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Atrophy and Neuron Loss: Effects of a Protein-Deficient Diet on Sympathetic Neurons

Silvio Pires Gomes,[1] Jens Randel Nyengaard,[2] Rúbia Misawa,[3] Priscila Azevedo Girotti,[3] Patrícia Castelucci,[3] Francisco Hernandez Javier Blazquez,[4] Mariana Pereira de Melo,[5] and Antonio Augusto Coppi Ribeiro[1]★

[1]Laboratory of Stochastic Stereology and Chemical Anatomy (LSSCA), Department of Surgery, College of Veterinary Medicine, University of São Paulo (USP), São Paulo, Brazil
[2]Stereology and Electron Microscopy Research Laboratory and MIND Centre, University of Aarhus, Aarhus, Denmark
[3]Laboratory of Metabolic Dysfunctions, Department of Anatomy, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil
[4]Department of Surgery, College of Veterinary Medicine, University of São Paulo, São Paulo, Brazil
[5]Department of Statistics, Institute of Mathematics and Statistics, University of São Paulo, São Paulo, Brazil

Protein deficiency is one of the biggest public health problems in the world, accounting for about 30–40% of hospital admissions in developing countries. Nutritional deficiencies lead to alterations in the peripheral nervous system and in the digestive system. Most studies have focused on the effects of protein-deficient diets on the enteric neurons, but not on sympathetic ganglia, which supply extrinsic sympathetic input to the digestive system. Hence, in this study, we investigated whether a protein-restricted diet would affect the quantitative structure of rat coeliac ganglion neurons. Five male Wistar rats (undernourished group) were given a pre- and postnatal hypoproteinic diet receiving 5% casein, whereas the nourished group (n = 5) was fed with 20% casein (normoproteinic diet). Blood tests were carried out on the animals, e.g., glucose, leptin, and triglyceride plasma concentrations. The main structural findings in this study were that a protein-deficient diet (5% casein) caused coeliac ganglion (78%) and coeliac ganglion neurons (24%) to atrophy and led to neuron loss (63%). Therefore, the fall in the total number of coeliac ganglion neurons in protein-restricted rats contrasts strongly with no neuron losses previously described for the enteric neurons of animals subjected to similar protein-restriction diets. Discrepancies between our figures and the data for enteric neurons (using very similar protein-restriction protocols) may be attributable to the counting method used. In light of this, further systematic investigations comparing 2-D and 3-D quantitative methods are warranted to provide even more advanced data on the effects that a protein-deficient diet may exert on sympathetic neurons.

Key words: stereology; malnutrition; coeliac ganglia; neurons; rats

The rat autonomic nervous system comprises nerves and ganglia, which are topographically divided into paravertebral, prevertebral, paravisceral, and intramural types (Gabella, 2004). One of the most relevant prevertebral ganglia groups is located in the abdominal cavity forming the abdominal plexus, which includes coeliac and superior mesenteric ganglia that are located next to the abdominal aorta. Two of the coeliac ganglion's target organs are the stomach and intestine (Gabella, 2004), and this ganglion plays an important role in the neural regulation of absorption mechanisms during the digestion process (Simmons, 1985).

Protein deficiency is one of the biggest public health problems in the world, accounting for about 30–40% of hospital admissions in developing countries (Bistrian and Blackburn, 1976). To some extent, all tissues can be affected by a hypoproteinic state, and the most protein-deficiency-affected tissues are those that possess a high cellular turnover, such as the intestinal mucosa (Deo, 1978). In early life or later, malnutrition may seriously affect either body mass or brain formation, especially the connectivity in the cerebellar cortex of adult rats. Some of these dysfunctions are irreversible, whereas a few others disappear when animals are given adequate renutrition (Yucel et al., 1994).

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★Correspondence to: Prof. Antonio Augusto Coppi Ribeiro, Departamento de Cirurgia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Av. Prof. Dr. Orlando Marques de Paiva, 87 CEP 05508-270 São Paulo-SP, Brazil. E-mail: guto@usp.br

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Apart from structural changes in the central nervous system (CNS), nutritional deficiencies may also lead to alterations in the peripheral nervous system (PNS) and digestive system. In the latter, they cause absorption failure as well as gastrointestinal motility disturbances. Most studies focusing on the effects of denutrition on the intrinsic innervation of the digestive system have reported no losses of enteric neurons after pre- or postnatal protein restriction (Sant'ana et al., 1997; Castelucci et al., 2002; Gomes et al., 2006). Understandably, all previous investigations used two-dimensional quantitative methods, which are currently used for layered-tubular organs such as the intestine, and, unfortunately, few studies have investigated the extrinsic sympathetic inputs to the digestive system, e.g., coeliac or superior mesenteric ganglia, and their structural changes by nutritional disturbances (Conboy et al., 1987).

