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Effect of age on the myosin-V immunoreactive myenteric neurons of rats ileum

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ABSTRACT: Alterations in the gastrointestinal neuromuscular function related to age have been demonstrated in human and animal models. This study analyzes the effects of the aging process on the area of the neuronal cell bodies of the myenteric plexus in the antimesenteric and intermediate regions of the ileal circumference of Wistar, 12 month-old in comparison 3 month-old animals. The ileum was removed and whole-mount preparations immunostained by the antibody anti-myosin-V were processed. The morphometric analyses were performed using a computerized image analysis system, with a subsequent distribution of neurons by size in intervals of 100 μm^2. The cellular body morphometry revealed a significant increase in the size of the myosin-V- immunoreactive myenteric neurons from 12 month -old animals when compared with 3 month-old animals. However, significant differences between the regions were not observed; these observations were not age-dependent. The implications of these results in relation to the increase of the body weight, size of the small intestine, general organization of the myenteric plexus, staining method of neurons and the possible factors involved in the regulation and/or control of the volume of neronal cells due to aging, are discussed.

Introduction

The aging process at the level of gastrointestinal tract involves structural and functional changes such as in the reduction in the frequency and amplitude of the peristaltic movements, in the digestion and absorption of nutrients and in the intestine immunity. According to Hall (2002), Wade (2002), and Wade and Cowen (2004), several gastrointestinal disorders become more common in the elderly, especially motor dysfunctions.

Since the Enteric Nervous System (ENS) is responsible for coordinating and integrating the intestinal activities, several studies have been conducted in this system to establish an etiology for the motor disorders are typical in elderly . The myenteric plexus neurons, located between the circular and longitudinal muscular layers, are the most studied intestinal motor modulators. Progressive reduction in the number of these neurons due to aging has been reported in the esophagus (Meciano-Filho *et al*., 1995), stomach (El-Salhy *et al*., 1999), small intestine (Santer and Baker, 1988; Gabella, 1989; El-Salhy *et al*., 1999; Cowen *et al*., 2000; Phillips and Powley, 2001; Phillips *et al*., 2003, 2004) and large intestine (Santer and Baker, 1988; Gomes *et al*., 1997; El-Salhy *et al*., 1999; Phillips and Powley, 2001; Phillips *et al*., 2003, 2004) of guinea pigs, rats, mice and human beings, suggesting these neurons are implicated in motor dysfunctions due to the aging process.

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Besides the loss of neurons, changes in the organization of the myenteric plexus and in the intestinal size have been observed during the ontogenesis (Gabella, 1971; Dunlap *et al*., 1988; Gabella, 1989; Amenta, 1993; Santer, 1994; Johnson *et al*., 1998; Schäfer *et al*., 1999; Cowen *et al*., 2000; Phillips and Powley, 2001; Phillips *et al*., 2003, 2004). Morphometrical analyses on the neuronal cell bodies of the myenteric plexus has revealed an increase in the size of neurons related to the age in the small and large intestine of rats (Gabella, 1971; Santer and Baker, 1988; Schäfer *et al*., 1999; Cowen *et al*., 2000; Phillips *et al*., 2003) and in the human esophagus (Meciano-Filho *et al*., 1995).

Morphological and quantitative studies of the myenteric plexus usually focus on a specific segment of the digestive tube. However, several authors have been pointing out the occurrence of a variability in the neuronal density, in a single gastrointestinal segment, when different regions of the stomach or of the intestinal circumference were compared (Santer, 1994; Fregonesi *et al*., 1998; Miranda-Neto *et al*., 2001). This fact should be considered of fundamental importance in quantitative and morphological analyses because phylogenetic and pathophysiological comparisons as well as those related to the aging process can be erroneously interpreted due to random selection of neurons along the intestinal circumference (Miranda-Neto *et al*., 2000).

Therefore, taking into consideration precious reports this study analyzes the effects of the aging process on the area of the neuronal cell bodies immunoreactive to myosin-V of the myenteric plexus located in antimesenteric and intermediate regions of the ileal circumference of Wistar rats, aged 3 and 12 months.

