent with the hypothesis that NO is an important neurotransmitter of the recto-anal motility in the ageing gut.

Key words: anal canal, distal rectum, histochemistry, morphometry, myenteric neurons, rat

Introduction. The distal part of the digestive tract takes part in the formation of the fecal mass and its periodic expulsion through the anus. The maintenance of continence is a complex process of interrelating factors which includes the sphincter muscles, rectal and anal sensation and stool composition. The movement of the bowel content is regulated by the enteric nervous system (ENS) via specific neurotransmitters. Functionally, the neurons in the gastrointestinal tract have been divided into two major subgroups, excitatory and inhibitory, and it is believed that the enteric inhibitory neurons in many animal species contain nitric oxide synthase (NOS) and release nitric oxide (NO) [1].

NO is a gaseous molecule which acts as a neurotransmitter in the central and peripheral nervous system [2,3]. The enzyme responsible for the synthesis of NO is NOS. Reduced nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) and the Ca^{2+} /calmodulin-dependent enzyme NOS form NO by converting L-arginine to citruline [4]. In this reaction NADPH serves as a co-substrate. Thus, NOS-containing structures can be morphologically revealed via the NADPH-d histochemical staining technique (for a review, see [5]).

In the gastrointestinal tract NADPH-d-activity has been found in neurons predominantly located in the myenteric plexus, and in nerve fibres in the internodal strands, secondary and tertiary plexuses. The reactive fibres are reported to be particularly more abundant in the deep circular muscle layer than in the outer longitudinal layer [6,7]. In addition, it has been suggested that NOS-containing neurons in the myenteric plexus of the rectum and anal canal produce NO, which mediates the recto-anal inhibitory reflex [8].

Aging is often accompanied by behavioural changes, such as impaired memory, reduced sensory acuity and slow motor control. Physiological, pharmacological and morphological data have also shown that during aging neurodegenerative changes are observed in the gastrointestinal tract of many mammals, including humans [9–16]. The age-related alterations of the ENS have attracted the attention of investigators with the hope to understand the gastrointestinal disorders [17,18]. In this respect, little is known as yet about the changes in the distribution, size and frequency of the NADPH-d-positive myenteric neurons in the recto-anal region of the rat in time.

In order to specify the presence of age-dependent alterations in the expression and distribution of NOS-containing structures in the rat rectum and anal canal, we examined the distributional patterns of NADPH-d-positive myenteric neurons and their density in the hind gut of rats of different ages.

Material and methods. All procedures were carried out according to a standard protocol established by the Bioethical Commission of the Biomedical Research at the Institute of Neurobiology of the Bulgarian Academy of Sciences. Nine male Wistar rats at different ages (15-day-, 3-month-, and 26-month-old) were used in this study. The animals were maintained under standard conditions at 21 °C, with a 12 h light-dark cycle. The rats were supplied with water ad libitum and fed on a normal diet of standard rat chow, providing 30 g of food daily.

Under deep anaesthesia the animals were transcardially perfused first with 0.05 M phosphate buffered saline (PBS) followed by a fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. The hind gut was dissected out and then postfixed in the same fixative overnight and after that soaked in 0.1 M PB containing 20% sucrose for 24 h at 4 °C. Longitudinal tissue sections of 30 µm from the distal rectum and anal canal were serially cut on a Reichert-Jung freezing microtome and washed overnight in PBS. The sections were collected in a subset of two. Every first section of the subset was mounted on an objective slide and stained by the Nissl method. Every second section of the subset was stained for NADPH-d employing a modification of the procedure of SCHERER-SINGLER et al. [19]. Briefly, free-floating sections were rinsed for 30 min in 0.1 M Tris-HCl buffer pH 7.4 with 0.8% Triton X-100, and then incubated for 120 min at 37 °C in 10 ml 0.1 M Tris-HCl buffer, pH 7.4 containing 4 mg reduced β -NADP (Sigma, USA) and 10 mg nitroblue tetrazolium (NBT, Sigma, USA). The sections were washed in Tris-HCl buffer, mounted on chrome-gelatin-coated glass slides, air-dried overnight, dehydrated in absolute ethanol, cleared in xylene and embedded in Entellan (Merck, Darmstadt, Germany).