Hence, in this study we investigated whether a protein-restricted diet would affect the quantitative structure of rat coeliac ganglion (CG) neurons by using more robust, state-of-the-art, design-based stereological methods (Nyengaard, 1999). Awareness of these data may provide a better morphological understanding of denutrition-related neuropathies and help clinicians improve the diagnosis and treatment of the disorder in human and nonhuman patients.

MATERIALS AND METHODS

The whole study (including the hypoproteinic diet) was conducted according to the current legislation relating to animal experiments of the Biomedical Science Institute and College of Veterinary Medicine of the University of São Paulo (application No. 897/2006). In addition, the pre- and postnatal denutrition protocol used here was similar to that published by one of the coauthors, Patrícia Castelucci (Castelucci et al., 2002; Gomes et al., 2006).

Young male and female Wistar rats (200–240 g body weight) were mated. After conception, which was assumed to have occurred when vaginal plugs or sperm were found, the female rats were placed in individual cages and divided into two groups, i.e., nourished mothers (n = 5), which received a 20% casein normoproteinic diet (AIN-93G), and undernourished mothers (n = 5), which received a 5% casein hypoproteinic diet (AIN-93G; Rhoster Indústria e Comèrcio Ltda., São Paulo, Brazil) during the whole gestation period. Pregnant rats were kept under standard conditions at 21°C with a 12-hr light-dark cycle, and all groups were supplied with water ad libitum. After birth, mothers and pups received the same diet given to them during gestation according to their group. Only male pups were used for this study, and they were also divided into two groups. The first group (nourished rats) was maintained on the normoproteinic diet in both the prenatal and the postnatal periods (until day 21; n = 5). The second group (undernourished rats) was protein deprived throughout gestation and for the first 21 days of the postnatal period (n = 5).

Biochemical Examinations (Plasma Concentration Dosing)

Glucose, leptin, and triglyceride plasma concentrations were measured in mothers immediately before they received the above-specified diets and also in mothers and their pups on day 21 after birth. After a fast of 4 hr, blood samples were collected, between 11 hr and 13 hr, from the retroorbital plexus of the eye with a heparin-coated glass capillary. Samples were separated by centrifugation and stored frozen at –20°C until analysis (Seidel et al., 2006).

Plasma Concentrations

Leptin was measured by using a ''Quantikine'' assay (R&D Systems, Minneapolis, MN). The lowest detectable dose of the assay was 22 pg/ml. Plasma glucose concentrations were determined with the Glucoquant assay (Roche Diagnostics GmbH, Mannheim, Germany), and a triglyceride assay (TG GPO-PAP; Roche Diagnostics GmbH) was used (Kratzsch et al., 1986).

Histological Study

Twenty-one days after birth, the rats were weighed and euthanized with an intraperitoneal overdose of sodium pentobarbital, i.e., Thiopental (10 mg/100 g bw). The thoracic cavity was opened, and a bulbed cannula was inserted into the left heart ventricle up to the aorta ascendens, and about 100 ml of a prewash fluid (PBS, Na nitrite, and heparin) was perfused, followed by about 200 ml of fixative: 5% glutaraldehyde (Merch) and 1% formaldehyde (Sigma, St. Louis, MO) in 100 mM Na cacodylate buffer (EMS; pH 7.4) injected at room temperature. Drainage of blood and perfusate (prewash fluid and fixative) was obtained by slitting the right auricle. The abdominal cavity was then incised by a midline incision (celiotomy), and some organs such as stomach, intestines, and the left adrenal gland were slightly pushed to one side to facilitate identification of the left coeliac ganglion (LCG) and its connections to the spinal cord and also to allow for visualization of the intermesenteric plexus and main vessels of the abdomen (abdominal aorta and caudal cava vein). The LCG was chosen because this ganglion innervates the stomach and small intestine, whereas the right CG provides sympathetic input to the liver (Miolan and Niel, 1996).

