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Developmental changes in submucosal nitrergic neurons in the porcine distal colon☆

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Nitrergic innervation;
Ganglia density;
Ganglion cell number;
Ganglion cells size;
Enteric nervous system

Abstract

Background/Purpose: As our understanding of the enteric nervous system improves, it becomes clear that it is no longer sufficient to simply determine whether enteric ganglion cells are present but also to determine whether correct number and types of ganglion cells are present. Nitric oxide is recognized as a potent mediator of inhibitory nerves responsible for the relaxation of the smooth muscle of the gastrointestinal tract. The aim of this study was to determine the normal nitrergic neuronal density and morphology in the submucosal plexus of the porcine distal bowel from fetal life to adulthood.

Methods: Distal large bowel specimens were obtained from porcine fetuses of gestational age E60 (n = 5), E90 (n = 5), 1-day-old piglets (n = 5), 4-week-old piglets (n = 5), 12-week-old piglets (n = 5), and adult pigs (n = 5). Whole-mount preparations of the submucosal plexus were made and stained with NADPH diaphorase histochemistry. The ganglia density, the number of ganglion cells per ganglia, and nucleus and cytoplasmic area were measured.

Results: Ganglia density decreased progressively and markedly with age until the adulthood ($P < .001$). On the contrary, ganglion cells increased their size over time predominantly because of increase in cytoplasm ($P < .001$). The number of ganglion cells per ganglia increased significantly during the fetal life. However, there was a significant reduction in the number of ganglion cells per ganglia during the period from birth to 4 weeks, remaining constant thereafter ($P < .001$).

Conclusions: The quantitative and qualitative morphometric analysis of the colonic submucous plexus shows that significant developmental changes occur during fetal and postnatal life. These findings indicate that the age of the patient is of utmost importance during histopathologic evaluation of enteric nervous system disorders.

Normal intestinal motility depends on the interaction of the enteric nervous system and the smooth muscle cells in the gut wall. As our understanding of the enteric nervous system improves, it becomes clear that it is no longer sufficient to simply determine whether enteric ganglion cells are present or absent but also to determine whether the correct number and type of ganglion cells are present at different developmental stages.

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Meier-Ruge [1] in 1971 described a malformation of the enteric plexus that clinically resembled Hirschsprung's disease, called *intestinal neuronal dysplasia* (IND). Intestinal neuronal dysplasia is characterized by the presence of giant ganglia in the submucosal plexus, enlarged parasympathetic nerve fibers in the submucosa, and increased acetylcholinesterase activity in the mucosa [2,3]. However, the criteria for diagnosis of IND remain controversial, and several authors have raised doubts about its existence as a distinct histopathological entity and its real correlation with clinical symptoms [4,5]. In particular, it has been suggested that proposed diagnostic criteria relating to ganglion cell density may overlap with age-related changes [2,3]. An important reason for this confusion is insufficient knowledge of the morphology of the normal enteric nervous system through life.

Nitric oxide is the most important inhibitory neurotransmitter in the gastrointestinal tract in various species and can be clearly shown by nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) histochemistry [6,7]. Whole-mount preparation is an elegant technique for visualization of the myenteric and submucosal plexuses. It provides a method for the detailed study of the 3-dimensional morphology of the meshwork of nerves and neurons and, therefore, is far superior to standard tissue sections in the investigation of the enteric nervous system [6]. NADPH diaphorase–positive ganglion cells have been estimated in whole-mount preparations to represent about 34% of all neurons in the human myenteric plexus [8]. Because the organization of the porcine enteric nervous system possesses functional and histologic similarities with the human one, the pig intestine is the most suitable experimental model for studying the human enteric nervous system [9,10].

The aim of this study was to investigate the normal nitrergic ganglia density, neuron number and morphology in whole-mount preparations of the submucosal plexus of the porcine distal bowel from fetal life to adulthood.

1. Materials and methods

1.1. Tissue sampling and whole-mount preparation

Bowel specimens were obtained from porcine fetuses of gestational age 60 days (n = 5) and 90 days (n = 5) and 1-day-old piglets (n = 5), 4-week-old piglets (n = 5), 12-week-old piglets (n = 5), and 1-year-old adult pigs (n = 5). The animals were provided from the Institute of Experimental Clinical Research, Skejby Sygeh, University of Aarhus, Denmark. The study was approved by the Danish authorities of animal protection, permission no. 200 601-068. The large bowel was removed from the piglets and placed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (PBS) for 48 hours at 4°C. Afterward, specimens were transferred into sterile containers filled with PBS and stored at 4°C until further use. The distal bowel was opened along the mesenteric border and rinsed. Four samples of distal bowel from each specimen were cut for further processing. Samples were pinned flat on a silicone plate, and whole-mount preparations were made under a dissecting microscope (Leica S8 APO; Heerbrugg, Switzerland). Initially, the mucosal layer was scraped out along with the inner submucosal plexus (Meissner's plexus) using fine-pointed forceps. After that, the submucosal layer containing the outer submucous plexus (Schabadasch or Henle plexus) was carefully lifted from the muscle layer with a fine forceps starting in one corner of the tissue sample.