The control sections were treated in the same way except for the omission of the substrate (either NADPH or NBT) and the electron acceptor. This way, the specific NADPH-d reaction was eliminated in the control sections. In order to avoid day-to-day-variations, the NADPH-d histochemical reactions in all the samples were performed on the same day with identical solutions and protocols. The sections were examined with a Nikon research microscope and photographed with a digital camera Cool pix 4.500, Nikon.

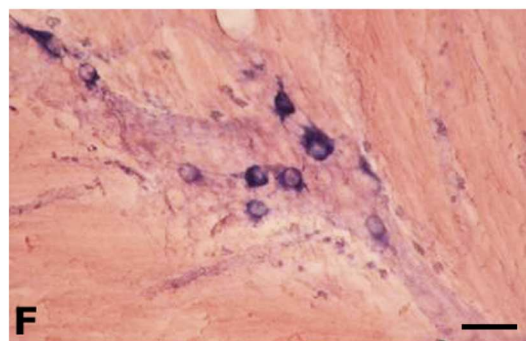
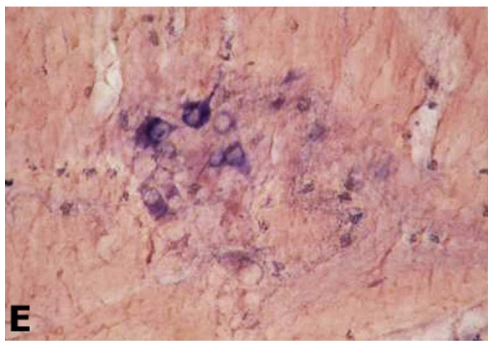
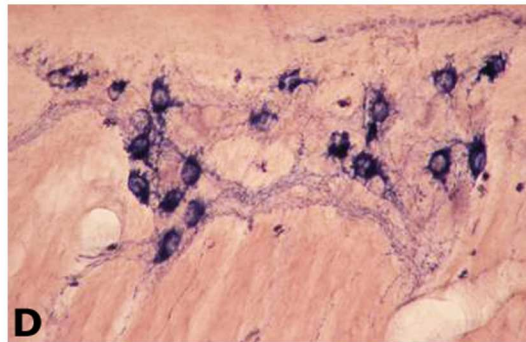
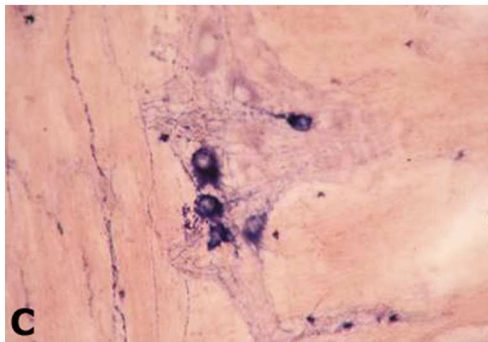
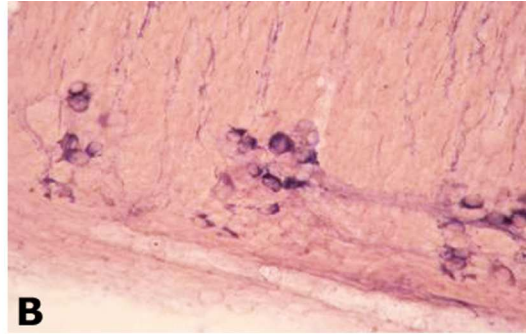
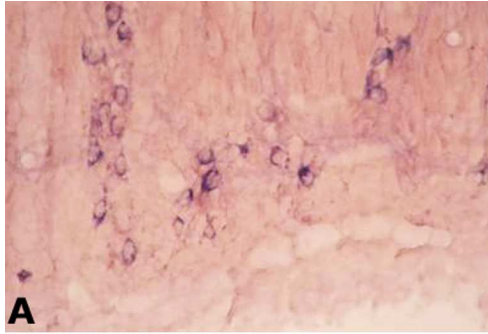
Image analysis. To assess the effect of aging on NADPH-d-positive neurons in the myenteric ganglia of the distal rectum and anal canal, 100 perikarya per group were identified under a magnification of $\times 40$. Thereafter, selected images were taken and gray-scale conversion was performed. The staining intensity