Material and Methods

Animal treatment

All the procedures of this study regarding the use of animals were in agreement with the ethical principles adopted by the Brazilian School of Animal Experimentation (COBEA) and approved by the Ethics Committee in Animal Experimentation of the State University of Maringá.

For this study, we isolated the ileal segments of 10 male Wistar rats (*Rattus norvegicus*). The animals were kept in individual cages, at constant temperature, with a photoperiod of 12 hours, receiving standard chow NUVILAB-NUVITAL® and water *ad libitum*.

After being anesthetized intraperitoneally with sodium Thiopental (Thionembutal®) (40mg/kg of body weight), five animals aged 3 months and five aged 12 months were sacrificed. Laparotomy was performed, followed by perfusion, collection and measurement of the small intestine, specifically the ileum.

Immunohistochemistry of the myenteric plexus

The antibody anti-myosin-V used in this study was, recently described for the immunostaining of neurons of the enteric nervous system by Drengk *et al*. (2000); it is specific and has the advantage of staining only neurons (sensorial, motor or interneuron) and their processes.

Animals were perfused with saline solution (1 ml/g body weight) followed fixative solution containing 10 mM sodium periodate, 75 mM lysine, 1% paraformaldehyde in 37 mM phosphate buffer, pH 7.4. Immediately after perfusion, each ileum was removed and the fixative solution was gently injected into the lumen, distending the muscular layer. After applying ligatures to maintain the distension, the samples were postfixed in the same solution as above for 1 hr, dehydrated in ethanol (50%, 70%, 80%, 90%, 95% and 100%), cleared in xylol, rehydrated in ethanol (100%, 95%, 90%, and 80%) and stored in ethanol 70%. The ileal fragments were opened at the mesenteric border and dissected under a stereomicroscope with trans-illumination. The mucosa and submucosa layers were removed to obtain the whole-mount preparations of the muscular layer containing the myenteric plexus. The tissues were washed four times in PBS (0.1M, pH 7.4) and blocked for 1 hr in PBS with 2% BSA, 2% goat serum and 0.5% Triton X-100 at room temperature. Immunostaining proceeded with the incubation of tissues in 0.89 μg/ml of affinity-purified antibody specific to the myosin-V medial tail domain (Espreafico *et al*., 1992; Buttow *et al*., 2003) diluted in the PBS with 2% BSA, 2% goat serum and 0.1% Triton X-100, at room temperature and under shaking (24 hr). After incubation, the fragments were washed twice in PBS with 0.1% Triton X-100 and twice in PBS with 0.05% Tween 20. Soon after, the tissues were incubated with 10 μg/ml secondary antibody conjugated with peroxidase for 24 hours at room temperature under agitation and washed four times during 15 min in PBS with 0.05% Tween 20. The immunoreaction was developed with 0.75 mg/ml Diaminebenzidine in PBS and 0.03% H_2O_2 for 10 min. Samples were placed in a gel mounting medium containing 50% glycerol, 0.07 g/ml gelatin in PBS and 2 μl/ml phenol.

Neuronal morphometrical analysis

For this analysis, images of the cell bodies of myosin-V- immunoreactive myenteric neurons of the whole-mount preparations were previously taken with a high-resolution digital camera coupled to a optical microscope Olympus BX50 with 40X objective. Area of the cellular bodies from Fifty neurons in the antimesenteric region and fifty neurons in the intermediate region of the ileal circumference from each animal were photographed, 250 cell bodies per region and 500 cell bodies per age group, therefore, were analyzed. They were measured with aid of a computerized image analysis system (Image-Pro Plus® 4.5 – Media Cibernetics). Then, the neurons of each age group were classified by size at intervals of 100 μm^2; and after that the corresponding percentage for each age and region were calculated.

Statistical analysis

The quantitative data obtained were analyzed by the test "t" of Student, with a significance level of 5%. The statistical analysis was accomplished in the statistical program GraphPad Prism® (GraphPad Software, Inc.).

Results

Body weight and length of the small intestine

Animals aged 12 months presented a significative increase in their body weight compared with 3 month-old animals (3 months: 404.50 ± 21.21 g; 12 months: 465.80 ± 40.52 g). However, the length of the small intestine was not altered by age (3 months: 127.80 ± 3.96 cm; 12 months: 124.50 ± 11.05 cm).