In addition, about 100 ml of a cleaning solution of PBS (Sigma; 0.1 M, pH 7.4) containing 2% heparin (Roche) and 0.1% sodium nitrite (Sigma) was reverse-perfused through the abdominal aorta close to the emergence of the coeliac artery; at the same time, the caudal cava vein was slit to drain the circulatory system. This was followed by perfusion of 100 ml of a fixative consisting of 5% glutaraldehyde (Merch) and 1% formaldehyde (Sigma) in sodium cacodylate buffer (EMS; 100 mM; pH 7.4). The LCG was identified with a surgical microscope Leica M651 (×6–40) and dissected out together with its connections with the left major splanchnic nerve and the intermesenteric and coeliac plexus. Then LCGs were immersion fixed in the same fixative for 2–3 days at 4°C, and fixed (wet) ganglia were then measured for their length, width, and thickness with a digital pachymeter (Digimess). Therefore, a total of 10 LCGs was used in this study, i.e., five LCGs from

nourished animals (group I) and five LCGs from undernourished animals (group II).

Subsequently, LCGs (from groups I and II) were 7% agar-embedded in spherical rubber molds to generate isotropic planes in them using the isector method (Nyengaard and Gundersen, 1992). In the next step, all agar-embedded LCGs were washed in sodium cacodylate buffer (EMS), postfixed in 2% osmium tetroxide (EMS), stained en bloc with a uranyl acetate-saturated aqueous solution (Reagen), dehydrated in graded ethanols and propylene oxide (EMS), and reembedded in Araldite 502 (Polysciences, Warrington, PA). The resin was cured at 60°C for 3 days, and 0.2-μm, 0.4-μm, or 1-μm IUR sections were cut from Araldite-embedded specimens with a glass knife. Sections were produced with an RMC MTXL calibrated ultramicrotome and were then stained with toluidine blue (nuclear), collected onto glass slides, dried on a hot plate, and mounted under a coverslip with a drop of Araldite. Each LCG was systematically, uniformly, and randomly sectioned and sampled (SURS; Gundersen et al., 1999; Nyengaard, 1999; Mayhew, 2008).

Stereology (Design-Based)

A modification of the disector (Gundersen, 1986) was used in the present study. Our approach, which used one reference and two look-up sections and therefore two disector heights, including a stack of serial sections between them, was used in this study to sample cell nucleoli and, finally, to estimate cell volume (see LCG mean perikaryal volume). The second look-up section, which is far apart from the first look-up section, was used to sample the perikarya and estimate the total number of LCG neurons.

Numerical density of LCG neurons N_V(LCG neuron, LCG). The modified physical disector was used to estimate the neuron numerical density, in which the whole perikaryon (soma) was the counting unit for estimating the total number of LCG neurons. In practical terms, the design comprised two disectors with two heights, i.e., 7.4 μm (large disector height) and 1.0 μm (small disector height) for nourished rats and 4.2 μm (large disector height) and 1.0 μm (small disector height) for undernourished rats. The method consists of counting the number of particles (in this case neurons) that are present within unbiased counting frames on the reference sections and that do not touch either the forbidden lines of the frames or their extensions and disappear on parallel look-up sections or vice versa.

The formula for N_V estimation is: $N_V := \Sigma Q^- (\text{LCG cell})/\Sigma V(\text{dis})$ where ΣQ^- represents the cell count and $\Sigma V(\text{dis})$ is the volume of all disectors sampled. The latter was estimated as $\Sigma P \cdot (a/p) \cdot h$, where P is the number of test points, a/p the area associated with each test point, and h the distance between disector planes (disector height). The 2-D unbiased counting frame area used was 13,200 μm^2 (Gundersen, 1977). For nourished rats, on average 116 disectors were used per animal to count 213 neurons. In undernourished rats, 207 disectors were used per animal, on average, to count 209 neurons.

LCG volume, V(LCG). The total volume of LCG was estimated using the Cavalieri principle. Briefly, LCG were exhaustively and serially sectioned, and every fortieth section (K = 40; nourished rats) or every twentieth section (K = 20; undernourished rats) was sampled and measured for cross-sectional area. Then, $V(\text{LCG}) := T \cdot \Sigma A$, where T is the intersectional distance, which was 336 μm for nourished rats and 104 μm for undernourished rats, and ΣA is the sum of the delineated areas of the LCGs, which were calculated using test points hitting the whole reference space (P ~ 270 in nourished and P ~ 200 in undernourished rats).

Total number of LCG neurons, N(LCG neuron). The total number of LCG neurons was estimated by multiplying N_V by LCG volume: $N = N_V \cdot V$.

Volume density of LCG neurons, V_V(LCG neuron, LCG). The fractional volume of the LCG occupied by LCG neurons was determined by point counting. A SUR sample of fields was obtained, and test points were randomly superimposed on the fields. We counted the total number of points falling within the LCG [ΣP(ref)] and the total falling on neurons [ΣP(LCG) neurons]. Volume density was then estimated simply as $V_V := \Sigma P(\text{LCG neurons})/\Sigma P(\text{ref})$.