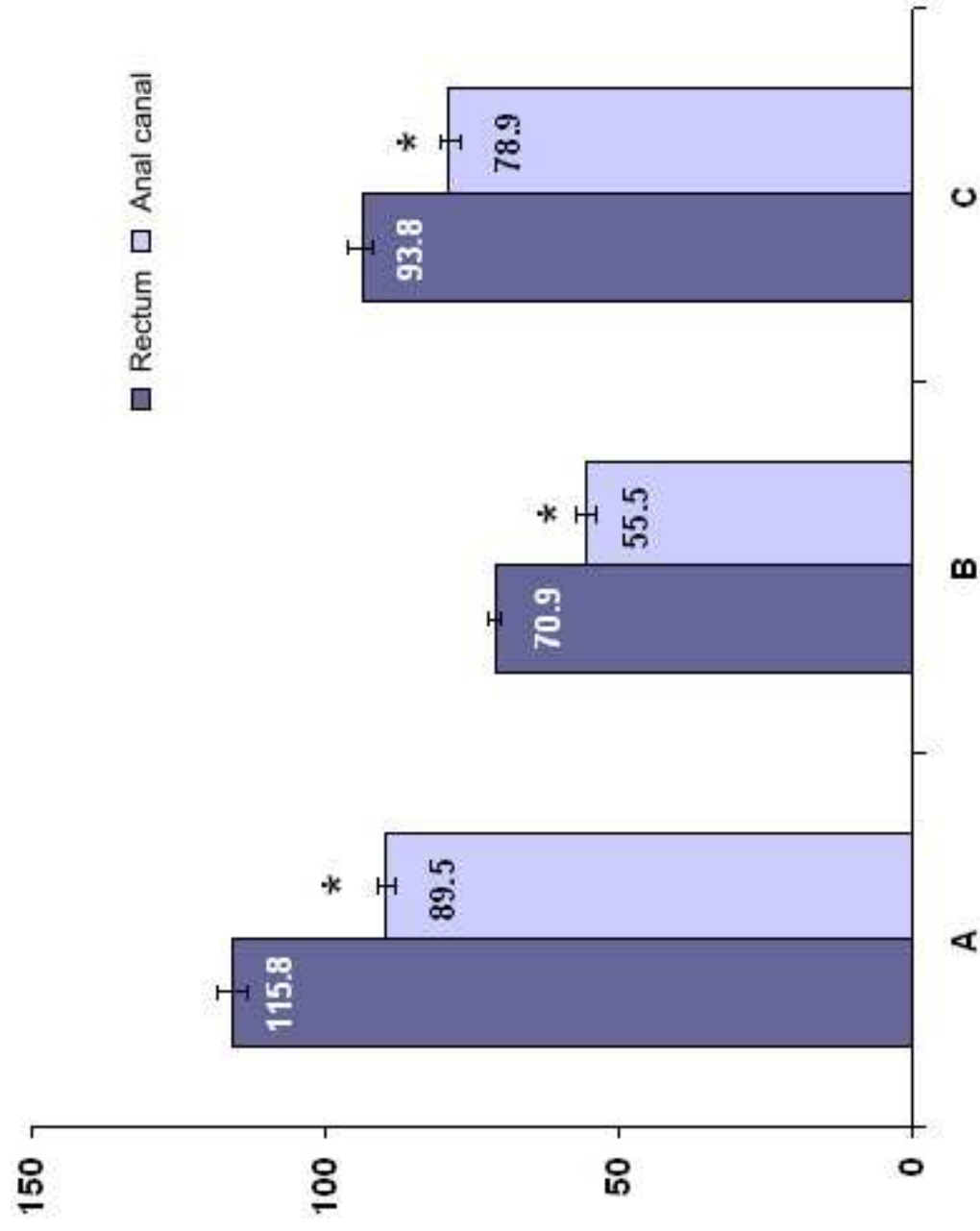
of myenteric neurons was evaluated by their optical density (OD). The OD was measured with a computer-based imaging system consisting of a Jenaval photomicroscope, a digital Hitachi video camera, an IBM computer and image analysis software, and was expressed in arbitrary units (AU) ranging from 0 (absolutely black) to 256 (OD of the glass slides). Low AU corresponded to high OD, respectively to high intensity of staining, while high values indicated low OD. Data were shown as mean $\pm$ S.E.M. according to the Student-Newman-Keuls multiple comparison test or the Student's *t*-test. Statistical significance was accepted at $p < 0.05$.

