VIA MEDICA

ORIGINAL ARTICLE

Folia Morphol.
Vol. 75, No. 2, pp. 188–195
DOI: 10.5603/FM.a2015.0097

ISSN 0015–5659
www.fm.viamedica.pl

Age-related changes in the myenteric plexus of the human jejunum

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[Received: 15 August 2015; Accepted: 21 October 2015]

Background: *Myenteric ganglia are an important regulator of peristaltic activity of the digestive tract. Getting to know its normal morphological changes during aging are of great importance for the interpretation of intestinal motility disorders. The aim of our histomorphometric study was to reveal detailed characteristics of myenteric plexus, cell number, orientation, properties of as well as age-related changes in the human jejunum.*

Materials and methods: *We examined the myenteric ganglia in the proximal jejunum specimens obtained from 30 human cadavers aged from 20 to 84 years. Tissue samples were classified into three age groups: 20–44, 45–64 and 65–84 years. After standard histological preparation, specimens were stained with haematoxylin and eosin method, Cresyl-Violet and silver nitrate ($AgNO_3$) by the method of Masson Fontana. In addition to standard staining methods we use and enzyme histochemical method for acetylcholinesterase. Morphometric analysis of all the specimens was performed, using a multipurpose test system M42. The data were subjected to the Tukey post hoc HSD significance test using one-way ANOVA. There is a significant decrease ($p < 0.001$) in the number of neurons in persons aged between 65 and 84 years.*

Results: *It was found that the reduction in number of neurons is 25.93% in oldest compared to the youngest. It was also noted a significant increase ($p < 0.05$) of the surface profile of ganglion neurons.*

Conclusions: *This increase in the size of neurons can be explained as compensation for the loss in the number of neurons in order to maintain the normal function of innervations of the jejunum.* (Folia Morphol 2016; 75, 2: 188–195)

Key words: myenteric plexus, jejunum, human, aging

INTRODUCTION

Normal functioning of the gastrointestinal tract is dependent on the interaction of the enteric nervous system (ENS), the smooth muscle layers and the submucosal and mucosal layer of wall of the digestive tube. The ENS is also the most complex part of the peripheral nervous system. Its complexity is reflected in the phenotypic diversity of neurons and the existence of a wide range of neurotransmitters [5, 6, 16] that are present and act in these neurons. The nerve junctions of the intestinal wall are responsible for peristaltic contractions and coordination of

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the motor activity of smooth muscles, which does not disappear even in the complete absence of external innervations [10, 11]. Neurons of the enteric system play an important role in coordinating the activities of other gastrointestinal cells and influence the processes such as absorption, secretion, and microvasculature. Recent evidence shows that the enteric neurons also influence the function of the epithelial barrier [23]. Two basic plexuses of the ENS are interconnected and arranged in the form of concentric circles in the wall of the intestine [12, 18]. The first is the myenteric plexus, which lies between the longitudinal and circular smooth muscle layers. The second is the submucosal plexus, which is located in the layer of connective tissue between the mucosal epithelium and the layers of circular smooth muscle. However, there are recent views that the ENS consists of three different ganglionated plexuses, myenteric plexus, outer submucosal plexus and inner submucosal plexus [2, 3]. In addition to the ganglion plexuses, in the wall of alimentary tract, there are a large number of aganglionated nervous plexuses [35, 37]. Myenteric plexus mainly regulates motility, and submucosal plexus mainly regulates secretion and local blood flow. Researches on aging ENS are largely focused on the analysis of changes in the number of neurons in the myenteric plexus. These studies were carried out in a number of animal species [27, 29, 31]. The literature that has been documented support varying data from those which indicate the significant reduction (30–50%) in the number of neurons [17, 33] to those where there is no significant loss of the neurons [14, 24, 36] in the aging process. There are a number of studies [7, 34] which describe changes in ENS due to the introduction of certain dietary restrictions in experimental animals. In addition to the loss of enteric neurons in the aging process, other signs of neurodegeneration such as dystrophic nerve fibres, the accumulation of lipofuscin, a change in the size of the neurons and their nuclei are occurring. It should also be noted that a large number of phenotypically and functionally different subpopulations of enteric neurons showed various levels of susceptibility to age-related changes. Some of the researchers [20] suggest that in terms of aging, myenteric neurons show age-related phenotypes. The aim of this study is detailed examination of myenteric plexus in the proximal part of the human jejunum using specific histological, morphometric, and stereological methods. Using methods that allow quantification, we sought to determine the presence of individual differences and verify certain changes in the structure of myenteric plexus that occur during the aging process. In order to achieve this, we determined the number of neurons of the myenteric plexus per unit area of the jejunum, the surface area of the nerve structures, as well as the surface of the individual neurons and their nuclei.

