Morpho-quantitative study of NADH-diaphorase positive myenteric neurons of the ileum of rats of Holtzman lineage (*Rattus norvegicus*)

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ABSTRACT. The myenteric plexus has a regular characteristic morphological pattern for each segment of the digestive tube and for each species of animal. Considering the lack of data pertaining to the mentioned plexus in rats of Holtzman lineage, the objective of this investigation was to carry out a morpho-quantitative study of the myenteric neurons in the ileum, by means of histological sections and whole-mount muscular preparations treated by the NADH-diaphorase method. The profiles of the cell bodies (CB) of the neurons in the mesenteric and antimesenteric regions were counted and measured. The neurons were classified according to the dimensions of the CBs. NADH-dp myenteric neurons were observed grouped together into ganglia in the muscular tunica. The mean neuronal density was 985.8 ± 195.4 neurons/8.96 mm^2 in the antimesenteric region and 1267.8 ± 259.92 neurons/8.96 mm^2 in the mesenteric region. The incidences of small, medium and large neurons were 14.4, 82 and 3.6% in the antimesenteric region and 14.6, 70.8 and 14.4% in the mesenteric region, respectively. It was concluded that ganglionated arrangements and medium-sized NADH-dp neurons predominated in the myenteric plexus of adult Holtzman rats. The results observed indicated that the NADH-dp myenteric neurons of the ileum of Holtzman rats are similar to those of rats of Wistar lineage with respect to their localization, ganglionated arrangement and the predominance of neurons with medium-sized CBs.

Key words: myenteric plexus, ileum, Holtzman rat, intestine.

RESUMO. Estudo morfoquantitativo dos neurônios mioentéricos NADH-diaforase positivos do íleo de ratos (*Rattus norvegicus*) da linhagem Holtzman. O plexo mioentérico possui um padrão morfológico regular característico para cada segmento do tubo digestório e para cada espécie animal. Considerando a escassez de dados pertinentes ao referido plexo em ratos da linhagem Holtzman, o presente estudo como objetivo o estudo morfoquantitativo dos neurônios mioentéricos do íleo, por meio de cortes histológicos e preparados de membrana, tratados pelo método da NADH-diaforase. Foram contados e mensurados os perfis do corpo celular (PC) de neurônios nas regiões mesentérica e antimesentérica. Os neurônios foram classificados segundo as dimensões do PC. Foram observados os neurônios mioentéricos NADH-dp, reunidos em gânglios na túnica muscular. A densidade neuronal média foi de 985,8 ± 195,4 neurônios/8,96 mm^2 na região antimesentérica e 1267,8 ± 258,92 neurônios/8,96 mm^2 na região mesentérica. As incidências de neurônios pequenos, médios e grandes foram 14,4, 82 e 3,6% na região antimesentérica, e 14,6, 70,8 e 14,4% na região mesentérica, respectivamente. Concluiu-se que, em ratos Holtzman adultos, predominam o arranjo ganglionado no plexo mioentérico e neurônios NADH-dp de tamanho médio. Os resultados encontrados indicam que os neurônios mioentéricos NADH-dp do íleo de ratos Holtzman são similares aos dos ratos da linhagem Wistar no que diz respeito à localização, arranjo ganglionado e predominância de neurônios com PC de tamanho médio.

Palavras-chave: plexo mioentérico, íleo, rato Holtzman, intestino.

Introduction

The final motor neurons for the segments of the gastrointestinal tract are located, mainly, in the myenteric ganglionated plexuses and submucosa of the enteric nervous system. Theses plexuses carry out complex functions, which include the control of

motility and gastrointestinal secretion, as well as control of the local blood flow (Gabella, 1979; Wood, 1981; Sternini, 1988). Although receiving nerve impulses from the central nervous system through the sympathetic and parasympathetic systems, the neurons of the myenteric plexus receive impulses mainly from other participating enteric neurons; that is, from the circuits responsible for the motor behavior of the gastrointestinal tract (Costa and Brooks, 1994).

Generally, in mammals, the myenteric plexus is located between the longitudinal and circular layers of the muscular tunica, or slightly displaced to the interior of one of these layers (Irwin, 1931). Their neurons are found organized into ganglia or isolated between bundles of interconnected nerve fibers. These intra- and interplexus neuronal connections allow the neural reflexes in the interior of the tract to be independent of the central nervous system, despite being subject to its influences (Guyton, 1997).

