Arq Neuropsiquiatr 1997;55(3-A):460-466

NEURON NUMBER IN THE MYENTERIC PLEXUS OF THE ASCENDING COLON OF RATS

A COMPARATIVE STUDY USING TWO STAINING TECHNIQUES

DÉBORA M.G. SANT'ANA; MARCÍLIO H. MIRANDA-NETO**; SONIA L. MOLINARI**; MARCO ANTÔNIO SANT'ANNA****

ABSTRACT - We carried out this study with the purpose of comparing the neuronal density in antimesocolic and intermediate regions of the colon of rats. We used the ascending colon of ten seven-months old Wistar rats. With the Giemsa method we found 29046 neurons/cm^2 on the antimesocolic region and 30968 neurons/cm^2 on the intermediate regions. With the NADH-diaphorase technique 12308 neurons/cm^2 on the antimesocolic regions and 8798 neurons/cm^2 on the intermediate regions were evidenced. The number of NADH-diaphorase positive neurons is significantly less than the number of Giemsa-stained neurons, and that this difference is enhanced on the intermediate regions of the intestinal circumference. Therefore, to compare the number of neurons of an intestinal segment of a same species at the same age, it is necessary to take into consideration the technique employed and the region of the intestinal circumference from where the sample was obtained.

KEY WORDS: myenteric plexus, ascending colon, enteric neurons.

Número de neurônios no plexo mientérico do cólon ascendente de ratos: estudo comparativo usando duas técnicas de coloração

RESUMO - Realizamos este estudo com o objetivo de comparar a densidade neuronal evidenciada nas regiões antimesocólica e intermediária do colo de ratos. Utilizamos o colo ascendente de 10 ratos Wistar com 7 meses de idade. Encontramos com o método de Giemsa 29046 neurônios/cm^2 na região antimesocólica e 30968 neurônios/cm^2 nas regiões intermediárias. Com a técnica histoquímica da NADH-diaforase encontramos 12308 neurônios/cm^2 na região antimesocólica e 8798 neurônios/cm^2 nas regiões intermediárias. O número de neurônios NADH-diaforase positivos é significantemente menor que o número de neurônios corados com o método de Giemsa, sendo que esta diferença se acentua nas regiões intermediárias da circunferência intestinal. Portanto, para comparar o número de neurônios de um segmento intestinal de uma mesma espécie e numa mesma idade, é necessário levar em consideração a técnica empregada e a região da circunferência intestinal em que se obteve a amostra.

PALAVRAS-CHAVES: plexo mientérico, colo ascendente, neurônios entéricos.

One of the features of the intramural innervation of the digestive tract is its large number of neurons, which exhibit a numerical magnitude as great as that of the spinal cord[9]. Many investigators, described their existence and morphology[27]. Many morphological, physiological and pharmacological studies demonstrated the great degree of independence of this system, that even in face of denervation (removal of nerve fibers from the central nervous system or of autonomic ganglia) maintains its

This study represents a part of thesis submitted of the Master degree in Biological Sciences. *Professor of Universidade Estadual de Maringá and Universidade Paranaense; **Professor of Universidade Estadual de Maringá; ***Professor of Universidade Paranaense. Aceite: 24-março-1997.

Dra. Débora M.G. Sant'Anna - Departamento de Ciências Morfofisiológicas, Universidade Estadual de Maringá - Av. Colombo 5690 Bloco H-79 Sala 01 - 87020-900 Maringá PR - Brasil.

functions in an almost unchanged pattern. The existence of distinguised histomorphological and physiological features on these neurons led the inclusion of the enteric nervous system as an independent division of the autonomic nervous system[16].

Morphologically the enteric nervous system is organized in plexuses ganglionated or not[10]. Among the ganglionated plexuses the most important are the submucous and the myenteric[13,16,18]. For the study of the enteric neurons many techniques have been employed, such as silver impregnation[22], methylene blue[2,4,18], histochemical techniques[1,11,14,30], immunohistochemistry[5], immunofluorescence[13], electronic microscopy[13,29] and others. For the quantitative analysis, the stainings with the Giemsa method[3] and the technique for nerve cells through the activity of the NADH-diaphorase enzyme[11] are among those most employed.