LCG mean neuron volume ($\bar{v}_N$; LCG neuron). The mean perikaryon volume of LCG neurons was estimated by the nucleator method (Gundersen, 1988) in the same reference sections used for neuron numerical density estimation; therefore, cells were unbiasedly selected using disectors (Fig. 1). The following formula was used: $\bar{v}_N = \Sigma(4\pi/3) \cdot \bar{l}_n^3$, where $\bar{l}_n^3$ is the mean cubed distance from a cell's central point (nucleolus) to the cell boundaries. For a two-nucleoli neuron profile, two approaches were used. 1) If the two nucleoli profiles disappeared simultaneously in the first look-up section, the average of $\bar{l}_n^3$ [mean cubed distance from a cell's central point (nucleolus) to the cell boundaries] per cell was used (Gundersen, 1988). 2) If only one nucleolus profile disappeared in the first look-up section, this nucleolus was therefore sampled and one $\bar{l}_n^3$ was obtained.

To achieve stable means for cell volume distribution, 200 neurons were measured in nourished rats and 197 neurons in undernourished rats (Gundersen, 1988; Sorensen, 1991; Howard and Reed, 2005).

Total volume of LCG neurons: V(LCG neuron, LCG). The total volume of LCG neurons was estimated by using two approaches: 1) directly, multiplying the mean perikaryon volume by the total number of neurons, and 2) indirectly, multiplying the perikaryon fraction volume by the total ganglion volume [V(LCG)] (Lima et al., 2007). The two approaches for estimating the total volume of LCG neurons, i.e., directly and indirectly, led to very similar results.

Precision of Stereological Estimates

The precision of stereological variables, i.e., ganglion volume, total number of neurons, and volume density of neurons, was assessed by estimating their coefficients of error (CEs) according to the formulas published by Gundersen et al. (1999) and Nyengaard (1999).

Statistical Analysis

Data were tested by two-sample *t*-test and one-way ANOVA in Minitab 15 statistical software. Normal distribution