Results. After applying the Nissl staining, it was possible to observe the muscle layers of the intestinal wall and the myenteric plexus. We found that the longitudinal muscle layer of the rectum and anal canal was much thinner than the circular muscle layer. The myenteric plexus consisted of a cluster of neurons situated between the two muscle layers. The histochemical staining for NADPH-d revealed the presence of positive myenteric neuronal perikarya identifiable by the formazan deposits (Fig. 1A, B). The reaction product was dark-blue in colour, homogeneous, filling the entire perikaryon, and often extending in the dendrites and axons. The reactive neuronal profiles were sharply outlined and the cell nuclei were unstained. In 15-day-old rats, NADPH-d-positive neurons showed a low staining intensity with certain differences in their distributional patterns between the rectum and the anal canal. The total number of reactive neurons in the rectum was relatively lower when compared with those in the anal canal. Such a constellation was also validated for the staining intensity of the myenteric neurons in the rectum and anal canal. Indeed, the mean staining intensity in the rectum was 115.8 ± 2.4 vs. 89.5 ± 1.6 (Fig. 2A). In the circular muscle layer of the recto-anal region single, thin varicose nerve fibres were seen more frequently than nerve bundles, running parallel to the muscle cells. However, they were more intensely stained in the circular muscle layer of the anal canal than in the rectum. In the longitudinal muscle layer thin varicose nerve fibres were occasionally observed. At this age, the positive nerve fibres in the internodal strands were stained more weakly.

In 3-month-old rats the muscle layers were usually thicker than in 15-days-old rats. In myenteric ganglia NADPH-d-positive perikarya were widely distributed

Fig. 1. Photomicrographs of the recto-anal region showing NADPH-d positive neurons in the myenteric ganglia of the rectum (A) and anal canal (B) in 15-day-old rats. Intensely stained myenteric neurons are also observed in the rectum (C) and anal canal (D) in 3-month-old rats. A population of reactive perikarya is visible in the myenteric ganglia of the rat rectum (E) and anal canal (F) at 26 months as well. Note the differences in the staining intensity and the relative number of positive neurons at different ages and regions. Scale bar = 50 μ m





(Fig. 1C, D) and, moreover, their OD was higher. Similarly to the situation in 15-day-old rats, the average number of reactive neurons and their OD in the rectum were significantly lower than in the anal canal. The measurements showed that the mean OD in the rectum was 70.9 ± 1.1 vs. 55.5 ± 1.5 in the anal canal (Fig. 2B) and this difference was statistically significant. The nerve bundles in the circular muscle layers were tortuous, intensely stained and the frequency of their occurrence was higher than in younger rats. The staining intensity of the positive nerve strands and fibres in the circular muscle layer of the anal canal was higher than in the rectum. The pattern of NADPH-d-activity in the longitudinal muscle layer was similar to the previous stage, but the staining intensity of the reactive neuronal structures was better pronounced.

In older adult rats, aged 26 months, the frequency of NADPH-d-positive perikarya occurrence decreased. Some of these cell bodies were observed as hypertrophied (Fig. 1E, F). The mean OD of positive neurons was lower in comparison with 3-month-old rats. Furthermore, the OD of the positive neurons in the rectum was 93.8 ± 2.0 vs. 78.9 ± 1.7 in the anal canal (Fig. 2C). In the circular muscle layer scattered nerve fibres and large nerve strands with variable staining intensity were visualized.