Criticism is found on the literature concerning the technique with methylene blue (Giemsa technique) because it stains enteric glial cells and other non-neuronal elements[1]. These authors propose the use of the NADH-diaphorase histochemistry, since it does not stain glial cells. In the care of the NADH-diaphorase method, for according with some authors the evidencing of the neurons is directly related with the time of exposition of the material to the reagents and can vary from no staining of small neurons to staining of some of these and glial cells, smooth muscle fibers and fibroblasts[30].

Besides variation on neuronal density, attributed to the different techniques, numerical differences according to the region of intestinal circumference from which samples has been obtained are also reported[1,18]. These facts prompted us to carry out this study, with the purpose of comparing the neuronal density observed with the Giemsa and NADH-diaphorase techniques on the antimesocolic and intermediate regions of the ascending colon of rats.

MATERIAL AND METHODS

We used the ascending colon of ten *Rattus norvegicus*, Wistar strain, from the Central Biotery of the Universidade Estadual de Maringá.

We selected male rats seven months old (429.1 ± 35.2 g body weight). The animals were raised in individual cages, receiving water and NUVILAB (Recommended by the National Council of Research and National Institute of Health, USA), ration *ad libitum*. The animals were killed under ether anesthesia. The interval of time from killing to the beginning of the technique preparation was about one minute and thirty seconds.

For quantitative analysis, samples of the ascending colon of five animals were to whole-mounted and stained with Giemsa, according to Barbosa[2]. The colon from the other five animals were used for NADH-diaphorase enzyme activity[11].

The quantitative counts on the antimesocolic region, included between 120° e 240°, and the intermediate region between 60° and 120° or 240° and 300°, considering 0° the region of insertion of the mesocolon[15,26]. The neurons present on 40 microscopical fields of each animal were counted in both techniques. We considered all the neurons of each field, discarding half-neurons in a field and considering in another. The area of microscopical field with 40X objective was 0.1735 mm^2.

We carried out the mean, standard deviation and variation coefficient of the number of neurons found in each region. Student's T test was used to compare differences between means. The significance level used was 5%.

We subjected the samples of the proximal segment of the ascending colon of three rats to fixation in solution of 10% neutral formol, dehydration in ascending series of alcohol, diafanization in xylene, inclusion in paraffin and transverse histological sectioning of 10 and 15 μm thickness. Sections were stained with hematoxilin-eosin.

Photographic documentation was obtained with photomicroscope Olympus BX50 and photographic equipment PM 10AK.

RESULTS

On the wall of the ascending colon we found the mucous, muscular and serous tunicas, the submucous net and the mucous muscular lamina. The muscular tunica of the mesocolic region (0°-60° and 300°-360°) was composed only of the circular layer. On the intermediate regions (60°-120°

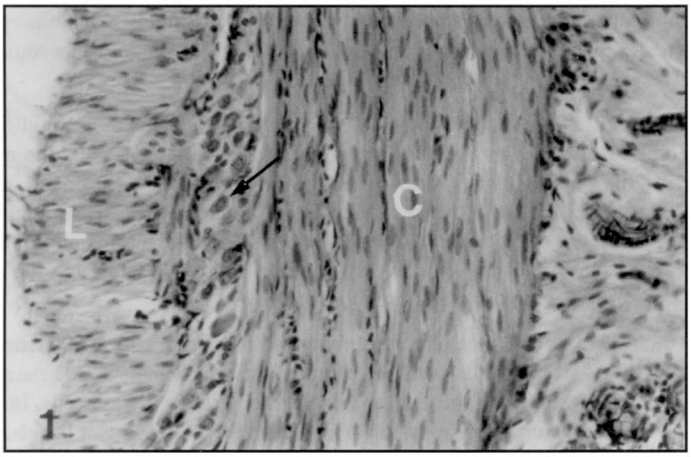

Fig 1. 15 μm transverse section of the intermediate region ascending colon: longitudinal (L) and circular (C) layers of the muscular tunica, neuron of ganglion of the myenteric plexus (arrow). H.E. Green filter. 183.7 X.

Table I. Incidence of myenteric neurons found on an area of 6.94 mm² of whole-mount preparations of the ascending colon of seven-month old rats, using the staining technique of Giemsa and histochemical technique of NADH-diaphorase. The significance level used was 5%.