1.2. Staining procedure

For NADPH diaphorase histochemistry, the submucous laminae were placed in a sterile 12-well plate (Corning, New York, NY) and were incubated in 2 mL of the staining solution containing 1 mg/mL β-NADPH (Sigma-Aldrich, St Louis, MO), 0.25 mg/mL nitrobluetetrazolium (Sigma-Aldrich), and 0.3% Triton X-100 (BDH Laboratory Supplies, Dorset, UK) in a 0.05 mol/L TRIS HCl buffer (pH 7.6) for 2 hours at 37°C and then left in the staining solution overnight at room temperature. After achievement of the desired staining intensity, specimens were rinsed in PBS for 15 minutes and then mounted on Polysine microscope slides (BDH) using Glycergel mounting medium (DakoCytomation, Glostrup, Denmark).

1.3. Morphometry

A ganglia was defined as a group of at least 3 ganglion cells with a distance between cells not exceeding 2-cell diameters as previously described [11]. Density of NADPH diaphorase–positive ganglia was measured by counting the total number of ganglia per 1 cm^2 under a light microscope (Leica DMLB) using ×200 magnification. For that purpose, a 1-cm^2 square graticule was drawn on the coverslip of the newborn, 4-week, 12-week, and adult specimens. Because E60 and E90 specimens were smaller than 1 cm^2, a smaller square graticule was drawn on them and the results were then converted to 1 cm^2. The number of NADPH diaphorase–positive neurons per ganglia was determined by counting them in 25 adjacent ganglia in each specimen under a light microscope (Leica DMLB). All profiles of positively stained cells were identified and counted. In some cell clusters, identification of cell profiles was confirmed by adjusting the focal depths of the objective. Size of NADPH diaphorase–positive neurons was determined by analyzing the photographs of at least 85 neurons from 10 different ganglia with clearly delimitated individual neurons from each specimen. The border of each neuron and its nucleus were marked by hand in the digitalized image, and the total area of the neuron and the nucleus was measured using software for image analysis (Image J 1.5 Beta 1; Research Services Branch, National Institute of Mental Health, Bethesda, Md, USA). The area of the cytoplasm was calculated by subtracting the area of the nucleus from the area of the whole neuronal body.

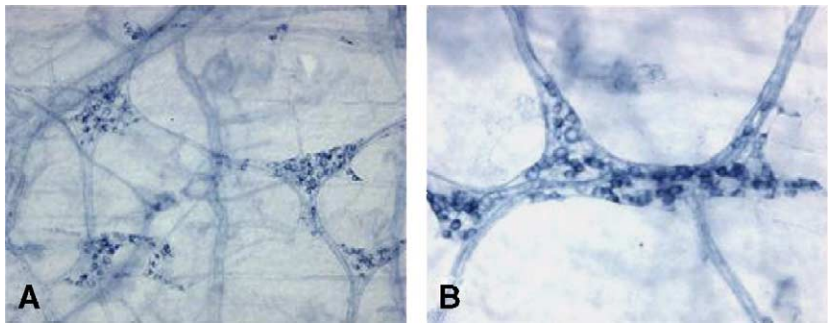

Fig. 1 Whole-mount preparations of the submucosal plexus of the distal pig colon. A, A mesh of nerve bundles with ganglia containing NADPH-d–positive ganglion cells at the intersections is clearly seen (original magnification ×100). B, Typical NADPH-d–positive submucosal ganglia (original magnification ×200).

1.4. Statistical analysis

All numerical data are expressed as mean ± SD. The normal distribution of each group was assessed with Kolmogorov-Smirnov test. After that, analysis of variance test was used to compare ganglia density, number of ganglion cells per ganglia, and ganglion cell size among the different age groups, with the Student-Newman-Keuls test being used for pairwise comparisons. A P value of less than .05 was considered statistically significant. All statistical tests were performed using a commercially available software package (SPSS 11.0 Statistical Analysis Software, Chicago, IL).

