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REGULAR ARTICLE

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Effects of pre- and postnatal protein deprivation and postnatal refeeding on myenteric neurons of the rat large intestine: a quantitative morphological study

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Abstract We investigated the effect of protein deprivation and refeeding on weight gain, the size of the colon, and the numbers and sizes of enteric neurons. Neurons were located by reduced nicotinamide adenine dinucleotide (NADH) diaphorase staining. Protein deprivation of the mother throughout pregnancy, and the mother and unweaned rat pups in the first 21 postnatal days, reduced the weights of pups to about 50% of control. The size of the colon was also reduced, by about 40%. Despite this, total numbers of neurons in the colon were not reduced. However, there was a small, but significant, 15% reduction in the areas of neuron profiles. After 21 days the remaining pups were removed from the mothers, and either maintained on the control diet, maintained on the protein-deprived diet, or changed from the protein-deprived diet to a normal diet (refed group). These rats were examined after a further 21 days. Refeeding restored body weight to 20% below control, restored colon size, and restored nerve cell size. After a total of 42 days of protein deprivation, nerve cell numbers were not significantly different from control. In undernourished rats at 21 and 42 days, neurons were less well stained than control for NADH diaphorase. Refeeding between 21 and 42 days restored the normal appearance of the neurons. It is concluded that enteric neurons are protected from loss even when there is a substantial reduction in body weight and organ size caused by protein deprivation. The neurons become smaller, but recover size after refeeding.

Keywords Enteric nervous system · Nutrition · Intestine · Rat (Wistar)

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Introduction

The effects of undernutrition on the central and peripheral nervous system have been extensively studied (Cordero et al. 1986; Cornblath and Brown 1988; Bedi 1994; Rushmore et al. 1998). In the central nervous system, severe undernutrition causes nerve cell loss in some regions (e.g., dentate gyrus, Bedi 1991), whereas other regions, such as the cerebral cortex, seem protected from cell loss (Bedi 1994). In the case of peripheral neurons, undernutrition causes a significant decrease in protein content in the superior cervical ganglion of young rats (Gaetani et al. 1977), and a period of maternal undernutrition during pregnancy causes a reduction in neuron size and in noradrenaline content in the coeliac-superior mesenteric ganglion (Conboy et al. 1987). These effects are observed early in the offspring and persist into adult life. In the enteric nervous system, a 27% decrease in the number of enteric neurons in the jejunum of rats submitted to severe prenatal undernutrition was reported (Santer and Conboy 1990). Similarly, Sant'ana et al. (1997) described a mean neuronal loss of 13% in the myenteric plexus of the ascending colon of protein-deprived adult rats.

The effects of refeeding on neurons of the central nervous system have been investigated (Wiggins et al. 1984; Rana et al. 1991; Andrade et al. 1995). The data indicate that if neurons are lost, they are not replaced when the animals are returned to a normal diet (Andrade et al.

1995). The effect of refeeding on enteric neurons has not yet been fully evaluated. In one previous study, mothers were food deprived, and then the offspring were examined after having a normal diet (Santer and Conboy 1990). The animals were not examined before the restoration of normal diet, although the study does suggest that if neuron numbers are reduced by malnutrition they do not recover (Santer and Conboy 1990). In the present work, the effects of pre- and postnatal protein malnutrition and postnatal refeeding on myenteric neurons in the large intestine of rat were analyzed.

Materials and methods

The study was conducted according to current legislation on animal experiments of the Biomedical Science Institute of the University of São Paulo. 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 females were placed in individual cages. The nourished mothers received an AIN-93G normal protein diet and the undernourished mothers received the AIN-93G diet free of protein (0% casein) (Hoster Indústria e Comércio Ltda, São Paulo, Brasil). The rats were maintained under standard conditions at 21°C, with a 12 h light-dark cycle, and all groups were supplied with water ad libitum.