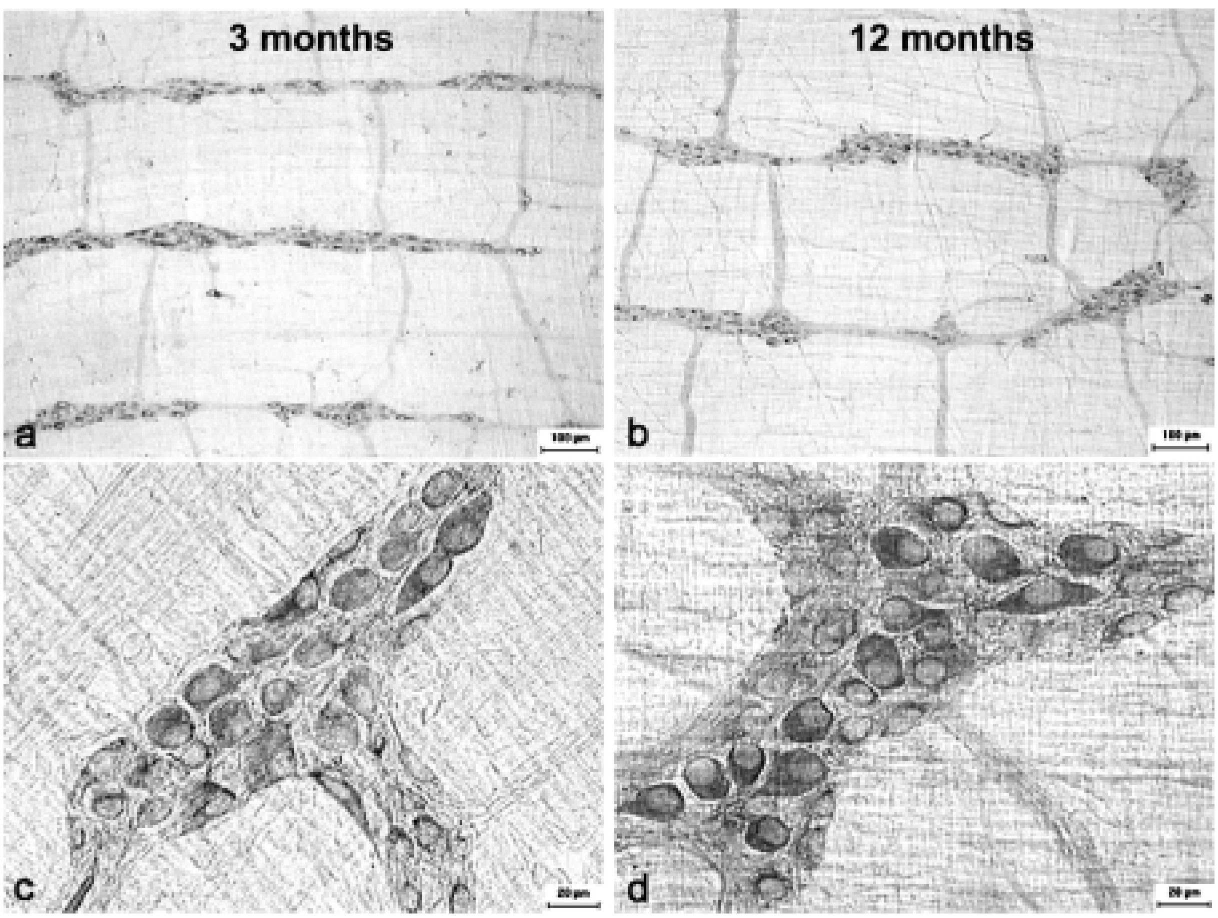

FIGURE 1. Rectangular ganglion organization interconnected with nervous fibers in the myenteric plexus of the ileum of rats aged (a) 3 months and (b) 12 months. Different sizes and color intensity of the myosin-V-immunoreactive neurons of a myenteric ganglion in rats aged (c) 3 months and (d) 12 months.