MATERIALS AND METHODS

Tissue samples of the human jejunum were obtained from autopsy material at the Institute of Forensic Medicine in Belgrade from 30 cadavers (17 men and 13 women), aged from 20 to 84 years, who were divided into three age groups (20–44 years, n = 10; 45–64 years, n = 10; 65–84 years, n = 10). The cause of death of each was ischaemic heart failure or intracranial bleeding. None had undergone previous surgery, and in their medical documents there was no information about gastrointestinal diseases. Fragments of the jejunum were taken during the first 24 h from the moment of death. The collection of materials is satisfied ethical requirements of the institutions from which is obtained (the Ethics Committee of the Medical Faculty in Belgrade). Samples were taken always from the same topographic places (antimesenteric wall of the jejunum, 80 cm from duodenojejunal flexure) and sorted by age in groups. Immediately after taken, tissue slices of preparations the size of 1 × 1 cm were fixed in 4% buffered paraformaldehyde for 48 h. After the routine processing in an increasing series of alcohol, samples were embedded in paraffin blocks, which were then cut in two ways: sections perpendicular to the longitudinal axis of the jejunum (classical) and sections parallel to the longitudinal axis of the jejunum (from serosa until myenteric plexus and through it). Histological preparations were stained with the routine haematoxylin and eosin method (H&E), and to ensure reliable identification of ganglion, cells and structures were stained with silver nitrate ($AgNO_3$) by the method of Masson Fontana and Cresyl-Violet (CV) staining.

Enzyme histochemistry for acetylcholinesterase (AChE) was used to stain the myenteric plexus in one part of the samples of jejunum for the reason that this enzyme occurs non-specifically within intrinsic nerves and ganglia. The hyaluronidase-treated part of the tissue samples were incubated for 1–3 h at 4°C in the incubation medium (pH 5.6) containing the following: sodium acetate, 60 mM; acetylthiocholine iodide 2 mM;

sodium citrate 15 mM; $CuSO_4$, 3 mM; $K_3Fe(CN)_6$, 0.5 mM; Triton-X100 supplemented the incubation medium up to 1% and hyaluronidase (0.5 mg/100 mL). All fats from the surface of gut which are almost completely non-permeable for the incubation media were previously removed. Following staining, the preparations were fixed in neutral 4% formalin in 0.1 M phosphate buffer. The preparations were stored in the same formalin. After that tissues were embedded in paraffin blocks, cut and mounted on glass slides.

Silver nitrate staining by the method of Masson Fontana was performed as follows: the hydrated preparations were put in a previously prepared solution of silver nitrate for 2 h at 56°C, rinsed in distilled water and tones with 0.2% gold chloride solution for 2–3 min, rinsed again with distilled water and 1 min down to 5% sodium thiosulfate. Preparations were again rinsed with distilled water and 5 min immersed in nuclear-fast red and then mounted on glass slides and covered the outer husks. The result of staining was following: argentaffin granules in neural cells were black, nuclei were pink-reddish, and cytoplasm pale-pink.

Cresyl-Violet staining for nerve cells was performed as follows: hydrated preparations were left in the previously prepared 0.5% solution of CV stain for 30 min. They were then discoloured in 96% alcohol, to which was added 1 drop of HCl, and bleaching was controlled under the microscope. When they got the desired colour, preparations were dehydrated and mounted on glass slides. The result of staining was: nucleus was dark blue, cytoplasm was lighter, and nerve fibres were not stained.