Different neuronal densities may be encountered in the same segment of the digestive tract in animals of the same species at different ages (Ali and McLelland, 1979; Santer and Baker, 1988; Santer, 1994) or when submitted to different experimental conditions (Torrejais *et al*., 1995; Natali and Miranda-Neto, 1996; Romano *et al*., 1996; Mello *et al*., 1997; Sant'ana *et al*., 1997; Zanoni *et al*., 1997; Araújo *et al*., 2006). Furthermore, neuronal density may vary in the same segment of the intestine when different regions of the intestinal circumference are compared (Irwin, 1931; Ali and McLelland, 1979; Santer, 1994; Sant'ana *et al*., 1997).

As in other tissues, quantitative and morphological alterations in the nerve cells of the myenteric plexus are observed when the animal is submitted to experimental conditions of ageing (Santer and Baker, 1988), mellitus diabetes (Hernandes *et al*., 2000; Zanoni *et al*., 1997 and 2003; Fregonesi *et al*., 2005; Alves *et al*., 2006) and malnutrition (Mello *et al*., 1997; Natali and Miranda-Neto, 1996; Araújo *et al*., 2006). Albino rats (*Rattus norvegicus*) of Wistar lineage have normally been used in these model experiments.

Innumerable studies have been developed to characterize the myenteric plexus in humans and other animal species with the aim of supporting theories proposed about the functional and pharmacological mechanisms of action of the myenteric plexus in the digestive processes (Burnstock, 1959; Ali and McLelland, 1979; Souza *et al*., 1982; Gabella, 1990; Bor-Seng-Shu *et al*., 1994; Molinari *et al*., 1994; Sant'ana *et al*., 1997).

There is growing interest in studying the myenteric plexus of arthritic rats has been awakened because the degenerative alterations characteristic of this condition are found in other systems of the body, including the central nervous system (Pearson and Wood, 1963; Gardner, 1965). Rats of Holtzman lineage are chosen for use in model experiments for the study of this condition because they are proven to be more susceptible to adjuvant-induced arthritis (Pearson, 1956; Pearson and Wood, 1963; Bersani-Amado *et al*., 1990).

Therefore, the aim of this study was to characterize some morphometric and quantitative aspects of the NADH-diaphorase positive neurons in the mesenteric and antimesenteric regions of the ileum of rats of the Holtzman lineage.

Material and methods

Five male Holtzman rats at 110 days of age and weighing 352 ± 10.16 g from the Central Vivarium of the State University of Maringá were used. After weaning, from 21 days of age, the animals were kept in individual plastic cages and received water and commercial laboratory chow *ad libitum*.

The cages were maintained in a temperature-controlled room (24 ± 2ºC) with a 12-hour light/dark photoperiod.

At 110 days of age and after fasting for 12 hours, the animals were weighed and anesthetized, and then underwent a laparotomy in order to retrieve the gastrointestinal tract and other abdominal viscera. These procedures resulted in the death of the animals by exsanguination.

The small intestine was separated from the gastrointestinal tract and the ileum – the most distal portion from the duodenum – was retrieved. The other segments and viscera were used in other studies.

Location of the myenteric plexus

A 1 cm segment was isolated from the proximal extremity of each ileum which, after washing in physiological solution and fixing in 10% formaldehyde solution, underwent routine treatment for paraffin embedment and the obtaining of transversal histological sections of 10 μm thickness, which were stained by the Hematoxylin-eosin and Azan methods, diaphanized and mounted between slide and cover glass with Permount resin (Fisher Chemical, USA). The sections were later analyzed using an Olympus BX40 light microscope to locate the myenteric plexus and observe the bundles of collagenous fibers connected to it.