2. Results

Whole-mount preparations of the submucosal plexus facilitated visualization of a regular mesh of nerve bundles with ganglia containing NADPH diaphorase–positive ganglion cells at the intersections. The ganglia were clearly separated one from another, and no neurons were seen along the bundles. Staining of NADPH diaphorase–positive neurons within each ganglia was not uniform. About one third of the neurons showed very strong staining, whereas the remaining neurons were moderately or weakly stained (Fig. 1).

2.1. Ganglia density in different age groups

The gross morphology of the submucous plexus varied with age. The meshwork became progressively less dense with increasing age. The highest number of ganglia per square centimeter was found at E60 (1912.5 ± 279.69 ganglia per square centimeter). After that period, ganglia density fell markedly and constantly with age until adulthood (E90, 775 ± 85.79 ganglia per square centimeter; newborn, 412.5 ± 50.51 ganglia per square centimeter; 4 weeks old, 264.28 ± 28.78 ganglia per square centimeter; 12 weeks old, 71.33 ± 18.14 ganglia per square centimeter; adult, 29.8 ± 5.61 ganglia per square centimeter; values in each age group are statistically different from the previous and the following age group, $P < .001$) (Fig. 2).

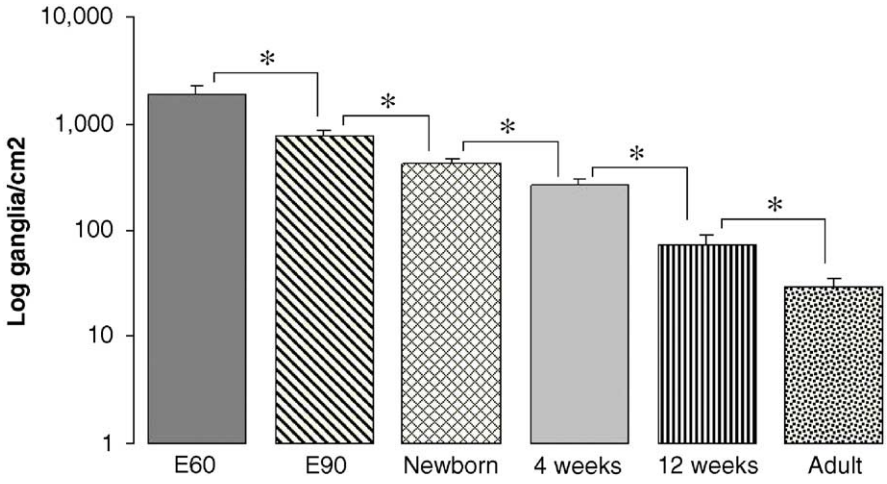

Fig. 2 Ganglia density in the submucosal plexus of the distal pig colon at different ages (logarithmic transformation). Ganglia density decreases progressively with age (mean ± SD). E60 indicates pig fetus of gestational age 60 days; E90, pig fetus of gestational age 90 days; newborn, newborn piglet; 4 weeks, 4-week-old piglet; 12 weeks, 12-week-old piglet; adult, 1-year-old adult pig. *$P < .001$.

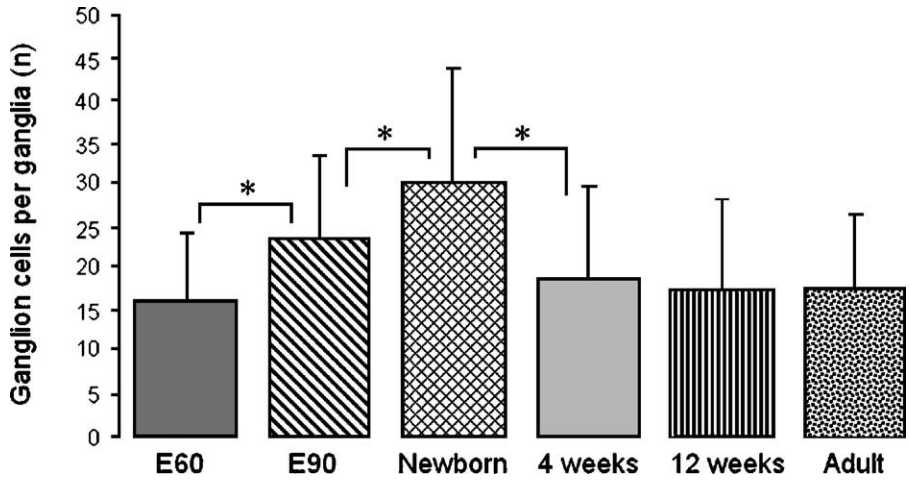

Fig. 3 Number of NADPH diaphorase–positive ganglion cells per ganglia in the submucosal plexus of the distal pig colon at different ages (mean ± SD). Note that the number of ganglion cells per ganglia increase significantly during the fetal life. After the newborn period, there is a marked reduction in the number of ganglion cells per ganglia, remaining constant thereafter. *$P < .001$.