The morphometrical analysis by applying the Student's *t*-test and Students-Newman-Keuls test demonstrated that the observed differences in the OD of the positive perikarya between the rectum and the anal canal at all ages studied were statistically significant. Likewise, the age-dependent changes in the OD in the rectum and the anal canal also showed statistical significance.

Discussion. The maintenance of the bowel contents and its evacuation is a complex process triggered by a number of neurotransmitters and controlled by the ENS. NO is known to mediate the recto-anal inhibitory reflex [8]. Our recent study showed that the ENS in the distal part of the digestive tract is well developed in 15-day-old rats and the myenteric ganglia appear as clusters of small neurons in the space between the longitudinal and circular muscle layers [16]. The circular muscle layer is predominately innervated by isolated nerve fibres whereas the nerve trunks are thin and rarely seen. In 3-month-old rats the circular muscle layer is supplied by a larger number of nerve fibres and dense nerve trunk running between the muscle cells. Thus, it is obvious that the process of development continues postnatally. Moreover, research on the age-related changes in the gastrointestinal tract has shown a significant decline in the density of the myenteric neurons by 40% in the small intestine and by 60% in colon of the

Fig. 2. A histogram showing the aging changes in the mean OD in arbitrary units of ← NADPH-d-positive perikarya in 15-day-old rats (A), 3-month-old rats (B) and 26-month-old rats (C). $n = 100$, $*p < 0.001$

rats [10,20]. In line with this, DE SOUZA et al. [11] and GOMEZ et al. [12] found a similar reduction (34–37%) in the number of the myenteric neuronal perikarya in the human small and large intestine(s). Such a loss of myenteric neurons in aging has been observed by conventional histological methods in the colon [12] and small intestine [11] in humans. GABELLA [10] found that the spatial density of myenteric neurons per unit of small intestine serosal surface fell dramatically in aging guinea pig, so it only comprises 40–60% of the value obtained in young adult guinea pig.

The present histochemical results demonstrate that NADPH-d-positive neurons are present in the myenteric ganglia of the rat recto-anal region 15 days after birth. However, their morphological features, i.e. small perikarya with relatively large nuclei and a discrete ring of cytoplasm around them, suggest that they are immature. The comparison of our data between 3-month-old rats and 26-month-old rats indicates that in aged rats the relative number of NADPH-d-positive perikarya is lower than in 3-month-old rats. Therefore, this may reflect a loss of myenteric neurons during aging. However, with respect to the age-dependent alterations in the density of reactive myenteric neurons the results of the present study are contradictory to the observations BELAI et al. [21] who found a relative increase in NADPH-d-positive neurons with increasing age in the myenteric plexus of the proximal colon. Our current data also contradict their findings in the ileum, where no considerable NADPH-d-positive neurons are seen in aged rats. Spatial discrepancies between the examined regions of the gastrointestinal tract can explain this difference in the age-related changes of the myenteric NADPH-d-positive neurons.

Apart from the problem of matching the relative number of nitrergic myenteric neurons in different regions of the hind gut, we used the parameter OD to assess their density along the distal part of the gastrointestinal tract during aging. In our previous investigation [22] in young adult rats, we found that the staining intensity of myenteric neurons in the rat rectum was lower than in the anal canal. We presently show that the mean OD of NADPH-d-containing neurons decreases with age in both the rectum and the anal canal. Furthermore, at identical age the OD of reactive neurons in the rectum is lower than that of neurons in the anal canal. It can be inferred that the OD of the NADPH-d-positive perikarya increases during the postnatal period from birth to middle age and later decreases in senescence.

In summary, two main conclusions can be drawn from the results of the present study. First, there are age-related changes in the density of occurrence of NADPH-d-positive perikarya and nerve fibres. Second, age is a key factor in the OD of the NADPH-d-positive perikarya. This allows us thus to surmise that NO and nitrergic transmission is important for the recto-anal motility in ageing.

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