Microscopic techniques

Tissue samples were cut with a slice thickness of 6 microns. Sections were then analysed by multipurpose test system M42, which was calibrated at the appropriate magnification light microscope (Leica DM LS2) and embedded in its ocular. At each section were analysed 10 fields. When using the test system respected the rule that when counting particle, count all particles whose profiles fall within the test system, except those that fall on "forbidden line", i.e. on the right and lower edge of the used system. So we avoid that one particle count twice, since the test system always moves to the right and down. In each visual field was counted the number of points of the test system, which falls on the area that occupies the ganglion and the number of points that fall on the profile of bodies of the myenteric nerve cells. In addition, it was counted the total number of neurons within each ganglion structure. At Figure 1 is illustrated how to use the test system M42. Surface area of the ganglion structure and surface area of the nerve structure were calculated by formulas for the appropriate test system and by adequate magnification of the lens.

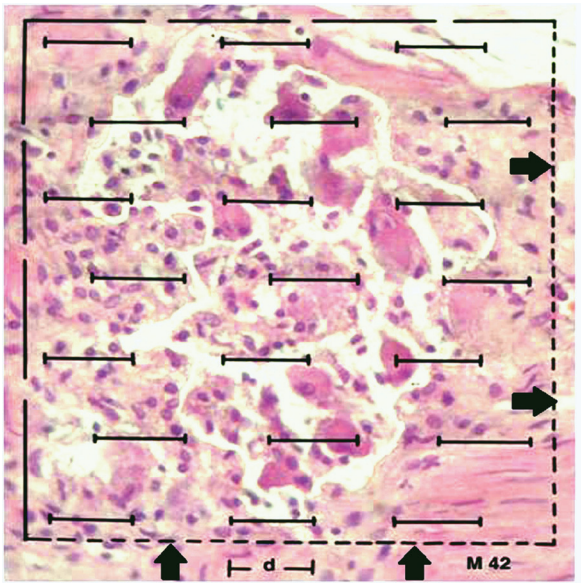

Figure 1. Manner of using the multipurpose system M42 (arrows indicate "forbidden line").

Based on the recorded surface of nerve structures and the total number of neurons within them was mathematically calculated number of neurons per cm^2 area of myenteric plexus. In addition, the micrographs of ganglion cells were acquired using a Leica zoom DM LS2 lighting microscope equipped with a camera and connected to the computer. Size of cells and the size of the nuclei are measured using ImageJ computer software. We measured the surface area profile of soma and nuclei of 50 randomly selected nerve cells from each sample. The results are presented in text and Table 1 and expressed as means ± standard deviation (SD).

Statictical analysis

For the analysis of primary data we used descriptive statistical methods and methods for testing statistical hypotheses. The descriptive statistical methods that were used are the measure of central tendency (mean) and the measures of variability (SD). Estimation of statistical significance between mean values was performed by the Tukey post hoc

Table 1. Influence of age on parameters of the myenteric plexus of the human jejunum

Parameters	Age [years]		
	20–44	45–64	65–84
Numbers of neurons/cm^2	57848 ± 2026.50	55883 ± 1940.58	42849 ± 2148.52*
Surface area of ganglion structure [mm^2]	0.0129 ± 0.0009	0.0142 ± 0.0020	0.0131 ± 0.0015
Surface phase of the ganglion structure belonging to the profiles of neurons [mm^2]	0.0025 ± 0.0002 19.6 ± 0.8%	0.0024 ± 0.0003 17.6 ± 0.9%	0.0018 ± 0.0002* 14.1 ± 0.6%*
Surface of neurons [μm^2]	284.2 ± 24.9	306.5 ± 33.3	324 ± 23.9**
Surface of nuclei [μm^2]	35.1 ± 3.6	36.6 ± 3	40.6 ± 2.6**
Nucleo-cytoplasmic ratio	0.124 ± 0.012	0.121 ± 0.019	0.125 ± 0.011

*$p < 0.001$; **$p < 0.05$

HSD significance test using one-way ANOVA. For statistical analysis, we used the statistical software package SPSS 21. Statistical hypotheses were tested for statistical significance level of 0.05.