2.2. Number of ganglion cells per ganglia in different age groups

The number of NADPH diaphorase–positive ganglion cells per ganglia increased significantly during the intrauterine life until the piglets were born (E60, 16.33 ± 7.79; E90, 23.41 ± 9.96; newborn, 30.29 ± 13.37; values in each age group are statistically different from the previous and the following age group, $P < .001$). From the newborn period until the piglets were 4 weeks old, the mean number of neurons per ganglia decreased significantly (30.29 ± 13.37 for newborn vs 18.89 ± 10.61 for 4 weeks old, $P < .001$). The number of ganglion cells per ganglia remained constant from 4 weeks until adulthood (4 weeks old, 18.89 ± 10.61; 12 weeks old, 17.51 ± 10.55; adult, 17.62 ± 8.67; P = not significant). In the adult pig, the mean number of NADPH diaphorase–positive ganglion cells was almost the same that in E60 piglets (Fig. 3).

2.3. Ganglion cell size in different age groups

A marked increase in the size of NADPH diaphorase–positive neurons was seen with increasing age (E60, 71.91 ± 14.67 μ^2; E90, 102.51 ± 28.52 μ^2; newborn, 157.21 ± 48.02 μ^2; 4 weeks old, 239.94 ± 88.81 μ^2; 12 weeks old, 257.19 ± 102.45 μ^2; adult, 392.51 ± 194.71 μ^2; values in each age group are statistically different from the previous and the following age group, $P < .001$). However, the increase in cell size was found to be predominantly caused by an increase in cytoplasm (E60 cytoplasm area, 28.71 ± 5.11 μ^2; E60 nucleus area, 43.2 ± 9.56 μ^2; E90 cytoplasm area, 51.23 ± 15.23 μ^2; E90 nucleus area, 51.28 ± 13.29 μ^2;

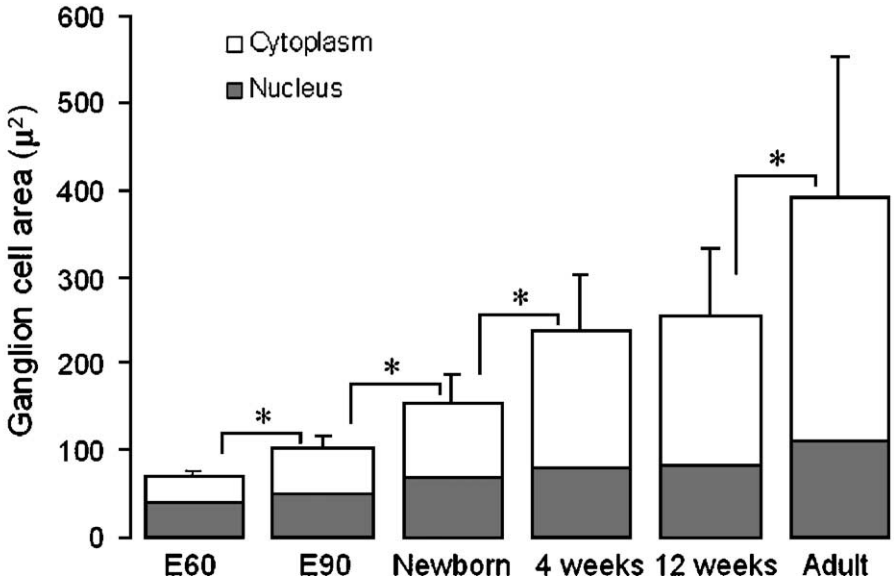

Fig. 4 Ganglion cells size in the submucosal plexus of the distal pig colon at different ages. A marked increase in ganglion cells size is seen with increasing age (mean ± SD). However, this increase is predominantly because of the growth of the cytoplasm. *$P < .001$.

