

Protein Restriction Produces Alterations in Nitroergic Myenteric Neurons in the Proximal Colon in Rats

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Abstract

Aim: This study aimed to investigate the effects of severe protein restriction on the morphometric and quantitative aspects of neurons in the myenteric plexus of the proximal colon in rats. **Methodology:** Ten rats were divided into two groups: (i) a normally fed group (NG) that received commercial chow with 26% protein for 90 days and (ii) a protein restriction group (RG) that received chow that contained a reduced amount of protein (4%) for 90 days. Nitroergic neurons were evaluated by nicotinamide adenine dinucleotide phosphate (NADPH-diaphorase) histochemistry. Intestinal segments were dissected. The number of neurons was counted, and the area of cellular bodies was measured. **Results:** A significant (58.92%) increase in the number of neurons that expressed NADPH-diaphorase and significant decreases in the area of cellular bodies, nuclei, and cytoplasm were found in the RG compared with the NG. **Conclusion:** In conclusion, protein restriction (from 26% to 4%) increased neuronal population density and nitroergic myenteric neuron atrophy in the proximal colon in rats.

Keywords: protein deficiency, malnutrition, large intestine, NADPH-diaphorase, enteric neurons

Introduction

Malnutrition, especially the restriction of protein and energy, has been a problem for humanity and appears frequently as a cause of multiple changes in human development.

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It is considered a clinical manifestation that results from the adoption of an inappropriate diet or diseases that prevent the proper biological utilization of food intake.⁽¹⁾ Compromised digestive system function leads to malabsorption, alters intestinal motility,⁽²⁾ and may cause diarrhea in children who are less than one year old.⁽³⁾

Clinical symptoms, such as abdominal pain, constipation, fecal incontinence, and diarrhea, in malnourished individuals⁽⁴⁾ are indicative of morphofunctional changes in enteric neurons. Malnourished animals display changes in the myenteric plexus, but few studies have correlated the effects of malnutrition and neurochemical pathways specifically in the enteric nervous system (e.g., the overall population density of myenteric neurons).⁽⁵⁻¹³⁾ Moreover, few studies have investigated the effects of protein deficiency on the two predominant myenteric neuronal subpopulations:⁽¹⁴⁾ nitrergic neurons and cholinergic neurons.⁽¹⁵⁾ Therefore, when evaluating myenteric neurons, considering both subpopulations is important to correlate the results with the physiology of intestinal smooth muscles. The labeling of cholinergic neurons is quite complex,⁽¹⁶⁾ but they may be quantified by subtracting nitrergic neurons from the total neuronal population.⁽¹⁵⁾

The present study evaluated the population density and plasticity of nitrergic and cholinergic neurons in the myenteric plexus in the proximal colon in rats subjected to severe protein restriction.

Material and Methods

Experimental Design

All of the procedures in this study were approved by the Ethical Committee on Animal Experimentation of the University of Paraná. Ten adult (90-day-old) male Wistar rats (*Rattus norvegicus*) were used. They were randomly divided into two experimental groups: a normally fed group (NG; $n = 5$) and a group with restricted protein (RG; $n = 5$).

The rats were individually housed in plastic cages with metal grid lids in our room under controlled temperature and a 12 h/12 h light/dark cycle with food and water available *ad libitum*.

The NG received commercial rat chow (Nuvilab) that contained 26% protein for 90 days. During the same period of time, the RG received a special chow that containing a reduced amount of protein (4%) but maintained the recommended levels of vitamins and minerals. ⁽¹⁷⁾

After 90 days and 12 h of fasting, all of the rats were euthanized with an overdose of anesthetic according to the following protocol: 1.26 ml/kg Acepran, 1.26 ml/kg of 10% ketamine, 0.42 ml/kg of 2% xylazine, and 0.22 ml/kg of 1% atropine ⁽¹⁸⁾ by intramuscular administration.