Following birth, the mothers and the pups received the same diet that the mother had during pregnancy. Only the male animals in the litters were used for experimentation. Females remained in the litters, but were not investigated. There were five experimental groups. The first group was caged with the mothers and normal feed was supplied until the pups were killed at 21 days postnatal (N21 group, *n*=9). The second group were undernourished (deprived of protein) during pregnancy and until 21 days postnatal, when they were taken for examination (D21, *n*=9). All rats that were kept beyond 21 days were weaned, by removal from the mothers, at 21 days postnatal. The third group of rats was maintained on normal feed throughout pregnancy and until they were taken for examination at 42 days (N42, *n*=9). The fourth group was protein deprived throughout pregnancy and for 42 days postnatal (D42, *n*=9). The fifth or refeeding group (DR42, *n*=9) used undernourished rats from the first phase (until 21 days old) that then received the AIN-93G normal protein diet from day 22 to day 42.

After they were weighed, the animals were sacrificed with an overdose of ether and the anterior abdominal wall was opened. The entire large intestine was removed and washed in Krebs solution. The surface area of the entire large intestine was measured using a planimeter. Thereafter, each piece was ligated with cotton thread at the proximal end and was gently distended with Krebs solution introduced with a syringe in the distal end. When the intestine was sufficiently distended, the syringe needle was withdrawn and the ligature was simultaneously tightened. The following steps were then performed to reveal neurons using the NADH diaphorase histochemical technique (Gabella 1987). After incubation in Krebs solution at room temperature for 15–30 min, the large intestines were transferred to a permeabilizing agent (0.3% Triton X-100 in Krebs solution) for 15–90 s and then submitted to three changes of Krebs solution, each of 10 min. The specimens were incubated for 30–90 min at 20°C in a 20-ml incubation medium containing 0.5 mg/ml nitroblue tetrazolium (Sigma Chemical Co., St. Louis, MO) in distilled water (25 parts), 0.1 M sodium phosphate buffer, pH 7.3 (25 parts), distilled water (50 parts) and 0.5 mg/ml of the reduced form of β-nicotinamide adenine dinucleotide (NADH). The reaction was stopped by immersion in 10% buffered formalin solution in which the tissue samples were fixed for a minimum of 24 h. Whole-mount preparations were then prepared as follows: the large intestines were opened, the mucosa was removed and the longitudinal muscle, with the myenteric plexus attached, was lifted at one corner and gently removed from the entire strip. After several washes in distilled water, portions (2 cm^2) from the mid-parts of the proximal, middle and distal thirds of the large intestine were prepared as whole-mounts in glycerol on microscope slides and sealed with Entellan (Merck).

The myenteric neurons were identified by the presence of formazan reaction product filling the perikaryon and by their large round and unstained nuclei. The number of neurons and the profile areas of the nerve cell bodies were measured by examining the whole-mount preparations under a binocular microscope at a magnification of ×400. All neurons present in each 2-cm^2 portion were counted. The profiles areas of 100 nerve cell perikarya from each portion were obtained on a semiautomatic device for morphometry.

The results are expressed as means ± SEM. Data were compared by analysis of variance (ANOVA), Student's *t*-tests and Duncan's test for multiple comparisons, as appropriate (Johnson and Wichern 1992). The level of significance was set at $P<0.05$.

Results

Qualitative analysis

In all groups, the myenteric ganglia revealed with the NADH diaphorase technique were elongated with their long axis parallel to the circular muscle layer (Fig. 1). Generally they were continuous with each other and contained a variable number of neurons of different sizes. In samples from the normally fed group at 21 days the ganglia exhibited a regular and uniform aspect (Fig. 1). The ganglia appeared less regular in tissue from the undernourished animals at 21 days (Fig. 1). This appearance has not been investigated further. It might be due to a combination of decrease in nerve cell size, decrease in staining intensity and loss of intestine mass.