	Regions			
	Antimesocolic		Intermediate	
Animals	Giemsa technique	NADH technique	Giemsa technique	NADH technique
1	2226	895	1776	631
2	2030	961	2521	833
3	2306	721	2353	540
4	2104	902	1879	624
5	1413	792	2217	425
X	2015.8	854.2	2149.2	610.6
s	353.46	96.13	314.9	149.6

Antimesocolic region: 120°-240° considering 0° the region of insertion of the mesocolon.
Intermediate region: 60°-120° and 240°-300° considering 0° the region of insertion of mesocolon.

and 240°-300°) we verified a thick longitudinal layer external to the circular layer, while on the antimesocolic region (120°-240°) the longitudinal layer was reduced (Fig 1).

On the mesocolic region we found neurons and ganglia of the myenteric plexus between the muscular fibers and the subserous connective tissue. On the intermediate and antimesocolic regions we found myenteric neurons and ganglia between the circular and longitudinal muscular layers and sometimes among the muscular fibers of the circular layer (Fig 1).

In an area of 6.94 mm² we verified, through the quantitative analysis of the Giemsa-stained whole-mount preparations, an average of 2015.8 neurons (29046 neurons/cm²) on the antimesocolic region and 2149.2 neurons (30968 neurons/cm²) on the intermediate region. On the whole-mount preparations stained with NADH-diaphorase we found 854.2 neurons (12308 neurons/cm²) on the antimesocolic region and 610.6 neurons (8798 neurons/cm²) on the intermediate region (Table 1).

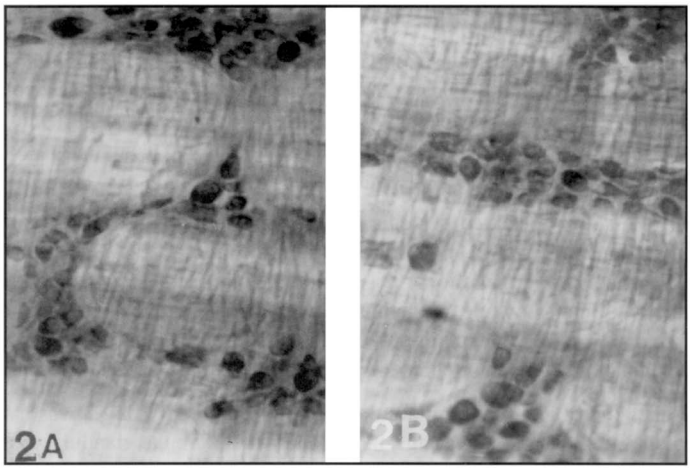

Fig 2. Whole-mount of ascending colon stained by Giemsa group of nerve cells forming ganglions of the myenteric plexus in the antimesocolic region (A) and intermediate region (B).Green filter. 183.7 X.

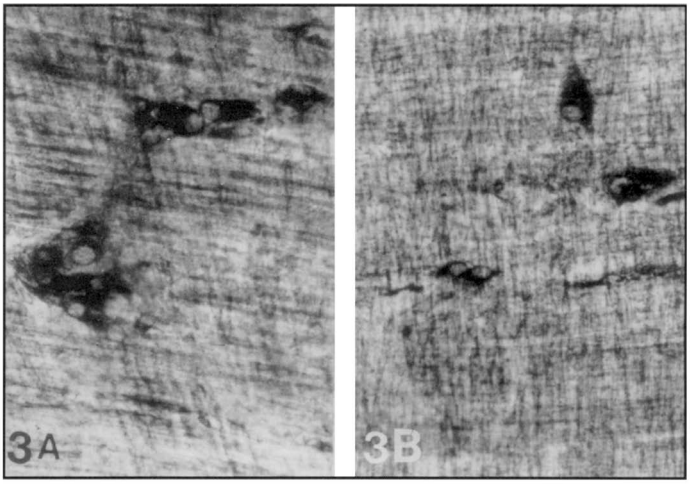

Fig 3. Whole-mount of ascending colon NADH-diaphorase group of nerve cells forming a ganglion of the myenteric plexus in the antimesocolic region (A) and intermediate region (B). Green filter. 183.7 X.