NADH-diaphorase histochemistry

The remaining segment of the ileum of each animal was washed and filled with Krebs solution (pH 7.3). The distension of the segments during their processing was maintained by ligature at both ends. The myenteric neurons were evidenced by the activity of the NADH-diaphorase enzyme, according to Gabella (1969), as follows:

The ilea were washed twice more in Krebs solution (for ten minutes each time). They were then kept in Triton X-100 dissolved to 0.3% in Krebs solution for five minutes and washed twice more (for ten minutes each time) in Krebs solution. Finally, they were transferred to an incubation medium for 45 minutes. For every 100 mL of distilled water, the incubation medium contained 25 ml of 5% Nitro Blue Tetrazolium solution (NBT, Sigma Chemical Company, St. Louis, Missouri, USA), 25 mL of phosphate buffer (0.1 M; pH 7.3) and 0.05 g of β-NADH (Sigma, Steinheim, Germany). In the laboratory of this research group, the reaction time is fixed at 45 minutes for all experiments that used the NADH-diaphorase technique, in order to enable direct comparison between experimental groups.

The reaction was stopped by immersing the ileum, released from its ligatures, in 10% formaldehyde solution in phosphate buffer, where it remained for a minimum of four days.

After fixing, the ilea were opened along the mesenteric insertion. Transversal samples of approximately 1 cm were isolated and microdissected using a stereomicroscope with transillumination (Olympus® JAP) to retrieve the mucous and submucous layers, while preserving the muscular tunica and the serosa. The obtained whole-mount muscular preparation was dehydrated in an ascending series of ethanol, diaphanized in xylene (Synth, São Paulo, Brazil) and mounted between slide and cover glass with buffered glycerine solution.

Quantification of NADH-diaphorase positive myenteric neurons

In each of the whole-mount muscular preparations of the ileum, a quantitative analysis was carried out in the antimesenteric (120° to 240°) and mesenteric (0° to 60° and 300° to 360°) regions, considering 0° as the mesenteric border (Miranda-Neto *et al*., 2001) (Figure 1).

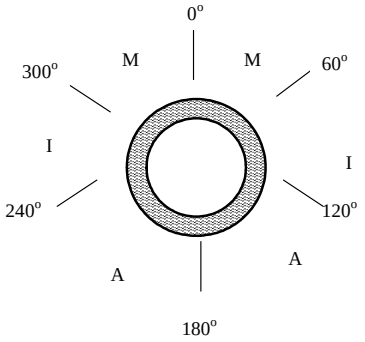

Figure 1. Scheme of the transversal section of the ileum, indicating the mesenteric (M), intermediate (I) and antimesenteric (A) regions. 0° corresponds to the mesenteric insertion.

In each whole-mount muscular preparation, the NADH-dp myenteric neurons were counted using an Olympus light microscope with a 40x objective lens in 80 random microscope fields, with 40 being from the mesenteric region and 40 being from the antimesenteric region of the ileum. Neurons straddling the boarders of the fields were counted in alternate fields.

The area of the field when using a 40x objective lens was 0.166 mm^2. The results were expressed as the mean number ± standard deviation of neurons present in 40 microscope fields; i.e. in 8.96 mm^2, of each region of the ileum.

Measurement of the cell body profile (CP) of the NADH-dp myenteric neurons

The Image-Pro-Plus 3.0.1. (Media Cybernetics, Silver Spring, Maryland, USA) image analysis software coupled to an Olympus BX40 light microscope was used in the morphometry. The images captured were separated and catalogued as mesenteric or antimesenteric according to the location of the neurons in the circumference of the ileum. In each region, the CPs (μm^2) of 50 neurons were measured, making a total of 100 neurons for each whole-mount muscular sample.

After obtaining the mean and standard deviation of the measurements, value intervals were established for the classification of the neurons according to the dimensions of the CPs. Neurons with CP dimensions belonging to the resultant interval of the mean ± standard deviation were considered of medium size. Those with dimensions larger and smaller than the interval were considered large or small, respectively.

Results

The myenteric plexus was located in the

muscular tunica of the ileum, between the longitudinal and circular layers, with a predominance of ganglia formations surrounded by bundles of collagenous fibers and with a varying number of neurons (Figure 2).