Although some neurons were relatively weakly stained in tissue from the control group at 21 days, most of them were intensely reactive (Fig. 1). However, in the undernourished group at 21 days the neurons were less reactive and there was empty space between them. Some neurons from these animals were very faintly stained (Fig. 1). The loss of staining was quantified by counting strong and weakly staining nerve cells in groups of 50 from 5 animals. In the normally fed rats, 30±5% of neurons were weakly stained, and in the nutritionally deprived rats the weakly stained neurons made up 59±7% of the population. The difference in proportions of weakly stained neurons is significant ($P<0.05$). The cytoplasm of most of the neurons in the normally fed group at 42 days was intensely stained, although neurons of various sizes with less intense staining were observed. Tissue from the undernourished group at 42 days showed a diffuse staining of some neurons. At high magnification it was verified that the staining was not uniform in the cytoplasm of these neurons. The appearance of neurons of the myenteric ganglia of the initially undernourished group that was normally fed from 22 to 42 days was similar to the group that was normally fed for the entire 42 days (Fig. 2). There were no signs of pyknotic nuclei in myenteric neurons from the protein-deprived rats at 21 or 42 days (or in tissue from normally fed rats).

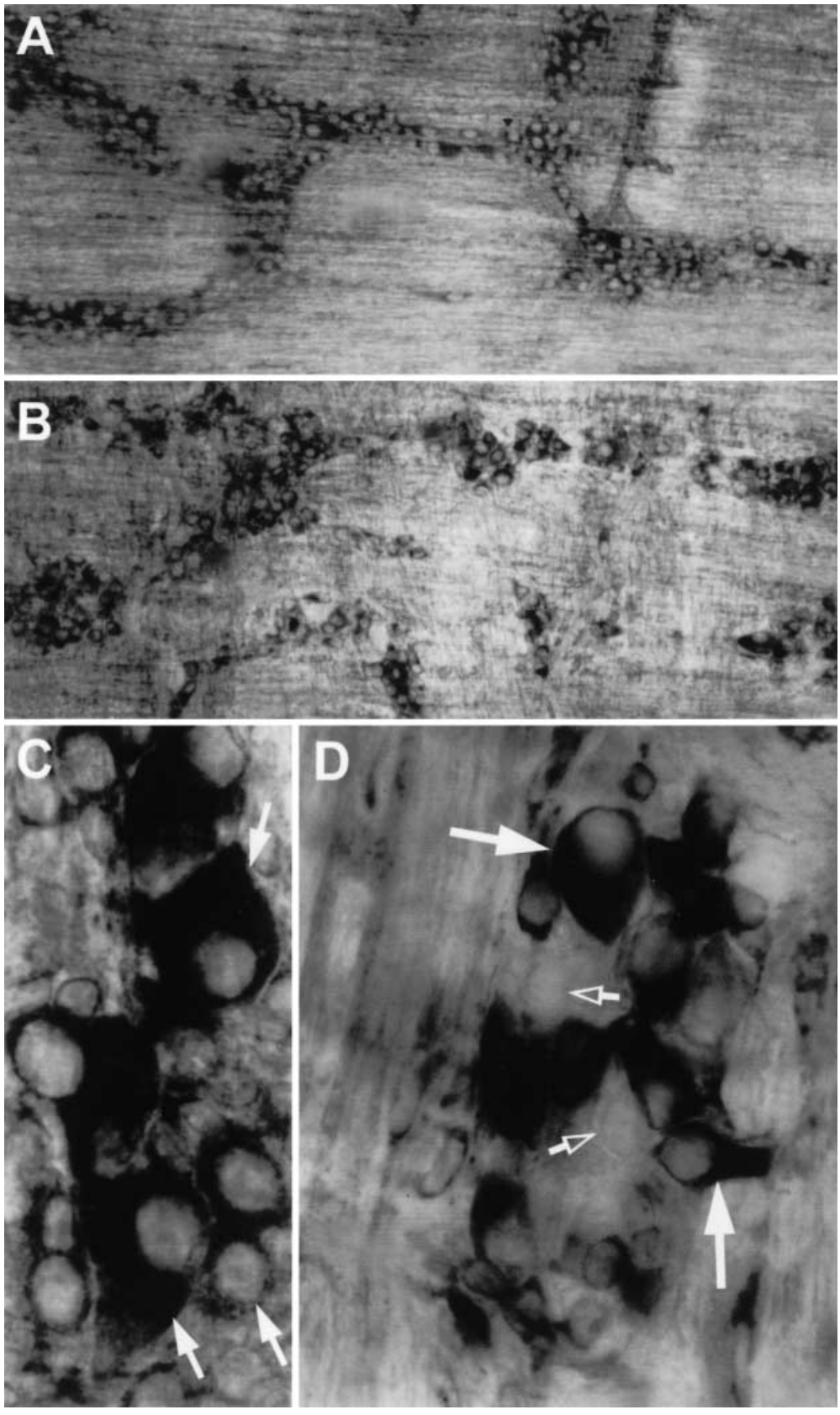

Fig. 1 The appearance of myenteric ganglia stained for NADH diaphorase in samples of colon taken from normally fed rats **(A, C)** and from rats whose mothers were protein deprived prenatally and for whom protein deprivation was continued to 21 days postnatal **(B, D)**. Note the less well organized appearance and the greater space between neurons after protein deprivation. In both the normally fed **(C)** and protein-deprived rats **(D)** small and large immunoreactive neurons are well stained (*arrows*), but in ganglia from the protein-deprived animals, the staining of some neurons is very weak (*open arrows*)