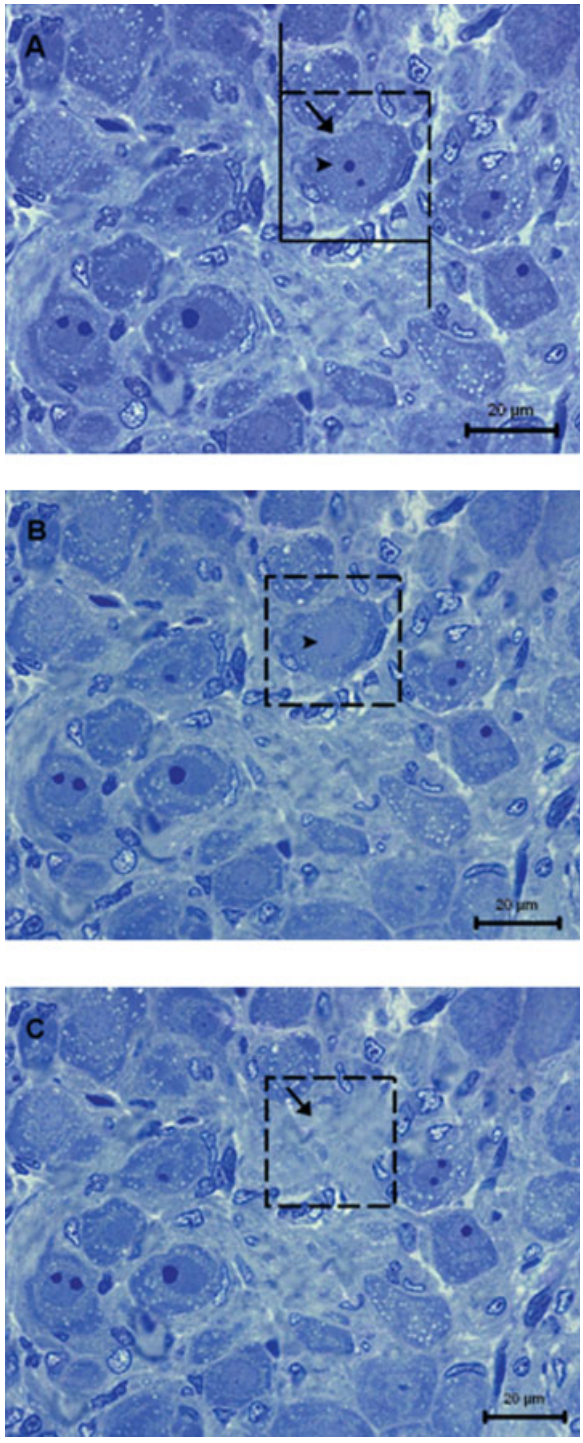

Fig. 1. Example of a disector-sampled LCG neuron of a nourished rat used for perikaryon volume estimation by the nucleator method. The reference section (**A**) and look-up 1 section (**B**) are separated by 1 µm. The reference section (A) and look-up 2 section (**C**) are separated by 7.4 µm. Arrowhead and arrow point to one nucleolus and one perikaryon, respectively, which disappear in the first look-up and in the second look-up sections, respectively. Toluidine blue. Scale bars = 20 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

TABLE I. Effects of a Hypoproteinic Diet on the Plasma Concentrations of Leptin ($pg \cdot ml^{-1}$), Glucose ($mmol \cdot l^{-1}$), and Triglyceride ($mmol \cdot ml^{-1}$) in Nourished (NM) and Undernourished (UM) Mothers and in Nourished (NP) and Undernourished (UP) Pups [Group Means (CV) for n = 5 Rats]

Variable	Group NM	Group UM	Group NP	Group UP
Leptin*,**	421 (0.17)	365 (0.15)	41 (0.21)	35 (0.14)
Glucose*,**	10.3 (0.15)	9.09 (0.15)	9.46 (0.17)	8.33 (0.22)
Triglyceride*,**	1.22 (0.22)	1.06 (0.23)	0.50 (0.13)	0.40 (0.17)

*Significant effect of prenatal protein deficiency ($P = 0.03$).
**Significant effect of postnatal protein deficiency ($P = 0.03$).

and equality of variances were ensured by Kolmogorov-Smirnov's and Levene's tests, respectively. Intergroup differences were considered significant at $P < 0.05$. Results are shown as mean (CV_{obs}), where the observed total coefficient of variation (CV_{obs}) = SD/mean.

RESULTS

Clinical Examination

Most of the symptoms of clinical malnutrition were present in pups, e.g., dehydration, skin folds, mucosa opacity, and loss of body weight. In addition, edema and fluid accumulation were seen in serous cavities. At 21 days after birth, animal body weights were 62.5 g (0.05) in nourished animals and 40.4 g (0.10) in undernourished rats. Differences between groups were significant ($P = 0.001$).

Biochemical Examinations

Intergroup differences were significant ($P = 0.03$) for leptin, glucose, and triglyceride plasma concentrations. The values are summarized in Table I.

Anatomy

In all animals, the LCG was located in the abdominal cavity and next to the coeliac and cranial mesenteric arteries. Dorsally, the ganglion was in contact with the abdominal aorta, and it was ''H-shaped'' in nourished and spherical in undernourished subjects. In nourished rats, LCG length, width, and thickness were 4.54 mm (0.03), 1.71 mm (0.03) and 1.62 mm (0.10), respectively. For undernourished rats, the figures were 1.26 mm (0.15), 0.86 mm (0.24), and 0.74 mm (0.12), respectively. Differences between groups were significant for ganglion length ($P = 0.0001$), width ($P = 0.016$), and thickness ($P = 0.001$).

Microstructure

In nourished rats, the LCG consisted of larger neurons, which were separated by nonneuronal tissue (Fig. 2). Conversely, the LCG neurons of undernourished rats were smaller and more densely distributed (Fig. 2; see Neuron size in LCG mean neuron volume).

Numerical density of LCG neurons, N_V(LCG neuron, LCG). The numerical density of LCG neurons was 41,000 mm^{-3} (0.03) in nourished rats and