Histochemistry for NADPH-diaphorase

The proximal colon of each animal in each group was removed, measured, washed, filled with phosphate-buffer saline (PBS; pH 7.4), fixed with 4% paraformaldehyde (Merck, Darmstadt, Germany) prepared in 0.1 M PBS for 30 min, immersed in 0.3% Triton X-100 in 0.01 M PBS, washed 10 times (10 min each) in PBS, and incubated for 60 min in the following (per 100 ml): 25 mg NBT, 50 mg β -NADPH (Sigma, Steinheim, Germany), and 0.3 ml of Triton X-100 and 0.1 M Tris-HCl (pH 6.0; GibcoBRL, New York, NY, USA). After incubation, the segments were washed three times in PBS (5 min each), opened at the insertion of the mesocolon, and immersed in 4% paraformaldehyde. ⁽¹⁹⁾

Whole-Mount Preparations

Whole-mount preparations of the muscular tunic that contained the myenteric plexus were obtained by dissection under a Motic SMZ-140 trans-illumination stereomicroscope, and the tunic mucosa and submucosa were removed. The samples were then dehydrated in serial aqueous solutions that contained increasing concentrations of ethanol, diaphanized in xylol, mounted on slides, and coverslipped with Permount (Fisher, Fair Lawn, NJ, USA).

Quantitative Analysis of NADPH-diaphorase-positive Neurons

All nicotinamide adenine dinucleotide phosphate-diaphorase-positive (NADPH-dp) neurons in 120 microscopic fields were counted, considering a homogeneous sample of the entire intestinal circumference. Half of the neurons were counted in alternate fields. The analyses were performed using an Olympus BX40 photonic microscope with a 40 \times objective. The total area of the colon analyzed per animal was 16.8 mm². The results are expressed as the number of NADPH-dpneurons per mm².

Estimation of NADPH-diaphorase-negative Neurons

The cholinergic subpopulation (NADPH-dn) of neurons can be obtained by subtracting NADPH-dp neurons from the total neuronal population. ⁽¹⁵⁾ Thus, we subtracted the number of NADPH-dp neurons obtained in the present study from the total number of myenteric neurons from the same animals reported previously. ⁽¹²⁾ The results are expressed as number of NADPH-dn neurons per mm².

Morphometric Analysis of NADPH-diaphorase-positive Neurons

The total area (μm^2) of cellular bodies and nuclei from 300 NADPH-dpneurons per animal per group was measured from images captured with a Moticom 2000 2.0 megapixel digital camera coupled to a MOTIC B trinocular light microscope using Motic Images Plus software, version 2.0. The proportion of the area of the nuclei relative to cellular bodies and coefficient of correlation were calculated. The neurons were classified in 50 μm^2 intervals according to the area of cellular bodies and 0.1 intervals according to the nucleus/cellular body proportion.

Statistical Analysis

The data were first analyzed using the D'Agostino-Pearson test or Shapiro-Wilk test for distribution analysis. Data with a normal distribution are presented as mean \pm standard deviation. In this case, Student's *t*-test for independent samples was used to compare groups. Data without a normal distribution are presented as medians and percentiles (25:75). In this case, the nonparametric Mann-Whitney test was used to compare groups. The Spearman test was used for correlation analyses. In all of the tests, values of $p < 0.05$ were considered statistically significant. All of the statistical analyses were performed using BioEstat 5.0 software.

Results

At the end of the experiment, the area of the proximal colon in the NG ($11.42 \pm 0.46 \text{ cm}^2$) was significantly different from the RG ($7.68 \pm 0.50 \text{ cm}^2$; $p < 0.0001$). The RG exhibited an increase in the density of nitrergic neurons compared with the NG. No group differences were found in the number of cholinergic neurons (Table 1).

Table 1: Mean \pm standard deviation of Giemsa-stained neuronal density (in mm^2), NADPH-diaphorase-positive (NADPH-dp) neurons, and estimation of NADPH-diaphorase-negative (NADPH-dn) neurons in normally fed rats (NG) and rats subjected to protein restriction (RG)

Group	Giemsa-stained neurons [†]	Nitroergic neurons (NADPH-dp)	Cholinergic neurons (NADPH-dn)
NG	242.84 \pm 29.71	112.78 \pm 12.67	130.06 \pm 34.70
RG	327.25 \pm 9.39*	179.23 \pm 30.67*	148.02 \pm 29.03

[†]Data from the literature (Hermes et al., 2008). * $p < 0.05$, significantly different from NG (Student's *t*-test).