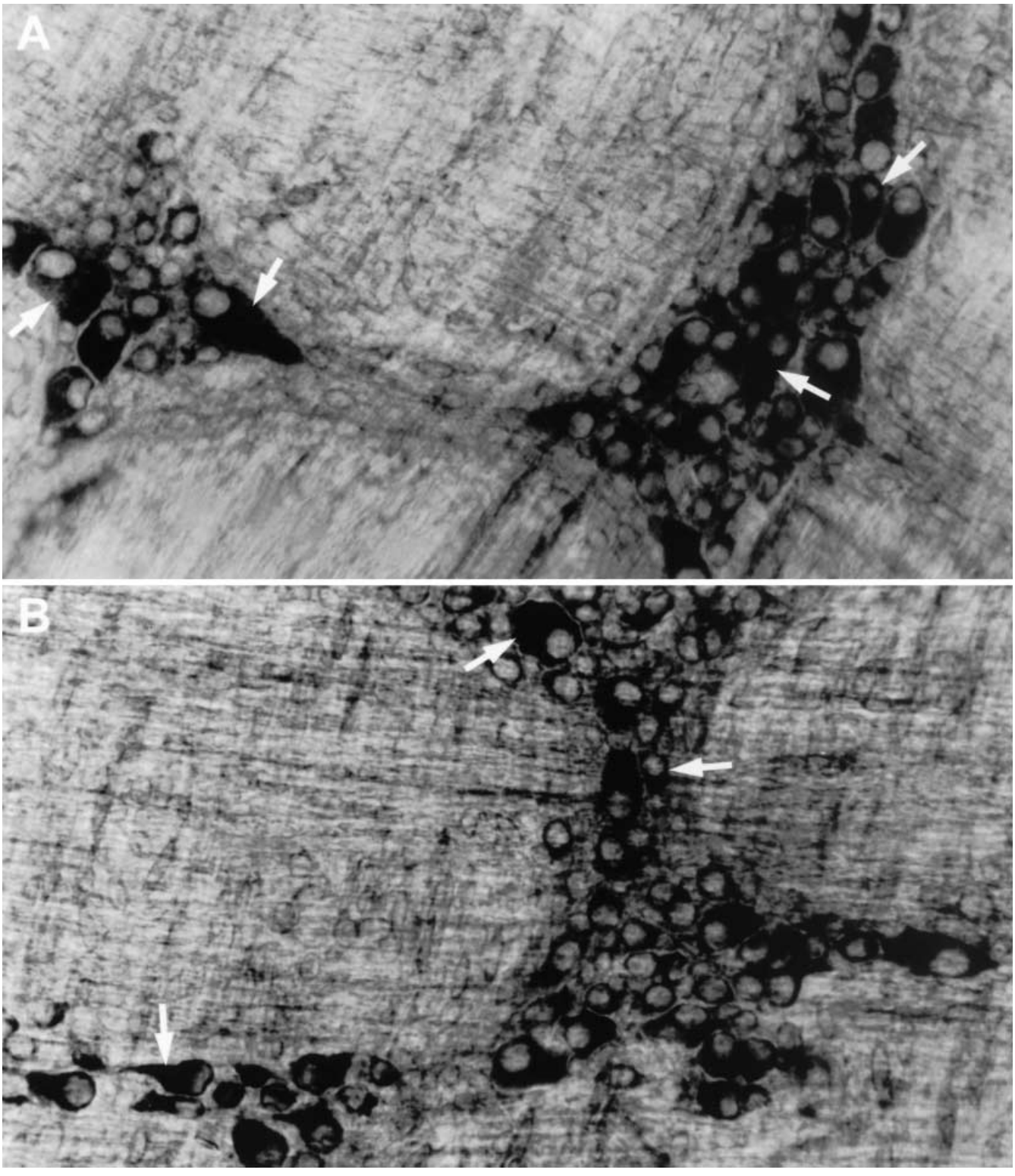

Fig. 2 Comparison of myenteric ganglia of the colon from rats that were fed normally through pregnancy and to 42 days postnatal **(A)**, when the tissue was taken, and ganglia from rats that were protein deprived through pregnancy and to 21 days postnatal, after which they had a normal diet to the time of sampling at 42 days **(B)**. The ganglia and neurons (*arrows*) had a similar appearance in both groups. This indicates that refeeding caused a recovery of the ganglion organization, which had been abnormal at 21 days after protein deprivation (Fig. 1)

Table 1 Body weight, total intestinal surface area, myenteric neuron cell profile area and neuron density of occurrence of normally fed and pre- and postnatal protein-deprived rats at 21 days postnatal. Values are means ± SEM

	Normally fed to 21 days (n=9)	Protein deprived to 21 days (n=9)
Body weight (g)	47.8±8.1*	24.8±5.9
Intestine, surface area (cm^2)	11.5±2.8*	7.1±1.9
Neuron cell profile (μm^2)	263±33*	233±44
Neuron density (nerve cells/cm^2)	25,000±41,000*	35,000±10,000
Neuron number	282,000±61,000	241,000±83,000

*Statistically different, P<0.05, compared to protein-deprived, Student's t-test

Table 2 Body weight, total intestinal surface area, neuron cell profile area, neuron density of occurrence and number of myenteric neurons in the colon of rats at 42 days postnatal. Values are means ± SEM

	Normally fed to 42 days (n=9)	Protein deprived to 42 days (n=9)	Protein deprived to 21 days, then normally fed to 42 days (n=9)
Body weight (g)	109±17*	68±18	90±22*
Intestine surface area (cm^2)	18.2±4.1*	12.9±2.8	17.5±3.6*
Neuron cell profile (μm^2)	320±39*	291±28	334±34*
Neuron density (nerve cells/cm^2)	19,500±2,500*	23,000±2,000	22,000±3,000
Neuron number	350,000±63,000	300,000±65,000	380,000±100,000

*Statistically different, P<0.05, compared to protein deprived to 42 days, Duncan's test for multiple comparisons

Quantitative analysis

Animals of the normally fed group had body weights almost double those of the protein-deprived rats at 21 days postnatal (P<0.05, see Table 1). The size of the large intestine was only 62% of the control value at this time (P<0.05).