Myosin-V immunoreactive myenteric neurons

Regardless of the region of the ileal circumference or the animal age the general organization of the myenteric plexus was not altered; the myosin-V immunoreactive myenteric neurons were arranged in ganglions forming predominantly rectangular meshes with interweaved nervous fibers (Fig. 1a, 1b). Neurons present in the ganglions were differentiated in both groups based on the myosin-V immunoreactivity. Neurons from 12 month-old animals, however, showed a more evident cell body staining and clearer nervous fibers (Fig. 1c, 1d).

The distribution of neurons by size at intervals of 100 μm² in the antimesenteric and intermediate regions from 3-month-old animals showed a variation in the cell body area ranging from 61.50 to 678.76 μm², with a larger percentage of neurons varying from 101 to 200 μm² in both regions (Fig. 2a). In 12 month-old animals the cell body area area ranged from 86.72 to 813.98 μm²; the neuronal predominance in the antimesenteric region ranged from 201 to 300 μm², while in the intermediate region did from 101 to 200 μm² (Fig. 2b).

The analysis of the overall mean of the cellular area in both ages revealed a statistically significant increase in the neuronal cell body size in the ileum of the 12 month-old animals. Table 1 shows that there was no significant difference in both age groups. Comparison

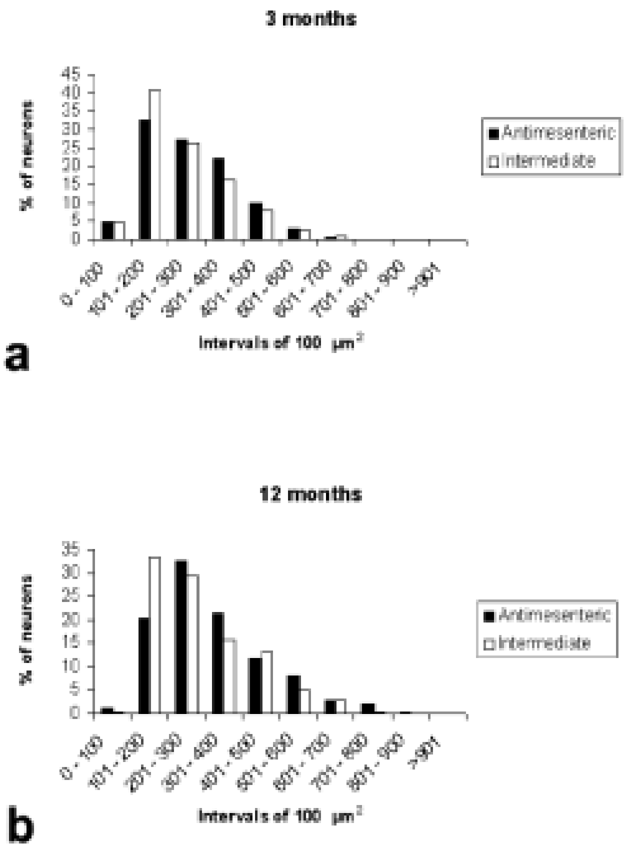

FIGURE 2. Distribution by size of myosin-V-immunoreactive myenteric neurons in the (✭) Antimesenteric and (✱) intermediate regions of the ileum of animals aged (a) 3 months and (b) 12 months, classified in intervals of 100 μm².

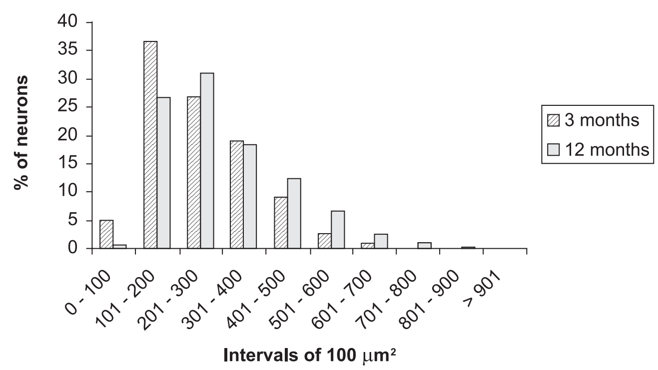

FIGURE 3. Distribution by size of myosin-V-immunoreactive myenteric neurons in both regions, classified in intervals of 100 μm². Animals aged 3 months (✭) and 12 months (✱).