Table 2 shows a reduction of the area of cellular bodies and nuclei in the RG compared with the NG.

Table 2: Median and percentile (25:75) of area (in μm^2) of cellular bodies, nuclei, and cytoplasm and ratio between the area of the nucleus and area of the cellular body of NADPH-dp myenteric neurons in the proximal colon in normally fed rats (NG) and rats subjected to protein restriction (RG)

Group	Area of cellular body	Area of nucleus	Area of cytoplasm	Nucleus/cellular body ratio
NG	307.65 (248.17; 368.74)	68.06 (56.48; 81.85)	236.55 (184.13; 293.14)	0.22 (0.19; 0.26)
RG	202.05 (156.84; 249.31)*	65.28 (50.08; 81.95)*	133.00 (100.66; 176.04)*	0.32 (0.27; 0.39)*

* $p < 0.05$, significantly different from NG (Mann-Whitney test).

Fig. 1 and 2 show the number of neurons distributed by size classification according to the area of cellular bodies and cellular body/nucleus ratio, respectively.

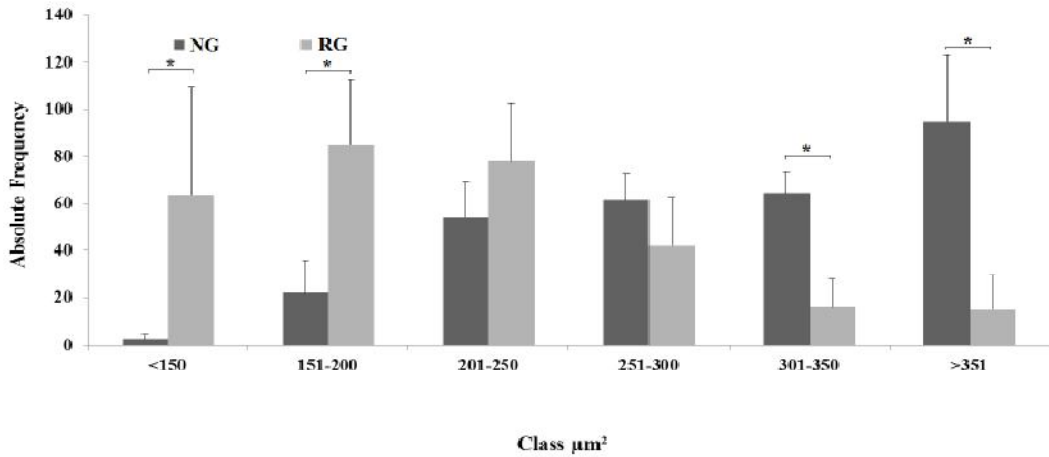


Fig. 1: Distribution of myenteric neurons stained with NADPH-diaphorase in the proximal colon in normally fed rats (NG) and rats subjected to protein restriction (RG) according to different size classifications based on the area of the cellular body. Columns with an asterisk in the same size classification are significantly different ($p < 0.05$; Student's *t*-test)