Figures 2 and 3 illustrate the neuronal density on the antimesocolic and intermediate regions with the Giemsa (Figs 2 A and 2B) and NADH-diaphorase (Figs 3A and 3B) techniques.

When we compared the averages of the antimesocolic and intermediate regions obtained with the Giemsa method (Table 1) we did not find significant difference (t=0.63; cv=2.31). We found a significant difference between the averages obtained with the NADH-diaphorase method (Table 1) on the two regions of intestinal circumference (t=3.09; cv=2.31). We also found a significant difference between the averages of neuronal number on the antimesocolic region obtained with the Giemsa (2015.8) and NADH-diaphorase (854.2) methods (t=7.12; cv=2.31). The difference between the averages obtained on the intermediate region with Giemsa (2149.2) and NADH-diaphorase (610.6) also attained significance (t = 9.91; cv = 2.31).

DISCUSSION

Like Mello[23] reported, on the mesocolic region we did not find the longitudinal layer of the muscular tunica, while on the intermediate (60°-120° and 240°-300°) and antimesocolic (120°-240°) regions the muscular tunica was composed of the circular and longitudinal layers, although not exhibiting taenias of longitudinal muscle, as described for humans[6,20] and domestic animals like pigs and horses[7].

Most of the authors consulted describe that the myenteric ganglia are found between the circular and longitudinal layers of the muscular tunica of the digestive tract[10,18,24]. On the ascending colon of rats we found a similar ganglionar position on the intermediate and antimesocolic regions of the intestinal circumference, while on the mesocolic regions the myenteric ganglia were located between the muscular fibers of the circular layer and the subserous connective tissue, as found by Iwanov[19] on the glandular stomach of chickens and by Mello[23] on the ascending colon of rats.

The presence of ganglia among muscular fibers of the circular layer was also reported by Irwin[18] on the pyloric regions of guinea-pigs, by Molinari et al.[24] on the glandular stomach of ducks and by Mello[23] on the ascending colon of rats.

Through the quantitative analysis we verified that on the ascending colon of seven months-old rats an average of 29046 neurons/cm^2 on the antimesocolic region and 30968 neurons/cm^2 on the intermediate region is found with the Giemsa method. Employing the same technique other authors, such as Mello[23] in 60 days-old rats, found on average 33323 neurons/cm^2, while Barbosa[2] found on the colon of adult rats 11082 neurons/cm^2.

With the histochemical technique of NADH-diaphorase we found, on average, 12308 neurons/cm^2 on the antimesocolic region and 8798 neurons/cm^2 on the intermediate region. On the other hand, Santer and Baker[27] with the same technique, one-hour exposure to the reagent and 10X objective, found on the mesocolic region 14124 neurons/cm^2 in six months-old rats and 5128 neurons/cm^2 in 24 months-old rats.

Our results, when compared with those of the literature, demonstrate divergence concerning the neuronal density on the colon of rats. These divergences are related to several factors, such as the different techniques of staining employed, which do no allow comparison of, for example, values obtained with Giemsa and those obtained with NADH-diaphorase; comparisons between results of research in which countings were carried out considering the mesocolic, antimesocolic and intermediate regions and those in which the author counts neurons regardless the area of the intestinal circumference are also impaired, a fact[1,12,26] call the attention to influences of aging also exist both in animals and humans, which provoke decrease on the number of enteric neurons[27,28]. Also deserves consideration the period of exposure to the reagents, as pointed out by Young et al.[30].

We verified that, independently of the region considered (antimesocolic or intermediate) the number of neurons found with the Giemsa method is more than two-fold that found with the NADH-diaphorase technique, and this difference is enhanced on the intermediate region. Ali and Mclelland[1] state that the histochemical method for detecting NADH-diaphorase activity used by Gabella[11,12] and themselves, consistently stains neurons, being possible, moreover, to restrict the staining to the cell bodies so that they are readily identifiable and easy to count. They comment that, unfortunately, comparison of their results with those of other workers is extremely difficult due mainly to the fact that widely different techniques have been employed to stain the nervous tissue. They say that most investigators have used the empirical staining methods of silver or methylene blue, which are notoriously capricious, frequently failing to stain all the nervous elements, and often staining non-nervous tissue. We agree with the authors mentioned concerning the fact that the Giemsa method stains other elements, like nuclei of muscular fibers and enteric glial cells. Using 40X objective during countings it becomes relatively easy to distinguish glial cells, which possess small and elongated

nuclei, non-stained nucleoli and cytoplasm that stains more intensely than the nucleus because of the staining affinity for polyribosomes and rough endoplasmic reticulum (Nissl corpuscules)[6,20].