TABLE 1.

Area of the neuronal cell bodies (μm²) in the antimesenteric and intermediate regions of the ileum of animals aged 3 and 12 months (n=5). Mean ± standard deviation.

	Animals	
Region	**3 months**	**12 months**
Antimesenteric	262.75 ± 27.59a [(1,2)]	316.42 ± 44.97a
Intermediate	243.06 ± 31.40a [(1,2)]	288.10 ± 39.66a
Total mean	252.90 ± 24.94a [(2)]	302.26 ± 39.55b

[(1)] Mean followed by the same letter (vertical), do not statistically different (test t of Student, p> 5%)
[(2)] Mean followed by the same letter (horizontal), do not statistically different (test t of Student, p> 5%)

of distribution of neurons by size in the whole ileum of 3-11-months-old animals showed an an evident increase at neurons with a size larger than 201 μm^2 in the older animals when compared to the younger (Fig. 3).

Discussion

Comparison of the body weight between animals from both groups of the two age groups (3 and 12 months) showed a significative increase in this parameter in the older animals, which may be justified by normal growth of the animal. According to Phillips and Powley (2001), the body weight varies with age, however, rats keep gaining weight until approximately 21-month-old, after that, they lose weight very fast.

Unlike the body weight, the length of the small intestine remained unaffected in both age groups. A similar result was observed by Phillips and Powley (2001) in their study with rats aged 3, 12, 21, 24 and 27 month-old. They observed that the intestinal length was maintained until the age of 12 months. After 21 months of age, a significative increase in the length was observed.

Other studies reinforce the concept that the age factor could interfere in the intestinal length leading to a gradual increase, (Dunlap *et al*., 1988; Johnson *et al*., 1998; Phillips *et al*., 2003, 2004) and guinea pigs (Gabella, 1989). In opposition to these studies, Stump (1999) supported that aging leads to a reduction in the organ size , which is an usual consequence of age and not an increase, due to the smaller metabolic rate observed with aging.

Myosin-V immunoreactive myenteric neurons

Among the different current techniques for staining of myenteric neurons, myosin-V motor protein immunolocalization is one of the most useful method and it has been used in many studies (Drengk *et al*., 2000; Buttow *et al*., 2003 and Zanoni *et al*., 2005).

According to Hasson and Mooseker (1997) and Langford and Molyneaux (1998), in the nervous cells, the myosin-V protein can be found in the pre-synaptic terminals, in organellas and in vesicles close to the plasmatic membrane. The protein seems to be related to the membrane dynamics (endocytosis and exocytosis), axoplasm transport and neurotransmitter release; thus, it is considered a useful and precise element for neuronal morphoquantitative demarcation and investigation.

The immunostained whole-mount preparations presented in this study show that the general organization of the myenteric plexus was maintained though the age. There was no variation between the antimesenteric and intermediate regions of the ileal circumference; the myenteric neurons were arranged in ganglions interconnected with fibers, forming predominantly rectangular nets.

Santer (1994), when studying the small intestine of 4, 24 and 30 month-old rats, observed (like we did) that the pattern of the plexus was preserved during the aging process. However, a different result was reported by Gabella (1989), who noticed changes in the ganglion structure and shape and in the architecture of the plexus in the small intestine of guinea pigs aged 26-30- months when compared to 3-4-month animals. The author pointed out the existence of larger distances between the ganglions and also fewer ganglions per area in the older animals. He attributed these differences to a necessary structural reorganization to accommodate the remaining elements in order to ensure the physiologic properties, since aging would lead to neuronal loss.

Comparison of neuronal staining intensity between both ages, showed heterogeneity. We also noticed a more evident staining in the neurons and nervous fibers of the myenteric plexus of the ileum of the older animals (12 months).

Despite of the use of different neuronal markers, Schäfer *et al*. (1999) also observed higher coloration intensity associated to the age in the duodenum and colon of rats. The intensity of the Cuprolinic Blue staining (total population) varies according to the age and the intestinal segment; staining will be more intense when the animal is older. Santer (1994), when studying 4, 24 and 30 month-old rats, observed higher intensity in the nervous fibers of the sub-population NADPH-diaphorase of the myenteric plexus from older animals.

