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Autonomic Neuroscience: Basic and Clinical 122 (2005) 77 – 83

AUTONOMIC NEUROSCIENCE: Basic & Clinical

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# Effects of a hypoproteic diet on myosin-V immunostained myenteric neurons and the proximal colon wall of aging rats

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Received 13 August 2005; received in revised form 13 August 2005; accepted 15 August 2005

**Abstract**

The objective of this work was to analyze the morphoquantitative behavior of neurons of the myenteric plexus, as well as the morphometry of elements of the proximal colon wall of Wistar rats (*Rattus norvegicus*) fed a normoproteic (22%) and a hypoproteic (8%) diet, and sacrificed at 360 days of age. To perform the neuronal evaluation, we used whole-mount preparations of the proximal colon immunostained with the antibody anti-myosin-V. The neurons were quantified in 80 microscopic fields (16.98 $mm^2$/animal). The neuronal cell body morphometry was performed in 100 neurons/animal. Samples of the proximal colon were weighed and measured, and then submitted to routine histological processing. They were later stained using the hematoxylin–eosin method in order to carry out morphometric analysis on the mucosa and external muscular layers. The number of neurons and the neuronal cell body morphometry did not present significant differences between the studied groups. A significant reduction in the weight and length of the proximal colon and in mucosa layer thickness was observed in the animals fed with the hypoproteic diet. We concluded that the neuronal and non-neuronal components of the proximal colon adapted to the imposed nutritional condition, which guaranteed the maintenance of their functions.


*Keywords:* Hypoproteic diet; Myenteric neurons; Proximal colon; Intestinal morphometry; Myosin-V; Aging

## 1. Introduction

The myenteric plexus, one of the main components of the Enteric Nervous System, is formed by a network of ganglia, arranged in regular rows and interweaved by nerve fibers, which extend from the esophagus to the anal channel. It is located between the circular and longitudinal layer of the external muscular layer and plays a major role in intestinal motility control. Changes in the myenteric plexus may result in the development of several gastrointestinal pathologies, due to nutritional imbalance.

As most biomolecular reactions require the participation of proteins, each cell and each tissue may be affected when submitted to proteic deprivation conditions (Deo, 1978). However, cellular response to protein deficiency is time-dependent, i.e. it depends on the period that the nutritional deprivation situation begins and the duration of this period (Firmansyah and Sunoto, 1989).

Studies of protein restriction alterations in the morphology and physiology of the nervous system are justified, since this system consists of tissues with a low rate of cellular renewal; thus, changes in the number, shapes or functions of these cells may lead to drastic alterations in the organism (Deo, 1978). The use of rats as experimental models in the study of neurons of the enteric nervous system submitted to situations of proteic restriction in different phases of animal development, mainly the gestation and lactation phases (Natali and Miranda-Neto, 1996; Miranda-Neto et al., 1999; Brandão et al., 2003) and the adult phase (Araújo et al., 2003; Natali et al., 2003), is rather frequent; however, studies that include more advanced ages are scarce.

According to Mitchell et al. (1978), proteic needs do not decrease with age, and the necessity of some amino acids, due

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doi:10.1016/j.autneu.2005.08.005

to age, may even increase to "compensate" for functional alterations in the organism. They also point out that the main physiological alteration due to aging is the reduction in the number of working cells, which slows down metabolic processes.

The myenteric plexus acts on the intestinal wall, and the muscular layer is the target-tissue responsible for its maintenance, development and plasticity (Saffrey and Burnstock, 1994). The variations that take place in the peripheral neurons are concomitant with the variations in the organs innervated by them. This plasticity is not limited to the initial periods of body growth. It persists in the completely differentiated nervous tissue, with the potential to promote increase or reduction in neuronal cell volume to desired levels, depending on different conditions (Gabella, 1987). We consider that assessing myenteric neurons in situations of proteic restriction associated with aging is an excellent way to investigate neuronal plasticity and the behavior of intestinal layers.

We employed the myosin-V immunostaining technique to study the myenteric plexus. The unconventional myosins are a superfamily of actin-based motors responsible for a rich array of intracellular motility events. The immunolocalization of myosin-V has been used in whole-mount preparations of the myenteric and submucous plexus of rats (Drengk et al., 2000; Buttow et al., 2003, 2004; Zanoni et al., 2003, 2005), which allows us to identify the whole neuronal population of the myenteric plexus (Drengk et al., 2000).

Thus, the present study aims at morphoquantitatively analyzing the myosin-V immunoreactive myenteric neurons and the morphometry of elements of the proximal colon wall of aging Wistar rats fed a hypoproteic diet.

## 2. Materials and methods

### 2.1. Animal treatment

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

We used male Wistar rats (*Rattus norvegicus*) distributed into two groups: *Control*: fed a standard rodent diet—NUVILAB-NUVITAL® (recommended by the National Research Council and National Health Institute—USA)—with a proteic value of 22%; *Proteic Restriction*: fed a diet with a proteic value of 8%, obtained with the addition of corn starch to the NUVILAB® chow (Natali et al., 2000), for 150 days, starting on the 210th day of life. The diet was supplemented with water-soluble B Complex vitamins and a saline mixture (American Institute of Nutrition, 1977; Natali and Miranda-Neto, 1996; Natali et al., 2000, 2003, 2005). The animals were kept in individual cages with a photoperiod of 12 h and a temperature of 22±2 °C. Water and chow were offered ad libitum.

Animal body weight was verified monthly and food intake controlled one week/month. This control consisted of a daily offering of 100 g of chow to each animal and weighing the leftovers, thus obtaining the daily consumption of each animal.

At 360 days of age, the animals were weighed and intraperitoneally anesthetized with sodic Tiopental (Thionembutal®) (40 mg/kg of body weight), followed by blood collection by heart puncture to assess the total proteins (Biureto Method—LABTEST®), albumin (Bromocresol Green—LABTEST®) and globulins. After performing a laparotomy and perfusion using a saline and fixative solution, the proximal colon (characterized by the end of the ileum–cecum–colic vial to the disappearance of the oblique mucosa folds) was collected, measured and weighed. Part of the samples was used for whole-mount preparations and part for routine histological processing.

### 2.2. Immunohistochemistry of the myenteric plexus

The antibody we chose, anti-myosin-V, recently described for the immunostaining of neurons from the enteric nervous system (Drengk et al., 2000), is specific and it has the advantage of staining only neurons (sensorial, motor or interneuron) and their processes.

The 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 proximal colon 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 h, 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 colon fragments were open in the mesocolic border and dissected under a stereomicroscope with trans-illumination through the removal of the mucosa and submucosa layer, thus, obtaining whole-mount muscular layer preparations containing the myenteric plexus. The tissues were washed four times in PBS (0.1 M, pH 7.4) and blocked for 1 h 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 (48 h). 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 h at room temperature under agitation and washed four times during 15