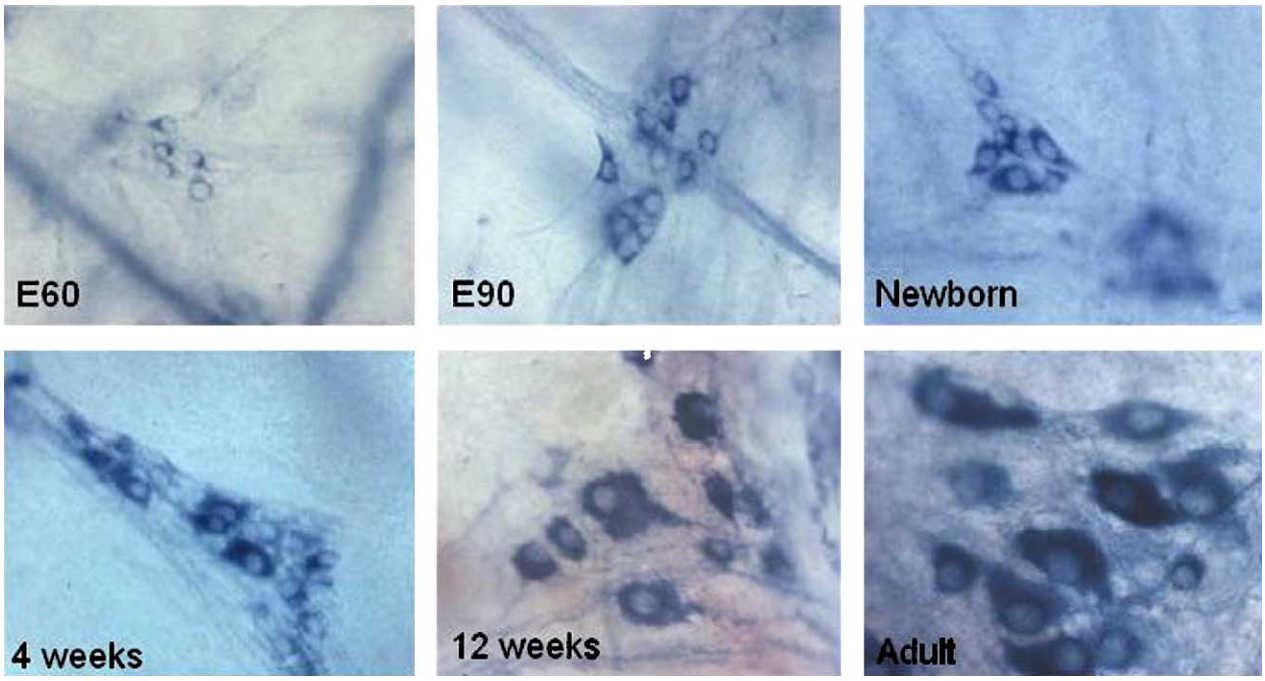

Fig. 5 Ganglion cells in the submucosal plexus of the distal pig colon at different ages. Ganglion cells increase their size throughout life predominantly because of an increase in cytoplasm. All photographs are taken with the same magnification (original magnification ×400).

newborn cytoplasm area, 88.74 ± 32.99 μ^2; newborn nucleus area, 68.47 ± 15.03 μ^2; 4-week-old cytoplasm area, 158.71 ± 64.81 μ^2; 4-week-old nucleus area, 81.23 ± 24.00 μ^2; 12-week-old cytoplasm area, 174.81 ± 77.67 μ^2; 12-week-old nucleus area, 82.38 ± 24.78 μ^2; adult cytoplasm area, 279.89 ± 162.14 μ^2; adult nucleus area, 112.62 ± 32.57 μ^2; values in each age group are statistically different from the previous and the following age group, $P < .001$) (Figs. 4 and 5).