Statistically, neuronal densities of occurrence and sizes were not different in the proximal, medial and distal portions of the large intestine (ANOVA, P>0.8) and so results from the three regions were pooled. At 21 days postnatal, the sizes of the neuron profiles were 13% greater in the normally fed group, compared to the protein-deprived group (P<0.05). As might be predicted from the reduction in organ size, the neuron density of occurrence was greater, by 38%, in undernourished compared with normally fed rats at 21 days (P<0.05). However, there was no significant difference between the normally fed and undernourished groups in the total numbers of neurons at 21 days (P>0.05, Table 1).

The body weights of animals of the normally fed group were about 60% more than the weights of the undernourished group at 42 days (P<0.05, Table 2). However, refeeding of undernourished rats between 21 and 42 days restored body weight to within 20% of normal at 42 days. The size of the large intestine was 35% less than control in undernourished rats (P<0.05), but this also returned to be no different from normal after 21 days of refeeding (Table 2). There was no significant difference in colon size between the group that was normally fed to 42 days, and the group that was undernourished to 21 days and then fed normally to 42 days (P>0.05).

Neuron size in protein-deprived rats was less than the control and the refed rats at 42 days (P<0.05). Interestingly, there was no size difference between neurons from normally fed rats, and rats that were protein deprived and then refed. There were differences in numbers of neurons per unit area that can be attributed to changed colon size, because absolute numbers of neurons were not different between the groups (Table 2). Numbers of neurons/cm^2 were 17% greater in the undernourished group when compared with the normally fed group at 42 days (P<0.05). There was no significant difference between the normally fed and refed groups at 42 days (P>0.05). Total numbers of neurons in the colon were not significantly different between normally fed, undernourished and undernourished and then refed groups at 42 days (P>0.05).

The distribution of neuron sizes in the myenteric plexus of normally fed and undernourished groups at 21 days and normally fed, undernourished, and undernourished and then refed groups at 42 days, is shown in the histograms in Figs. 3 and 4. In the undernourished group at 21 days, compared to the normally fed group, there is a shift in the peak of the distribution towards

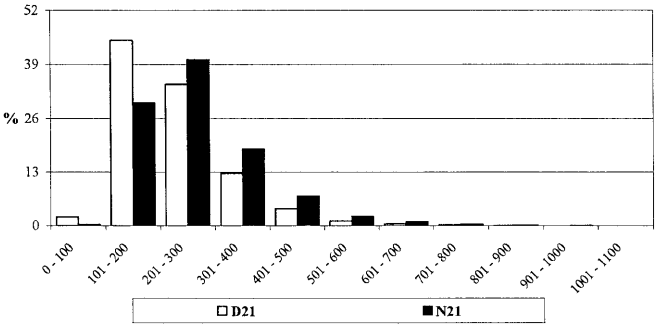

Fig. 3 Histogram showing the distribution of neuronal somata sizes (μm^2) in the myenteric plexus of 21-day-old rats that were protein deprived before and after birth (*D21*), compared with rats that were normally fed (*N21*)

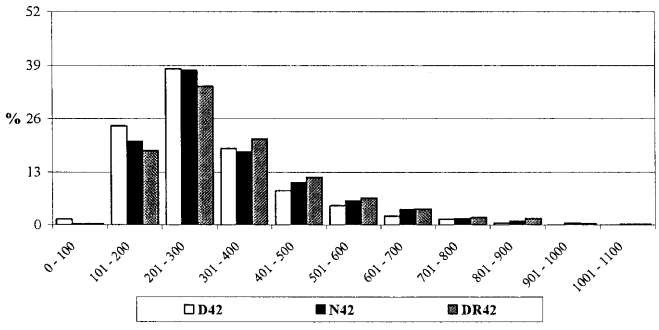

Fig. 4 Histogram showing the distributions of neuronal somata sizes (μm²) in the myenteric plexus of 42-day-old rats submitted to protein deprivation pre- and postnatal (*D42*) compared to normally fed rats (*N42*) and rats that were protein deprived to 21 days and then supplied with normal diet (refed) until 42 days (*DR42*)

smaller neurons (Fig. 3). Data from the normally fed, undernourished and undernourished and then refed groups at 42 days showed most of the neurons have nerve cell profile areas between 200 and 300 μm² (Fig. 4).