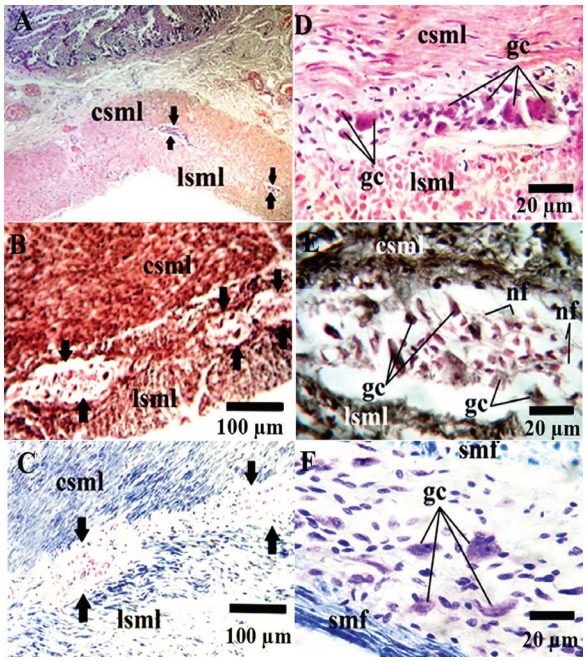

Figure 2. Nerve structures in the myenteric plexus of the jejunum; **A.** Cross-section of jejunum (woman 39 years), H&E (arrows indicate ganglia of the myenteric plexus; **B.** Cross-section of jejunum (man 51 years), $AgNO_3$ (arrows indicate ganglia of the myenteric plexus; **C.** Cross-section of jejunum (man 60 years), CV (arrows indicate ganglia of the myenteric plexus; **D.** Cross-section through the jejunum (woman 39 year), H&E; **E.** Cross-section of jejunum (man 75 years), $AgNO_3$; **F.** Cross-section of jejunum (woman 64 years), CV; gc — ganglion cells; lsml — longitudinal smooth muscle layer; csml — circular smooth muscle layer; nf — neuronal fibres; smf — smooth muscle fibres; $AgNO_3$ — silver nitrate; CV — Cresyl-Violet; H&E — haematoxylin and eosin.

RESULTS

Myenteric nervous plexus is a network of nerve fibres and nerve cells that are organised in the form of greater and smaller nerve structures that lie between the external longitudinal and internal circular muscle layers of smooth muscle of the jejunum (Fig. 2A, B). The myenteric plexus is made up of ganglion cells that connect bundles of nerve fibres, the muscle layers formed between the polygonal network. The examination of cross sections of the jejunal wall revealed an elongated structure, a tangle of myenteric ganglia with long axes. At very low magnification they appear as a series of varying thicknesses, and in those places are seen groups of nerve cells. Longitudinal sections reveal much richer morphology of the myenteric plexus. The series of longitudinal cuts allow a large number of section analyses through the myenteric plexus. In these sections this plexus takes the form of a smaller or larger cluster of neurons around which are irregularly scattered bundles of smooth muscle. Neurons of the myenteric plexus form a rather homogenous network and show very high neuronal density. The cross-sections of the nerve structures of the myenteric plexus are relatively small in size, and within each of them can be found a larger number of neurons (Fig. 2C, D). The neurons are interconnected, as well as with the muscle cells (Fig. 2E). The somata of neurons are most pronounced on slides stained by CV methodology (Fig. 2F).

Longitudinal sections made from serosa to mucosa, or through the longitudinal axis of the myenteric plexus, allow ganglion structure of various shapes and sizes to be seen. The shape and size of the nerve structures depend on the extent to which they are affected by the cut. Around the nerve structures are irregularly