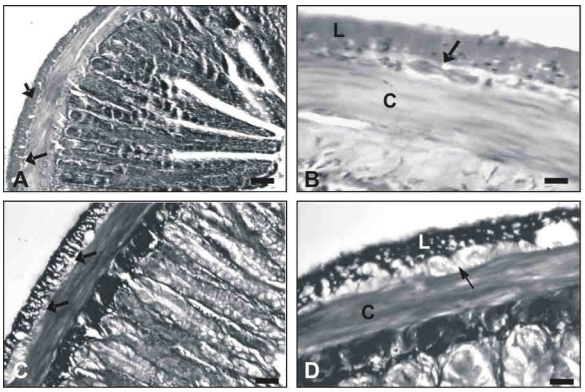

Figure 2. Transversal section of the ileum. **A**: showing the myenteric plexus (arrows), HE, (bar = 100 μm); **B**: myenteric plexus (arrows) located between the longitudinal (L) and circular (C) layers of the muscular tunica, HE, (bar = 10 μm); **C**: myenteric plexus (arrows), Azan, (bar = 20 μm); **D**: a ganglion of the myenteric plexus surrounded by bundles of collagenous fibers (arrows) between the longitudinal (L) and circular (C) layers of the muscular tunica, Azan, (bar = 10 μm).

Isolated neurons were observed less frequently. The majority of the ganglia showed a parallel arrangement among themselves and were transversely-oriented in relation to the main axis of the intestine (Figure 3).

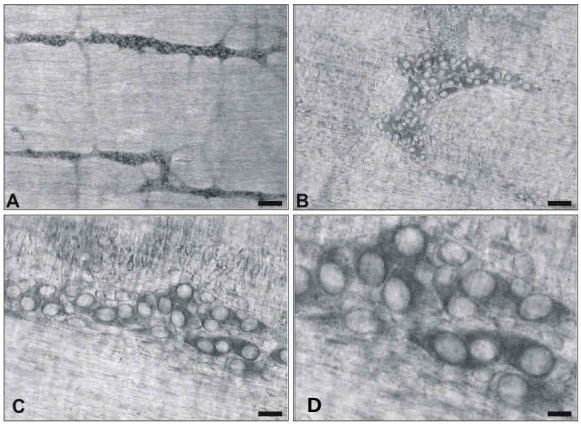

Figure 3. Ileum whole-mount muscular preparations. A and B: myenteric plexus, NADH-diaphorase; C and D: myenteric ganglia with NADH-diaphorase positive neurons (bar A = 100 μm, B = 50 μm, C = 20 μm, D = 10 μm).

There was varied neuronal density, with the greatest neuronal density being observed in the mesenteric region. The observed mean density ± standard deviation of the NADH-diaphorase positive myenteric neurons in the quantitative analysis of an area of 8.96 mm^2 of the ileum was 1267.8 ± 1267.8 in the mesenteric region and 985.8 ± 195.4 in the antimesenteric region (Table 1).

Table 1. Number of NADH-diaphorase positive myenteric neurons in 8.96 mm^2 of the whole-mount preparations from the mesenteric and antimesenteric regions of the ileum of rats of Holtzman lineage (n = 5).

Whole-mount preparation	Neuronal density/region	
	Mesenteric	Antimesenteric
1	1651	1111
2	1141	1208
3	1370	1025
4	1212	868
5	965	717
*Mean ± standard deviation of the mean	1267.8 ± 258.92	985.8 ± 195.4

*$p < 0.05$ significant different (Student t-test).

In the mesenteric region, the dimensions of the CPs of the myenteric neurons varied between 40.11 μm^2 and 240.79 μm^2. Neurons with CP dimensions within the interval of 85.83 μm^2 to 190.30 μm^2 were classified as medium sized. Neurons with dimensions greater than 191.92 μm^2 and smaller than 85.09 μm^2 were considered large and small, respectively.

In the antimesenteric region, the CPs of the myenteric neurons varied from 29.71 μm^2 to 279.26 μm^2. Neurons with CP dimensions within the interval of 89.7 μm^2 to 162.86 μm^2 were classified as medium sized. Neurons with dimensions greater and smaller than these figures were considered large and small, respectively.

The incidences of small, medium and large neurons in the mesenteric and antimesenteric regions of the ileum can be found in Table 2.

Table 2. Incidence of NADH-diaphorase positive myenteric neurons in the mesenteric and antimesenteric regions of the ileum of rats of Holtzman lineage according to cell body profile dimensions (n = 5).