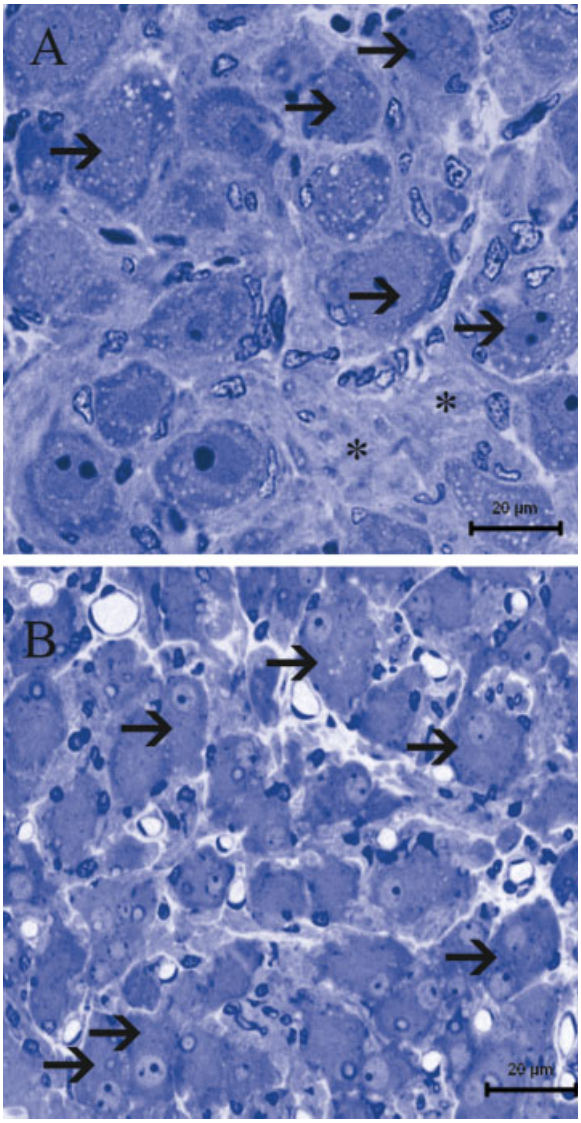

Fig. 2. Structure of the LCG of a nourished (**A**) and an undernourished (**B**) rat in semithin sections (1 µm) at the same magnification. In nourished rats, LCG neurons (arrows) were larger and separated by nonneuronal tissue (asterisk). On the contrary, in undernourished animals, LCG neurons (arrows) were smaller and more densely packed, with a higher neuron numerical density. Toluidine blue. Scale bars = 20 µm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

70,200 mm^{-3} (0.04) in undernourished rats. Intergroup differences were significant ($P = 0.0001$).

LCG volume, V(LCG). LCG volume was 1.02 mm^3 (0.17) in nourished rats and 0.22 mm^3 (0.21) in undernourished subjects. Differences between groups were significant ($P = 0.002$; Fig. 3). The error variance (CE) of the estimated ganglion volume was 0.04 for nourished and 0.06 for undernourished rats. Volume shrinkage (mean % ± SD) was estimated as 6.75 ± 1.23 for nourished rats and 5.07 ± 1.34 for undernourished rats. Although the global shrinkage was higher than

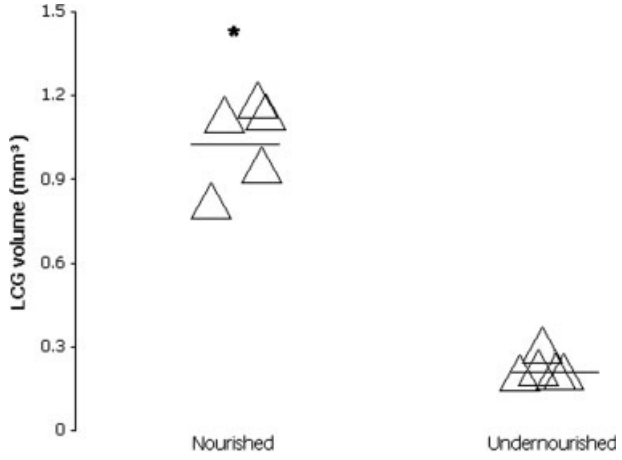

Fig. 3. LCG volume (mm^3) in nourished and undernourished rats. Intergroup differences were significant (*$P = 0.002$). Triangles indicate individual values, and horizontal bars show group means.

zero, no corrections were performed because intergroup differences did not attain statistical significance ($P = 0.44$).

Total number of LCG neurons, N(LCG neuron). The total number of LCG neurons was 41,800 (0.14) in nourished and 15,400 (0.20) in undernourished rats. Differences between groups were significant ($P = 0.005$; Fig. 4). The CE of the estimated number was 0.06 and 0.07 for nourished and undernourished rats, respectively.

Volume density of LCG neurons, V_V(LCG neuron, LCG). The volume density of LCG neurons was 0.29 (0.03) in nourished and 0.38 (0.05) in undernourished rats. Differences between groups were significant ($P = 0.014$). The CE of the estimated volume density was 0.06 and 0.09 for nourished and undernourished rats, respectively.

LCG mean neuron volume ($\bar{v}_N$; LCG neuron). The mean perikaryon volume of LCG neurons was 7,090 μm^3 (0.10) and 5,390 μm^3 (0.14) in nourished and undernourished rats, respectively. Intergroup differences attained statistical significance ($P = 0.014$).