With the Giemsa staining it is possible to distinguish neurons with greater or lesser basophilic intensity, a parameter employed to assess the desintegration of the Nissl corpuscules on the processes of neuronal chromatolysis, as described by Erhart[8]; Cormack[6]. Natali and Miranda Neto[25] employed the Giemsa staining to assess the proportion of weakly to strongly basophilic neurons in animals subjected to desnutrition.

Our results lead us to disagree with Ali and Mclelland[1] and Gabella[14] when they state that the histochemical technique to evidence nerve cells by the NADH-diaphorase activity stains all neurons, once with this technique the number of neurons we found was much smaller than that found with the Giemsa method. We believe that in our experiment the values obtained with Giemsa are nearer to the total amount of neurons on the area studied, for all neurons, independently of physiological properties, have Nissl corpuscules to be stained.

The smaller number of neurons we found with the NADH-diaphorase technique is probably related to the time of exposure we used, which was 40 minutes, an interval which, according to Young et al.[30] does not stain the totality of neurons. In experiments with exposures of one hour or more that we have carried out in experimental protocols, we verified that, not only neurons, but enteric glial cells, fibroblasts and smooth muscular fibers were also stained. Gabella[12] and Young et al.[30] also verified the staining of glial cells with the NADH-diaphorase technique.

The fact that the NADH-diaphorase technique does not stain all the neurons makes it innapropriate for quantitative studies where the investigator has the purpose of analysing the totality of neurons in a species, but, in quantitative studies of comparative nature, where the purpose is to assess the presence of NADH-diaphorase positive neurons, this technique can be used and the data compared with those obtained with another technique which stains all the neurons, like Giemsa or immunohistochemical techniques. It can also be used in studies where the purpose is to compare the incidence of NADH-diaphorase positive neurons in different regions of the digestive tract of the same species with a phylogenetic aim, as well as in comparisons of animals subjected to different experimental conditions. Our reseach group is now using the NADH-diaphorase technique to compare the amount of NADH-diaphorase positive neurons on the large intestine of animals subjected to desnutrition with that of normally fed animals.

The literature has emphasized the validity of employing the histochemical technique that evidence diaphorases to localize nitric oxide (NO) and vasoactive intestinal peptide (VIP) producing neurons, for that it is preferable to employ ß-NADPH as substrate[17,21]. Santer[26] comments that the distribution of such NADPH-diaphorase activity in enteric neurons is identical to that of NO sintase (NOS) -like immunoreactivity, and further states that there is considerable evidence that the NOS and the NADPH-diaphorase are co-located with VIP on the myenteric neurons of rats. Keränen et al.[21] demonstrated that on the myenteric ganglia several neurons, often forming clusters, show NADPH-diaphorase reactivity and NOS-immunoreactivity and states that both are co-located.

CONCLUSIONS

1. On the ascending colon of rats, the number of neurons NADH-positive is significantly lesser than the number of neurons stained with the Giemsa method, and this difference enhances on the intermediate regions of the intestinal circumference (60°-120° and 240°-300°, considering the mesocolon insertion as 0°).

2. To compare the number of neurons of an intestinal segment of a same species and at the same age, it is necessary to take into consideration the technique employed and the region of the intestinal circumference from where the sample was obtained.