3. Discussion

Our results show that significant morphological changes occur in the submucosal plexus of the porcine distal colon throughout life. We found an inverse relationship between ganglia density and ganglion cells size with age. Ganglia density decreased progressively with gestation and postnatal age, whereas ganglion cells size increased with age. On the other hand, the mean number of ganglion cells per ganglia showed a distinct pattern reaching the highest value at the neonatal period and decreasing thereafter to stabilize at 4 weeks in the pig. Comprehensive information regarding the prenatal and postnatal normal morphological changes of the enteric nervous system is scanty and is extremely important when interpreting histopathological findings in early childhood. The gold standard test when evaluating a child with chronic constipation and obstructive symptoms is a suction rectal biopsy, which normally comprises mucosa and submucosal layers of the rectum. Hirschsprung's disease, IND, and other dysganglionosis may be diagnosed by evaluating the submucosal innervation pattern. For this reason, our study focused on the submucosal plexus of the distal colon. We used whole-mount preparation technique, which produces a 3-dimensional picture, to better show the structure of neuronal networks and their relationship of branching and interconnecting nerve fibers to each other and to the neighboring tissues [6]. Quantitative morphological analysis of the enteric plexuses are therefore much more accurate with whole-mount technique than with standard tissue sections, which only partially show the morphology of the plexus and whose results may vary depending on the number and thickness of sections studied [6]. We chose the pig as our experimental model because it is a large mammal with an enteric nervous system that possesses striking similarities with the human one [9,10]. Nitric oxide is an important inhibitory neurotransmitter that mediates relaxation of the smooth muscle of the gastrointestinal tract [7]. The enzymes involved in the neuronal generation of nitric oxide are the constitutive neuronal isoform of nitric oxide synthase and NADPH diaphorase [6]. A one-to-one correlation between the 2 enzymes responsible for nitric oxide synthesis has been found in the enteric neurons. Consequently, neurons producing nitric oxide can be detected by using either nitric oxide synthase immunohistochemistry or NADPH-d histochemistry. It has been demonstrated that NADPH diaphorase–positive neurons account for 34% of the total number of enteric neurons [8]. NADPH-d–positive neurons of the porcine submucous plexus showed a gradation of reactivity for the enzyme. Other authors have previously shown similar findings [12,13].

We found the meshwork to become progressively and markedly less dense with increasing age, which was objectively assessed by quantifying the number of ganglia per surface area. Ganglia density significantly changed from one age point to the following one until the adulthood. This is a known phenomenon that has been studied in different species including human and is known to occur in both myenteric and submucosal enteric plexuses and also in the bladder [8,11,13-18]. Growth of the bowel with increasing surface area probably causes the originally densely packed network to expand and leads to a lower ganglia density with increasing age.

We were surprised by our findings on the variation of the number of ganglion cells per ganglia throughout life. They significantly increased during the intrauterine life until the piglets were born. Then, from the newborn period until the piglets were 4 weeks old, the number of neurons per ganglia decreased significantly, remaining constant after that until adulthood, when the number of ganglion cells per ganglia return to be the same as during early intrauterine life. If we consider that pig natural life span is approximately 17 years and they reach puberty at 7 months old, a 4-week-old piglet would be the equivalent to a 2-year-old infant [19]. If we extrapolate our findings to the human situation, that would mean that the number of ganglion cells per ganglia decreases progressively after birth until the child is 2 years old to remain constant thereafter. Similar findings have been shown in the chicken myenteric plexus, whose ganglion cells per ganglia increase around the perinatal period [17,18]. Coerdt et al [15] have recently studied sections of human colonic submucosal plexus. Their results are strikingly similar to the present study. They found the mean number of ganglion cells per ganglia to be highest in the group with a gestational age of less than 35 weeks, followed by the group of patients aged less than 1 year, and slightly decreasing thereafter. It is intriguing why neurons increase their number during fetal life to decrease then after birth. We speculate that this could be an adaptation phenomenon of the enteric plexuses to the greater demand for innervation at birth, when the actual bowel function starts. However, this may also be a purely developmental phenomenon.

Our measurements revealed an increase in ganglion cells size during life from relatively small cells with small cytoplasm in the early fetal period to large cells with enlarged cytoplasm in the adult. This finding has been previously reported in the human enteric nervous system [8,16,20] and also in the pig bladder [13] and has been considered a sign of maturation of the neurons. The increase in cell size is explained by the growth of the bowel: an increase in volume of an innervated organ is normally accompanied by an increase in the volume of intramural nervous tissue and particularly in the cytoplasm. Moreover, it has been demonstrated that neurons react upon an increase of muscle mass by increasing their size even in adult rats [21].

The present findings suggest that enteric nervous system is dynamic and developmental changes occur all throughout life but especially in the perinatal period and during the first years of life. Applied to the clinical setting, it implies that interpretation of enteric nervous system pathology is highly dependent on age of the patient. Intestinal neuronal dysplasia is characterized by the presence of hyperganglionosis and giant ganglia with more than 7 ganglion cells in the submucosa [2,3]. Our present findings may have implications for the understanding of pathophysiology of IND. We hypothesize that IND is a developmental maturation phenomenon, with histopathologic features of IND corresponding to the normal enteric plexus findings in the late gestational period. Further evidence supporting this hypothesis is that although IND is a recognized histopathological and clinical entity [2,3,22], most patients do well with only conservative treatment [23].

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