Total volume of LCG neurons: V(LCG neuron, LCG). The total volume occupied by LCG neurons in the LCG was 0.30 mm^3 (0.17) in nourished and 0.08 mm^3 (0.26) in undernourished rats. Differences between groups reached statistical significance ($P = 0.002$).

DISCUSSION

The main goal of this study was to investigate whether a protein-deficient diet would affect the quantitative structure of rat coeliac ganglion neurons, so the main findings to be discussed here are the changes in morphostructural parameters, which were estimated by using design-based stereological methods.

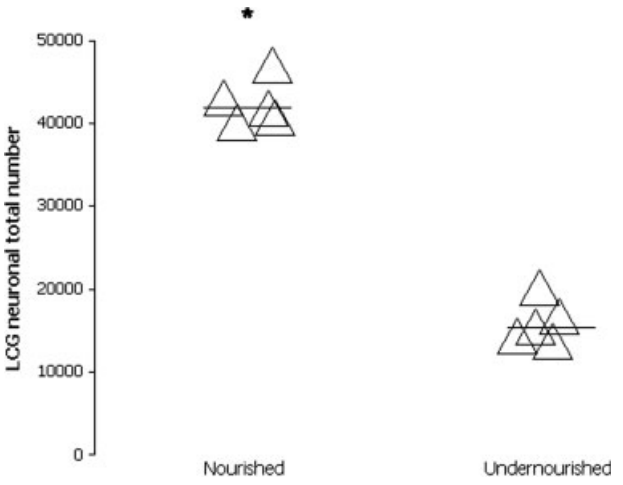

Fig. 4. LCG neuronal total number in nourished and undernourished rats. Intergroup differences were significant (*$P = 0.005$). Triangles indicate individual values, and horizontal bars show group means.

Main Structural Findings of This Study

A diet that was deficient in protein (5% casein) caused atrophy of coeliac ganglion (78%) and of coeliac ganglion neurons (24%) and neuron loss (63%).

Biochemical Data

In line with Seidel et al. (2006), this study foundd a 15% and 20% reduction in the plasma concentrations of leptin and triglycerides, respectively, in undernourished pups. Additionally, glucose levels were diminished (12%). The latter differs from the report by Seidel et al. (2006), in which plasma glucose levels did not change with food restriction. Decreases in biochemical parameters were accompanied by a reduction in body weight (35%), which was also reported by Sant'ana et al. (1997; 35%) and by Castelucci et al. (2002; 50%) for undernourished rats. However, the animals either recovered values partially (80% of initial body weight; Castelucci et al., 2002) or totally after they were refed (Sant'ana et al., 1997).

Protein and protein-calorie malnutrition affected the neural mechanisms believed to control defensive behaviors in rats (Da Silva Hernandes et al., 2005). The most noticeable behavioral changes were lower anxiety and/or higher impulsiveness rather than learning deficits. On the other hand, no motor deficits were registered in malnourished animals (Da Silva Hernandes et al., 2005). Prolonged induced protein deficiency (eight months) in adult rats produced a remarkable loss of hippocampal neurons and synapses and substantial damage to hippocampus-dependent behavior, which was restored after protein refeeding (Lukoyanov and Andrade, 2000). Unlike the efficiency of a mature CNS in recovering from the damage caused by prolonged malnutrition, the developing CNS seems not to be able to reverse such damage (Pedrosa and Moraes-Santos, 1987). For example, early postnatally induced undernutrition leads to a substantial deficit in the total number of rat dentate gyrus granule cells as assessed by stereological methods (Bedi, 1991). In addition, social behavior, e.g., mother-pup or pup-pup interactions, is also affected by malnutrition; this evidence highlights the possible effects of early malnutrition on the neural mechanisms involved in the regulation of adaptive strategies (Bedi, 1991).

Morphoquantitative Aspects: Methodological Appraisal

There are a plethora of studies reporting on the effects of malnutrition on the enteric nervous system (Sant'ana et al., 1997; Castelucci et al., 2002; Brandão et al., 2003; Zanin et al., 2003; Gomes et al., 2006; Moreira et al., 2008), though little attention has been paid to the sympathetic input to the digestive system, represented mainly by the CG. Until now, most quantitative data produced on the enteric nervous system rely on ratios using two-dimensional counting methods, which may have biases (Sant'ana et al., 1997; Castelucci et al., 2002; Brandão et al., 2003; Zanin et al., 2003; Moreira et al., 2008). For instance, the neuron profile cross-sectional area and neuron profile density are commonly assumed to be the true cell size and the true total number of cells, respectively. However, by using three-dimensional design-based stereological methods, it is now possible to detect several malnutrition-related differences either in number and size of coeliac neurons or in ganglion size. For instance, the LCG of undernourished rats was 78% smaller than that of nourished rats, which is typical of ganglion atrophy caused by protein deficiency.