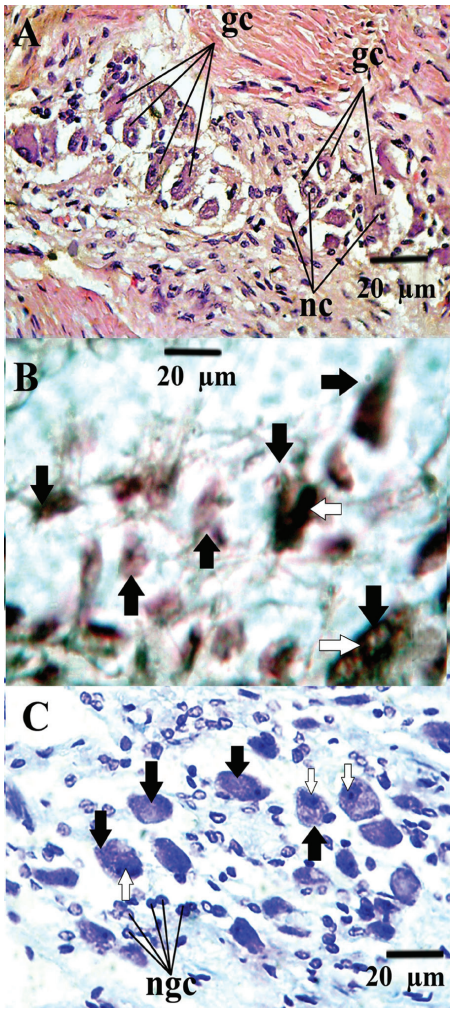

Figure 3. Longitudinal sections through the myenteric plexus of the jejunum; **A.** Longitudinal section (man 24 years), H&E; **B.** Longitudinal section (woman 68 years), $AgNO_3$; black arrows — neurons, white arrows — nuclei neurons; **C.** Longitudinal section (man 61 years), CV; black arrows — neurons; white arrows — nuclei of neurons; gc — ganglion cells; nc — cell nuclei; ngc — nuclei of glial cells; $AgNO_3$ — silver nitrate; CV — Cresyl-Violet; H&E — haematoxylin and eosin.

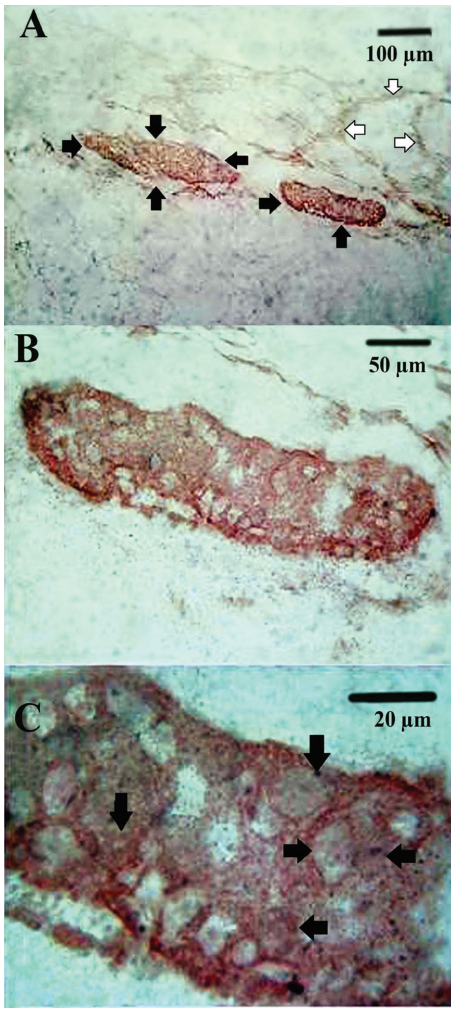

Figure 4. Nerve structures in the myenteric plexus of the jejunum in the man aged 44 years; **A.** Cross-section of jejunum, acetylcholinesterase (AChE); black arrows — ganglion of the myenteric plexus; white arrows — nerve bundles; **B.** Ganglion of the myenteric plexus, AChE; **C.** Cross section of ganglion of the myenteric plexus, AchE; black arrows — the neurons that show intense activity for AChE.

scattered bundles of smooth muscle fibres. In some ganglion only a few neurons are evident, while in others more tens of neurons are present. Neurons have different shapes and sizes: oval, round, spindle, or polygonal with vesicular nuclei, which contain very little chromatin. Nuclei are generally eccentric set (Fig. 3A, B), and contains nucleolus which is usually centrally placed. Around neurons are supporting cell whose cytoplasm is not apparent, because it was not stained by this method. However, it is clear to observe the oval nuclei, which are scattered irregularly, and often form string or wreath around neurons (Fig. 3C).