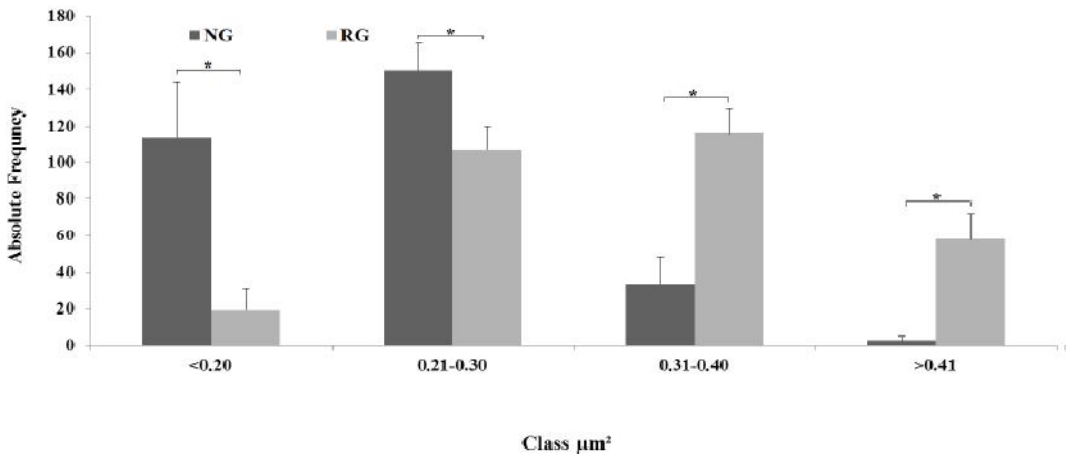


Fig. 2: Distribution of myenteric neurons stained with NADPH-diaphorase in the proximal colon in normally fed rats (NG) and rats subjected to protein restriction (RG) according to size classification based on the ratio between the area of the nucleus and area of the cellular body. Columns with an asterisk in the same size classification are significantly different ($p < 0.05$; Student's *t*-test)

Table 3 shows the correlations between the area of cellular bodies, nuclei, and cytoplasm of NADPH-dpmyenteric neurons in the proximal colon in the NG and RG.

Table 3: Correlation between areas of cellular bodies, cytoplasm, and nuclei of NADPH-dpneurons in the proximal colon in normally fed rats (NG) and rats subjected to protein restriction (RG)

Group	Area of cellular body vs. area of nucleus	Area of cellular body vs. area of cytoplasm	Area of nucleus vs. area of cytoplasm
NG	0.57	0.98	0.40
RG	0.65	0.95	0.39

All of the values are statistically significant ($p < 0.05$; Spearman's test).

Discussion

The present results showed that the proximal colon in the RG was 32.75% smaller than the proximal colon in the NG ($p < 0.05$). The reduced organ sizes in malnourished animals results from tissue alterations, and different tissues can be differentially affected, depending on their cellular organization and structure.⁽²⁰⁾

We observed an increase in the number of NADPH-dp neurons in the RG. This effect may be partially attributable to a deficit in the development of the area of the colon, thus producing a larger concentration of neurons per area. However, the increase in neuronal density was approximately 58.92%, whereas the size of the proximal colon was an average of 32.75% smaller, suggesting an increase in neuronal cells that began to express the NADPH-d because of malnutrition.

NADPH-d histochemistry has been used to evaluate enteric neurons because it detects neurons that express the enzyme nitric oxide synthase (NOS), which is responsible for producing nitric oxide (NO).⁽²¹⁾ Nitric oxide is an important mediator of intestinal relaxation, and diarrhea can be a cause of death in malnourished children.⁽²²⁾ Therefore, studies that investigate intestinal alterations produced by malnutrition are highly important.

We hypothesize that the greater density of nitrergic neurons may be attributable to a compensatory mechanism that results from an increase in oxidative stress that occurs during protein restriction. Previous studies reported that glutathione levels are reduced during protein restriction,⁽²³⁾ and glutathione is the main non-enzymatic cellular antioxidant.⁽²⁴⁾

A study by Fechner et al. ⁽²⁵⁾ corroborated these findings by showing decreases in antioxidants in the blood and glutathione in erythrocytes and increases in plasma NO concentrations in patients with the Kwashiorkor form of malnutrition.

Although some authors considered that nitrergic neurons are less vulnerable to cellular death during aging in animal, ⁽¹⁴⁾ other studies have reported a reduction of this enteric subpopulation in animals subjected to protein restriction. ^(8, 9, 26) Some studies have found a reduction of this subpopulation, but the present findings found an increase in NADPH-dp neurons. This may indicate that NO may exert either cytotoxic or cytoprotective effects, depending on the experimental conditions. ⁽²⁷⁾ Additionally, different organs and models of malnutrition can have different outcomes.