With 2-D counting methods, e.g., cell profile density per area (a ratio), 97% and 45% increases in the densities of rat myenteric neuron profiles have been reported for the small intestine after either prenatal (Zanin et al., 2003) or pre- and postnatal induced malnutrition (Brandão et al., 2003). Conversely, a 13% or 24–52% reduction in the ascending or descending colon neuron profile density occurred in undernourished adult rats (Sant'ana et al., 1997; Araújo et al., 2003). In our study, the numerical (or volumetric) density of LCG neurons (not neuron profiles) in a sampled volume increased by 71% after pre- and postnatal induced malnutrition. However, the total number of the "same neurons" decreased by 63% when estimated by design-based stereological methods. This numerical example confirms the danger of coming to conclusions based only upon ratios, without considering the reference space. This pitfall is known as a *reference trap* (Hunziker and Cruz-Orive, 1986; Cruz-Orive, 1994; Mayhew, 2008).

In perusing the literature another possible example of a reference trap came to light. No changes in the number of colonic neurons in rats were reported after a pre- and postnatal protein-restriction protocol, which was accompanied by a 40% reduction in the area of colon (Castelucci et al., 2002). By the same token, Schoffen et al. (2005) have also postulated no alterations

in the number of proximal colon neurons of rats after a postnatal protein-restriction protocol, although 14%, 31%, and 32% significant decreases in the length, weight, and mucosa layer thickness of the proximal colon were noted, respectively. In addition, Moreira et al. (2008) have shown increases of 26% in the density of NADH-diaphorase and NADPH-diaphorase myenteric neuron profiles per area in the ileum of rats subjected to a postnatal protein restriction, which were accompanied by 17% shortening and 41% reduction in ileum length and weight.

Given this, all the above-mentioned authors concluded that enteric neurons are protected from loss even when there is a substantial reduction in organ weight or organ size caused by protein deprivation. From our point of view, these data are questionable because the authors estimated not the total number of neurons but rather the number of neuron profiles per area. Because the area of the colon was reduced by 40% (Castelucci et al., 2002), the chances are that the organ shrank and, as a result, the number of neuron profiles may be considerably higher in this area (see above, our example for the concept of a reference trap). This fact might have misled authors to conclude that enteric neurons are protected against any loss subsequent to protein deprivation. If such protection does exist, it did not occur in this study, insofar as a 63% fall was observed in the total number of LCG neurons of rats subjected to pre- and postnatal protein deficiency.

Despite the controversies over the existence of a protection mechanism against malnutrion-related neuron loss, most authors agree on a reduction in neuron size (Castelucci et al., 2002; Brandão et al., 2003; Zanin et al., 2003). With an accurate local design-based stereological volume estimator, i.e., the nucleator, we have found a 24% decrease in neuron size (atrophy) in undernourished rats. Understandably, a reduction (atrophy) in cell dimensions is commonly observed when there is a shortage of protein in the diet. The only exceptions seem to be the data reported by Conboy et al. (1987) and Schoffen et al. (2005), who found no changes in the size of neurons from celiac-mesenteric ganglion and the proximal colon of rats subjected to prenatal or postnatal undernutrition, respectively. It has to be stressed, therefore, that Conboy et al. (1987) and Schoffen et al. (2005) employed a linear and an areal (and not volumetric) size estimator, respectively.

CONCLUSIONS

The present study has shown that the main effects of protein deficiency on sympathetic ganglion neurons are cell atrophy and cell loss. Cell size reduction has also been reported for enteric neurons from undernourished animals, although there is some disagreement on the total number of neurons in protein-restricted animals. From our point of view, the main reason for these divergences fall to the quantitative methods used by most authors hitherto, which invariably lead to a reference trap when two-dimensional counting methods are applied, especially in the case of layered-tubular organs, e.g., the digestive system. Along similar lines, the discrepancy in the number of estimated enteric neurons in experiments using very similar protein-restriction protocols is well known. In the light of this, further systematic investigations comparing 2-D and 3-D quantitative methods are warranted to provide even more advanced data on the effects that a protein-deficient diet may exert on sympathetic neurons.

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