REFERENCES

1. Ali HA., Mclelland, J. Neuron number in the intestinal myenteric plexus of the domestic fowl (*Gallus gallus*). Zbl Vet Med C Anat Histol Embryol 1979;8:277-283.
2. Barbosa AJA. Auerbach's plexus of the albino rat. I. Quantitative Study of the ganglia and nerve cells in the caecum and colon. Rev Bras Pesq Med Biol 1973;6:253-262.
3. Barbosa AJA. Técnica histológica para gânglios nervosos intramurais em preparados espessos. Rev Bras Pesq Med Biol 1978;11:95-97.
4. Barbosa AJA., Tafuri WL. Ganglion cell number in hypertrophic colon above experimental stenosis. Braz J Med Biol Res 1983;16:165-169.
5. Bornstein JC, North RA, Costa M, Furness JB. Excitatory synaptic potentials due to activation of neurons with short projections in the myenteric plexus. Neuroscience 1984;11:723-731.
6. Cormack DH. Ham histologia. Ed 9. Rio de Janeiro: Guanabara Koogan, 1995:273-287, 384-404.
7. Dellmann HD., Brown EM. Histologia Veterinária. Rio de Janeiro: Guanabara Koogan, 1982:200-201.
8. Erhart EA. Neuroanatomia. Ed 4. São Paulo: Atheneu, 1974:3.
9. Furness JB., Costa M. Types of nerves in the enteric nervous system. Neuroscience 1980;5:1-20.
10. Furness JB., Costa M. The enteric nervous system. Edinburg: Churchill Livingstone, 1987.
11. Gabella G. Detection of nerve cells by a histochemical technique. Experientia 1969;25:218-219.
12. Gabella G. Fine structure of the myenteric plexus in the guinea-pig ileum. J Anat 1972;111:69-97.
13. Gabella G. Innervation of the gastrointestinal tract. Int Rev Cytol 1979;59:129-191.
14. Gabella G. Fall in the number of myenteric neurons in aging guinea pigs. Gastroenterology 1989;96:1487-1493.
15. Gabella G. On the plasticity of form and structure of enteric ganglia. J Auton Nerv Syst 1990;30:59-66.
16. Gershon MD. The enteric nervous system. Ann Rev Neurosci 1981;4:227-272.
17. Hope BT, Vincent SR. Histochemical characterization of neuronal NADPH-diaphorase. J Histochem Cytochem 1989;37:653-661.
18. Irwin DA. The anatomy os Auerbach's plexus. Am J Anat 1931;49:141-165.
19. Iwanow IF. Die sympatische Innervation des Verdaungstraktes einiger Vogelarten. Z Mikrosk Anat Forsch 1930;22:469-492.
20. Junqueira LCU., Carneiro J. Histologia básica. Ed 8. Rio de Janeiro: Guanabara Koogan, 1995:245-267.
21. Keränen U., Vanhatalo S., Kiviluoto T., Kivilaakso E., Soinila S. Co-localization of NADPH-diaphorase reactivity and vasoactive intestinal polypeptide in human colon. J Auton Nerv Syst 1995;54:177-183.
22. Matsuo H. A contribution on the anatomy of Auerbach's plexus. Jpn J Med Sci Anat 1934;4:417-428.
23. Mello EVSL. Estudo da morfologia da parede e dos gânglios do plexo mientérico do colo proximal de ratos. Dissertação (Mestrado), Universidade Estadual de Maringá. Maringá, 1995.
24. Molinari SL, Pereira MS, Souza RR, Miranda-Neto MH. Estudo morfológico do plexo mientérico do estômago glandular do pato (*Anas* sp.). Unimar 1994;6:419-426.
25. Natali MRM., Miranda-Neto MH. Effects of maternal proteic undernutrition on the neurons of the myenteric plexus of duodenum of rats. Arq Neuropsiquiatr 1996;54;273-279.
26. Santer RM. Survival of the population of NADPH-diaphorase stained myenteric neurons in the small intestine of aged rats. J Auton Syst 1994;49:115-121.
27. Santer RM., Baker DM. Enteric neuron numbers and sizes in Auerbach's plexus in the small and large intestine of adult and aged rats. J Auton Nerv Syst 1988;28:59-67.
28. Souza RR., Moratelli HB., Borges N., Liberti EA. Age-induced nerve cell loss in the myenteric plexus of the small intestine in man. Gerontology 1993;39:183-188.
29. Tafuri WL. Ultrastructure of the vesicular component in the intramural nervous system of the guiena-pig's intestines. Z Naturforschg 1961;19:622-625.
30. Young HM, Furness JB, Sewell P, Burcher EF, Kandiah CJ. Total numbers of neurons in myenteric ganglia of the guinea-pig small intestine. Cell Tissue Res 1993;272:197-200.