Mesenteric region		
Cell profile	Frequency	Relative frequency %
Small (< 85.09 μm^2)	36	14.4
Medium (85.09 to 91.92 μm^2)	205	82
Large (> 191.92 μm^2)	09	3.6
Total	**250**	**100**
Antimesenteric region		
Small (< 89.7 μm^2)	36	14.8
Medium (89.7 to 162.86 μm^2)	177	70.8
Large (> 162.86 μm^2)	37	14.4
Total	250	100

Discussion

In the ileum of rats of Holtzman lineage, the NADH-dp neurons of the myenteric plexus are located between the circular and longitudinal layers

of the muscular tunica, as has also been reported for the jejunum and ileum of Wistar rats (Santer and Baker, 1988; Miranda-Neto *et al*., 2001; Tranin *et al*., 2001) and in guinea-pigs (Irwin, 1931; Matsuo, 1934), chickens (Gabella and Halasy, 1987), ducks (Molinari *et al*., 1994) and carp (Stabille *et al*., 1998; 1999 and 2000).

The location of the myenteric plexus in the wall of the intestinal segment is of prime importance. When whole-mount preparations are retrieved for the morphometric and quantitative analyses of intestinal neurons, the location of the myenteric plexus signals which membranes can be microdissected (Molinari *et al*., 1994). Thus, the presence of the myenteric plexus between the layers of the muscular tunica in the ileum of Holtzman rats indicates that it is possible to remove and discard the mucosal tunica and the submucosal tissue, and preserve the remaining parts, in order to obtain membrane samples, without the partial or total removal of the plexus.

The arrangement of the myenteric plexus did not vary along the circumference of the ileum, with a predominance of neurons grouped into elongated ganglia with the main axis orientated in a circular direction in a similar manner to the arrangement of the myenteric plexus of the ileum of Wistar rats, reported by Miranda-Neto *et al*. (2001).

Bundles of collagenous fibers were observed surrounding the ganglia of the myenteric plexus in a similar way to that described in the stomach of Wistar rats (Molinari *et al.,* 1994) and also in intestinal segments of fish (Stabille *et al.*, 1998; 1999 and 2000). According to Gabella and Halasy (1987) and Molinari *et al.* (1994), the presence of the collagenous bundles gives sustentation and protection to the plexus and to the blood vessels located in the muscular tunica of the gastrointestinal segments.

In the interior of the ganglia, the NADH-dp neurons are more disperse. It seems that sparse intraganglionic distribution of NADH-dp neurons is characteristic, as this was also observed in the myenteric plexus of the ileum of Wistar rats (Miranda-Neto *et al*., 2001).

The density of neurons distributed through the circumference of the ileum was visibly heterogeneous, with a greater frequency of neurons in the region close to the mesenteric insertion than in the antimesenteric region. The presence of a greater number of neurons in the mesenteric region than in the antimesenteric region of the ileum of Wistar rats and in the jejunum of cats was also observed by Miranda-Neto *et al*. (2001) and Leaming and Cauna (1961), respectively. The same was found in the ascending colon of rats of Wistar lineage, where Sant'Ana *et al*. (1997) found a greater density of NADH-dp neurons in the antimesocolic region than in the intermediate region.

The difference in neuronal density in the same intestinal segment may be related to the variation in the thickness of the muscular tunica, as stated by Tranin *et al*. (2001). In regions with thicker and more vascularized muscular layers, the number of myenteric neurons is greater (Saffrey and Burnstock, 1994). The fact that the distribution of neurons is not homogeneous along the circumference of the ileum in Holtzman rats must be taken into account in research on the myenteric plexus when using this lineage of rats in experimental models. Miranda-Neto *et al*. (2001) stated that the inobservance of such characteristics might lead to incorrect findings, especially if the interest is the quantitative comparison of neurons between experimental groups that deal with neuronal degeneration and its respective control.

In the intestinal segment analyzed, myenteric neurons from the mesenteric and antimesenteric regions of the ileum of Holtzman rats were classified into small, medium and large sizes. In this classification, medium-sized neurons predominated in both regions. The same was observed by Torrejais *et al.* (1995) in the ileum of Wistar rats. With regard to the presence of neurons of different sizes in the myenteric plexus, Gabella (1971) stated that the small neurons represent an intrinsic part of the plexus, responsible for motor activity, while, according to Burnstock (1959), the large neurons are sensitive to and related to the peristaltic arch reflexes.

Conclusion

It can be concluded that in the ileum of rats of Holtzman lineage, the NADH-diaphorase positive myenteric neurons have a similar location and similar morphometric and quantitative characteristics to those described in previous literature for rats of Wistar lineage.

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