Although cholinergic neurons exhibit higher vulnerability to neuronal death, ⁽¹⁴⁾ the results of the present study suggest that the estimated number of cholinergic neurons (NADPH-dn) was not significantly altered by protein deficiency. This subpopulation of neurons is predominant in rodents, ⁽²⁸⁾ which was found in the NG in the present study. However, nitrergic neurons predominated in malnourished animals, indicating a change in the chemical code of myenteric neurons in the proximal colon in the RG. The predominance of excitatory neurons over inhibitory neurons can cause disequilibrium in the mechanism of fecal motility in animals subjected to protein restriction. ⁽⁹⁾

The neuroplastic alterations observed in the present study indicate the occurrence of neuronal atrophy caused by malnutrition. However, an increase in the nucleus/cellular body ratio was observed, indicating that nuclei occupied a proportionately larger area of the cellular body of neurons in the RG. Neuronal atrophy is considered a basic mechanism of cellular response to injury. ⁽²⁹⁾ In response to insult, a neuron may adapt by reducing its metabolism and consequently reducing its volume. ⁽³⁰⁾ This effect may have occurred in the present study because of the lower bioavailability of amino acids.

Reports in the literature about neuronal size in experimental models of malnutrition have been disparate. Hermes et al. ⁽¹²⁾ studied the proximal colon in malnourished animals and found an increase in the area of cellular bodies and nuclei of enteric neurons.

Leite-Melo et al. ⁽³¹⁾ described a greater proportion of giant neurons in the total neuronal population in the colon in rats subjected to protein restriction during pregnancy and lactation. Experimental models of aging have shown an increase in the area of cellular bodies of both NADPH-dp myenteric neurons and cuprolinic blue-stained myenteric neurons in the colon and rectum in 24-month-old rats, suggesting that this hypertrophy is caused by a compensatory mechanism of neuronal loss. ⁽¹⁵⁾ Therefore, one may suggest that the amount of proteins in the diet, organ under investigation, duration of the experiment, and technique used to identify neurons can affect the outcome. Due to this complexity, the use of different experimental approaches is necessary to better understand the effect of malnutrition on the gastrointestinal tract.

Importantly, studies have shown a recovery of the normal size of neurons in the colon. For example, pre and postnatal protein restriction caused a reduction of neurons that was restored with postnatal feeding. ⁽³²⁾ These findings suggest the need for more studies that utilize this model of malnutrition to investigate the recovery produced by changes in nutrition.

We analyzed the distribution of neurons by size classification according to the area of cellular bodies in 50 μm^2 intervals and observed a wide-range of cellular body sizes. A higher frequency of neurons with cellular bodies $> 351 \mu\text{m}^2$ was observed in the NG, indicating that this subpopulation naturally displays larger neurons in the proximal colon. In the RG, we observed a reduced frequency of large-size neurons (i.e., 301-350 μm^2 and $> 351 \mu\text{m}^2$) and an increased frequency of smaller-size neurons (i.e., $< 200 \mu\text{m}^2$). No neuronal death was observed, and atrophy of the area of cellular bodies was found. This suggests that the greater frequency of neurons $> 301 \mu\text{m}^2$ in the NG was attributable to the greater frequency of neurons $< 200 \mu\text{m}^2$ in the RG. Hermes et al. ⁽¹²⁾ reported that the majority of neurons in the myenteric neuronal population were between 51 and 100 μm^2 .

Despite the atrophy of the areas of cellular bodies and nuclei found in the present study, the nucleus tended to occupy a larger proportion of the cellular bodies of myenteric neurons in the proximal colon in the RG (Fig. 2).

Notably, in the NG, the nucleus of most of the neurons occupied 30% of the cellular body, whereas the nucleus occupied more than 30% of the cellular body in the RG.

This result may be attributable to greater atrophy in the area of cellular bodies (34.42%) compared with the area of nuclei (4.08%). Thus, this may result in a drastic reduction of the area of the cytoplasm in the cellular body (43.77%), an effect virtually unaffected by malnutrition (NG: $r = 0.98$; RG: $r = 0.95$).

Conclusion

In conclusion, the reduction of protein levels in the diet from 26% to 4% for 90 days in adult rats resulted in an increase in the number of myenteric neurons in the proximal colon that expressed the enzyme NADPH-d and atrophy of these cells.

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