At the slides stained by histochemical method for AChE, on a smaller microscope magnification can be clearly seen the boundaries of ganglia (Fig. 4A, B). A number of neurons within myenteric ganglia showed an intense reactivity for AChE activity, with homogeneous cytoplasmic staining pattern (Fig. 4C). Opposite them were registered neurons with moderate AChE activity, and a portion of the neurons which exhibited a very low AChE activity.

Using the morphometric method of quantification, we determined the number of ganglion cells of myenteric plexus of the antimesenteric wall of

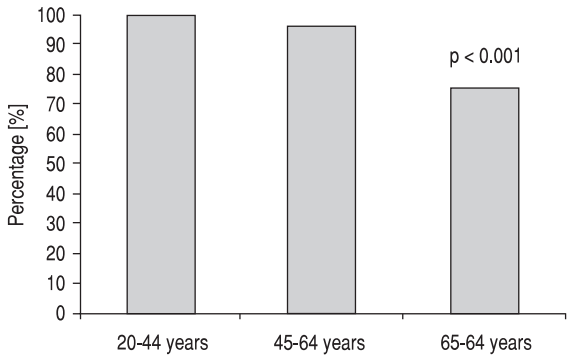

Figure 5. Decreasing in the number of neurons in percentages (20–44 years = 100%; 45–64 years = 97.39%; 65–84 years = 74.07%).

jejunum per unit area (cm^2) in specimens of all age groups. The results are expressed as mean ± SD and presented in Table 1.

There was a significant difference ($p < 0.001$) in the number of neurons between the age groups. Expressing the resulting values in percentage, shows decreases in the number of myenteric neurons by 25.93% in oldest (65–84 years) compared to the youngest (20––44 years), and decreases by 23.32% in relation to the middle-aged (45–64 years). The decrease (in percentage) of the number of neurons is illustrated in Figure 5.

During aging, a loss in the number of neurons occurs. In the elderly, ganglion structures are partly emptied of bodies of the nerve cells ("empty ganglions"). It is noticeable that the elderly have many neurons at a greater distance, which obviously is not typical for the younger population.

In addition to the number of neurons per cm^2 surface, the area of ganglion structure of the myenteric plexus in the jejunum was also measured. It should be noted that the surface area of the ganglion structure is an area of the visual field in which is present myenteric plexus (neurons, glia and nerve bundles). The surface area of ganglion structure was expressed in mm^2 as mean ± SD, and presented in Table 1. The results were subjected to statistical analysis. The data shows that the different values of the surface area of the ganglion structures based on age group is not statistically significant. Using the morphometric analysis method, the surface area occupied with the profiles of neurons within the ganglion structure, i.e. surface phase of the ganglion structure of myenteric plexus belonging to the profiles of nerve cells was measured. The obtained values expressed as mean ± SD, in mm^2 are shown in Table 1.

The data statistically showed that the total area of ganglion neurons structure of myenteric plexus of the jejunum in the oldest (65–84 years) were significantly different as compared with other age groups. The level of statistical significance was $p < 0.001$. The difference of the total area of ganglion cells within the ganglion structure between different age groups seems to be more apparent if the computation of the total area was expressed in percentage. The results are presented in Table 1. It can be seen that the percentage of surface in the ganglion structures occupying ganglion cells is the smallest in the oldest subjects (65–84 years), i.e. (14.1 ± 0.6%), and highest (19.6 ± 0.8%), in the young adults (20–44 years).

Our study indicates that aging does not decreased the surface area of ganglion structure of the myenteric plexus. The obtained values were approximately equal in all age groups. However, it was found that the total area of all the neurons that are present inside the ganglion structure is significantly decreased ($p < < 0.001$) in the oldest compared with younger groups.