Taking into account the method we employed in this study (immuno- localization of the myosin-V protein), the signal heterogeneity observed in neurons at both age groups might indicate different levels of neuronal activity (Drengk *et al*., 2000). A more intense staining of neurons and nervous fibers in 12 month-old animals might be related to an increase in the cellular activity and expression of the myosin-V that could be more concentrated in the myenteric plexus of these animals.

Azevedo *et al*. (2004), studied the expression of myosin-V thought the development of the chicken nervous system and reported a progressive increase in myosin-V expression during the several stages of the embryogenesis. This supports its general role in neuronal function and might demonstrate its temporary and target-site expression in the nervous cells, thus, suggesting that the expression of myosin-V is related to its recruitment for specific cellular tasks, that depends on the cellular demand. Calliari *et al*. (2002) have also described this distinct temporal pattern in the expression of myosin-V during the process of regeneration of the sciatic nerve after nervous injury.

We propose that this increase in the staining intensity of neurons and nervous fibers in the 12 month-old animals might indicate an adaptation of the neurons to a new situation: the cellular alterations promoted by the aging process. These could lead to a higher mobilization of intracellular components, including an increase in the transport of neurotransmitters to ensure the functional activity of the myenteric plexus after neuronal losses that may have taken place due to age. We infer, therefore, that the myosin-V protein may be involved in the plastic changes that take place not only during the developing stage, as already reported by Tilelli *et al*. (2003) for myosin-V in the rat brain, but also due to aging.

In regards to neuronal size, we observed that the obtained means in the antimesenteric and intermediate regions of the ileum circumference were not statistically different in any of the two age groups studied (3 and 12 months), suggesting the maintenance of the neuronal distribution between those regions.

A similar result was obtained by Miranda-Neto *et al*. (2000) when they analyzed the neuronal size in these same regions of the duodenum of 7 month-old rats.

Although the size and neuronal distribution between the two confronted regions were maintained (which allowed adding the two regions), the comparison of the mean of the neuronal areas between the 3 and 12 months-old animals revealed there was an increase in the area of the neuronal cell body due to age.

The increase in the cell body of myenteric neurons through the aging process was already observed by Gabella (1971), Santer and Baker (1988), Schäfer *et al.* (1999), Cowen *et al*. (2000) and Phillips *et al.* (2003) in the small and large intestine of rats, and by Meciano-Filho *et al*. (1995) in the esophagus of human beings. However, little is known about the factors responsible for such growth with age.

Among the factors that could lead to such increase, the literature mentions some hypotheses such as the increase in the length of the intestine and thickness of the muscular layer (Gabella, 1971, 1989; Meciano-Filho *et al*., 1995; Schäfer *et al*., 1999).

The reorganization of the remaining neurons – including growth of the cellular body due to the neuronal reduction common with age, ensuring its functional role (Santer and Baker, 1988; Gabella, 1989; Saffrey and Burnstock, 1994; Gavazzi and Cowen, 1996). We can also consider the increase in the neuronal size – might take place due to the stress and functional demand on certain kind of neurons remaining from a gradual neuronal loss, thus, allowing the survival and adaptation of the remaining neurons to help the intestinal functions maintenance (Phillips *et al*., 2003).

Orr and Chen (2002) stated that the degenerative processes resulting from the advance of age might be compensated by the plasticity of the ENS, and alterations such as a reduction in the number of neurons and dysfunction in gastrointestinal motility are observed.

Taking to account the hypotheses presented here and the increase in the area of the cell bodies of the myenteric neurons immunoreactive to myosin-V of the 12 month-old animals, we can dismiss the possibility of an increase in the intestinal length as the triggering factor of the neuronal hypertrophy; our results showed it did not change. However, we can infer that 12 month-old rats need a neuronal adjustment due to the larger functional demand, which might be associated to the age-related neuronal loss, as already proved in ileum of rats (Santer and Baker, 1988; Cowen *et al*., 2000; Phillips and Powley, 2001; Phillips *et al*., 2003; Phillips *et al*., 2004) and it is shown by the larger neuronal size, and by the higher intensity in the myosin-V protein staining observed in our experiment.

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