Discussion

The normally fed and protein-deprived groups showed differences in staining of the cytoplasm of the neurons with the NADH diaphorase technique. Although the neurons of the rats that were undernourished to 21 days showed more space between the neurons and the cytoplasm of many neurons had diffuse or less intense staining, there was no sign of changed nuclear appearance that might indicate dying cells.

The neuron numbers in the myenteric plexus of the large intestine were not statistically different between normally fed and undernourished groups at 21 days, and between normally fed, undernourished groups, and undernourished and refed groups at 42 days. The results agree with Leite-Mello et al. (1997), who did not find a statistical difference in the neuron numbers in the proximal portion of the colon between undernourished and nourished rats. In contrast, Santer and Conboy (1990) showed that prenatal undernutrition, by providing pregnant mothers with half the amount of food they would normally consume, decreased enteric neuron numbers in the jejunum by 27%, and that this decrease was measurable at 140 days postnatal, despite the animals being fed a normal diet. Our experiments imply that there is considerable resilience of enteric neurons, because neurons were not lost, even though body weights were halved. On the other hand, if there is sufficient food deprivation to kill enteric neurons, numbers do not recover even with several months of adequate feeding (Santer and Conboy 1990). This suggests that there is little or no ability of the enteric nervous system to generate new neurons. These data are consistent with studies on the central nervous system. In many regions of the central nervous system, severe undernutrition does not cause neuronal loss (Bedi 1994), but if there is loss, the neurons are not restored by refeeding (Andrade et al. 1995).

The data of Santer and Conboy (1990), which show that neuron numbers in the enteric nervous system do not recover with re-feeding, suggest that there is not a pool of enteric neuronal stem cells from which new neurons can be derived. This can also be concluded from the work of Gabella (1984), who investigated neurons in hypertrophied intestine. Despite a tenfold increase in muscle mass, that is, a considerable increase in a major target tissue for the enteric neurons, and an increase in nerve cell profile of about 2.5-fold, implying a nerve cell volume increase of almost fourfold, there was no indication of increased nerve cell number or neuronal mitosis. Furthermore, a recent extensive review of plasticity in the enteric nervous system (Giaroni et al. 1999) did not provide evidence of an enteric neuronal stem cell population.

The difference between our study, in which nerve cell loss did not occur, and that of Santer and Conboy (1990), in which nerve cells were lost and did not recover, is probably in the degree of undernutrition. In our study, pregnant dams and their offspring were protein deprived, but were unrestricted in the amount they could eat, whereas in the other study the rats were restricted to half the total food intake of the control, free-feeding, group. It is also possible that neurons in the colon (present study) and those in the jejunum (Santer and Conboy 1990) respond differently.

Similar to the present results, a decrease in neuron cell profile area with inadequate nutrition has been demonstrated for prevertebral sympathetic neurons and enteric neurons (Conboy et al. 1987; Natali and Miranda-Neto 1996; Torrejais et al. 1995). The neuron cell profiles of the undernourished group were smaller than those of the normally fed group at 21 and 42 days. We found that neuronal size (profile area) was restored in the protein-deprived then refed group. The distribution of the neuron cell profile areas for myenteric neurons from the undernourished 21-day-old animals was displaced towards smaller cell sizes compared to data from normally fed animals. At 42 days, data were similar for normally fed rats, undernourished rats, and undernourished and refed rats.