Surfaces of neuronal somata and their nuclei were studied in all age groups. The obtained results are shown in Table 1 as the means ± SD, and expressed in μm^2. Our research has shown that during the aging processes the decreasing in the number of neurons is accompanied by a significant increase in their size. Average size of neurons of the myenteric plexus ranged from 284.2 ± 24.9 μm^2 in the youngest to 324.0 ± ± 23.9 μm^2 in the oldest subjects.

The obtained data was tested. The surface of neurons and their nuclei in the oldest groups was significantly ($p < 0.05$) different compared with both of the younger groups.

Nucleo-cytoplasmic ratio (ratio of nucleus surface to cytoplasm) does not show any significant differences between observed groups (Table 1).

By observing and comparing all the parameters presented in Table 1, we noticed that there were no significant differences between the age groups of 20–44 years and 45–64 years.

DISCUSSION

There are a number of studies in accordance with our results that indicate considerable decrease in the number of myenteric neurons in the colon and small intestine, as well as in the human oesophagus during aging. A decrease of at least 34% in the number of neurons in the myenteric ganglia of older persons was recognised in all parts of the small intestine, especially

in the duodenum [8], where the number of neurons is decreased by more than 38%. Similarly, in the colon [4, 15] the total number of neurons in elderly subjects decreased by more than 37%. In the oesophagus [22], the reduction in the number of neurons during aging, ranges from 22% to 62%, and is most prominent in the upper third and at the junction with the pharynx.

In some animal studies, aging is accompanied by a significant loss of myenteric neurons. In all segments of rat small intestine [28], was observed a linear decrease in the number of neurons during aging, and in the duodenum was greater than 30%. In the rat oesophagus [39], the total neuronal loss amounted to 27%. Similar results were observed in the guinea pig small intestine [13], the stomach of sheep [26], and a mouse [9]. However, some recent research has shown that there is no significant loss in the number of neurons in the myenteric plexus associated with aging [14, 24, 36]. We assume that the reasons for these differences may be due to technical difficulties in the precise counting of the number of neurons. One reason is certainly the fact that during the aging process comes to changes in the size of the digestive tract. This is particularly evident in animal experimental models in which certain influences on cellular aging may include living conditions and diet [25, 32]. It also must be taken into account that the various types of animals used in the research have different natural life span.

It is noticeable that ganglions of the oldest within their borders have often completely blank spaces in which there are no nerve cells bodies. Ganglion spaces like this, some authors refer to as a "cavity" [17]. They also conclude that, in ganglia of the myenteric plexus, "cavities" occur more frequently with age. Some of the studies [21] showed that, in the rat small intestine, there was a significant age-related increase in the mean size of neurons. This finding is not confined only to the small intestine, but also in the colon and rectum in 3- and 24-month-old rats [27]. An increase in the size of neurons with age is not typical for the rat enteric nervous plexus [1]. A neuronal loss in intracardiac ganglia and increase of connective tissue was also accompanied by an increase in the size of the remaining neurons. We found that, in the process of aging, in addition to increasing the area of the myenteric plexus there is a certain increase in the nuclear area. The increase in the nuclear surface with age is more or less in accordance with the increase in cell size. Similar findings were also reported in human enteric nervous systems [38], and also in the nervous plexus of the pig urinary bladder [30]. A significant increase in the size of the somata of neurons and their nuclei followed by a decrease in the number of neurons is found in the ENS, as well as in the cardiac ganglia, with aging [19].

This finding supports the fact that, within the range of ganglion structures in the elderly, there is some reduction in the surface phase which belongs to surface of the neuronal soma located in a given structure. With aging, there is no significant change in the size of surface area of the ganglion. That leads to the logical conclusion that the decrease in neuronal density is compensated by the increase of fibrous components; therefore the size of the myenteric ganglia is practically unchanged.

CONCLUSIONS

An organism compensated the dramatic loss of neurons that comes with aging by increasing their size and the size of their nuclei, likely in order to maintain adequate functional innervations of the jejunum. Researchers have not yet fully investigated the changes in certain types of cells during aging, such as enteric neurons, but it is important in order to examine how these changes affect the neighbouring cells. Firstly, changes within the cells with which myenteric neurons have certain contacts should be investigated, then also those which are simply in the same vicinity.

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