The present observations demonstrate that there was recovery of body weight, intestine area and neuron cell profile areas of the animals of the food deprived and refed group. This suggests that enteric neurons can recover from undernutrition stress.

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References

Andrade JP, Madeira MD, Paula-Barbosa MM (1995) Effects of long-term malnutrition and rehabilitation on the hippocampal formation of the adult rat. A morphometric study. J Anat 187:379–393

Bedi KS (1991) Effects of undernutrition during early life on granule cell numbers in the rat dentate gyrus. J Comp Neurol 311:425–433

Bedi KS (1994) Undernutrition of rats during early life does not affect the total number of cortical neurons. J Comp Neurol 342:596–602

Conboy VB, Santer RM, Swift GL (1987) Effects of prenatal undernutrition on prevertebral sympathetic neurons in the rat: a morphological and fluorescence histochemical study. J Anat 154:47–53

Cordero ME, Trejo M, García E, Barros T, Rojas AM, Colombo M (1986) Dendritic development in the neocortex of adult rats following a maintained early prenatal and/or postnatal life undernutrition. Early Hum Dev 14:245–258

Cornblath DR, Brown MJ (1988) Influence of malnutrition on developing rat peripheral nerves. Exp Neurol 99:403–411

Gabella G (1987) The number of neurons in the small intestine of mice, guinea-pig and sheep. Neuroscience 22:737–752

Gabella G (1984) Size of neurons and glial cells in the intramural ganglia of the hypertrophic intestine of the guinea-pig. J Neurocytol 13:73–84

Gaetani S, Mengheri E, Rossi A, Spadoni MA, Tochi G (1977) Long term protein deficiency in adult rats. Effects on different proteins of a sympathetic ganglion. Neurochem Res 2:439–448

Giaroni C, De Ponti F, Cosentino M, Lecchini S, Frigo G (1999) Plasticity in the enteric nervous system. Gastroenterology 117:1438–1458

Johnson RA, Wichern DW (1992) Applied multivariate statistical analysis, 3rd edn. Prentice Hall, Englewood Cliffs, NJ

Leite-Mello EV, Stabille SR, Miranda Neto MH (1997) Effect of maternal protein deprivation on morphological and quantitative aspects of the myenteric plexus neurons of proximal colon in rats. Arq Neuropsiquiatr 55:106–113

Natali MRM, Miranda-Neto MH (1996) Effects of maternal proteic undernutrition on the neurons of the myenteric plexus of the duodenum of rats. Arq Neuropsiquiatr 54:273–279

Rana SV, Chopra JS, Mehta S, Chand UK, Sharma U (1991) Electrophysiological and histopathological changes in peripheral nerves of postnatally undernourished and rehabilitated young rhesus monkeys. Indian J Exp Biol 29:385–390

Rushmore J, Luebke J, Galler JR (1998) Electrophysiological properties of rat hippocampal principal cells are unaltered by prenatal protein malnutrition. Hippocampus 8:380–389

Sant'ana DM, Miranda Neto MH, De Souza RR, Molinari SL (1997) Morphological and quantitative study of the myenteric plexus of the ascending colon of rats subjected to proteic desnutrition. Arq Neuropsiquiatr 55:687–695

Santer RM, Conboy VB (1990) Prenatal undernutrition permanently decreases enteric neuron number and sympathetic innervation of Auerbach's plexus in the rat. J Anat 168:57–62

Torrejais MM, Natali MRM, Conegero CI, Miranda Neto MH (1995) Effects of proteic malnutrition after breast-feeding on the morphology of the intestinal wall and myenteric neurons of the ileum in rats. Rev Universidade de Maringá 17:315–327

Wiggins RC, Fuller GN, Brizzee L, Bissel AC, Samorajski T (1984) Myelination of the rat optic nerve during postnatal undernourishment and recovery: a morphometric analysis. Brain Res 308:263–272