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Organisation of Autonomic Nervous Structures in the Large Intestine of Chinchilla (*Chinchilla laniger* Molina)

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The organization of autonomic nerve structures in the large intestine of chinchilla was investigated using histochemical and immunocytochemical methods. The myenteric plexus formed network nodes of cholinergic neurocyte agglomerations connected with bundles of nerve fibres and localized between the circular and longitudinal layers of the smooth muscles. The highest density of myenteric plexus was observed in the rectum. The different densities of myenteric plexus in subsequent parts of the large intestine is connected with the disparate functions of this part of the gut. The submucous plexus was distributed at several levels of mucosa and was a more dispersed structure than the myenteric plexus. Characteristic varicose adrenergic fibres were observed within the myenteric and submucous plexus in different layers of the large intestine wall.

Key words: Autonomic ganglia, large intestine, rodents.

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Intestinal movement, blood flow and glandular secretion are controlled by the autonomic nervous plexuses localized in the wall of the gut. AUERBACH (1864) introduced the first classification of the intramural plexuses. Further investigations revealed the presence of these nerve structures in other species of vertebrates (GUN 1968; COSTA & GABELLA 1971; GABELLA 1979). Numerous studies examined changes in the structure and number of neurons in intestinal plexuses influenced by experimental infection (MAIFRINO *et al*. 1999). Later investigations showed the morphological and functional connections that distinguished layers of the nerve plexuses in the wall intestine. Most studies investigated the differentiation of neurochemical plexuses of the small intestine in livestock (TIMMERMANS *et al*. 1992; TIMMERMANS *et al*. 2001; HANSEN 2003). Comparatively few studies presented the organization of neurochemical plexuses in the large intestine (CHRISTENSEN *et al*. 1984; MAIFRINO *et al*. 2007).

The aim of this investigation was to present the morphology and topography of the myenteric plexus (Auerbach) and the submucous plexus (Meissner) in the wall of the large intestine in chinchilla. The results provide comparative anatomic data on the enteric nervous system in small mammals.

Material and Methods

The research was conducted on six adult chinchillas *Chinchilla laniger* of both sexes. All research procedures met the requirements of the 1st Local Ethical Commission in Kraków – 3/2011. Animals were anesthetized by intraperitoneal administration of Morbital (Biowet, Puławy, 20-40 mg/kg). Four individuals were used for macromorphological studies. Parts of the large intestine (cecum, ascending, transverse and descending colon, rectum) were dissected, rinsed in saline solution and fixed for 30 min. in 10% formalin. The mucosa, submucosa and external muscle were separated and whole-mount samples were prepared. Further proceedings were in accordance with the direct-colouring thiocholine method for cholinesterase (AChE) (KARNOVSKY & ROOTS 1964) and the thiocholine method modified for macromorphological studies (KOELLE & FRIEDENWALD 1949; GIENC 1977). The SPG method (sucrose-phosphate-glioxylic acid) was used for finding the adrenergic structures (DE LA TORRE 1980). The obtained histochemical specimens were examined under a stereoscopic microscope, Nikon SMZ-800 (Japan), and a light microscope, Nikon Eclipse E-400 (Japan). For the morphometric measure-

ments, fragments 1 cm in length of whole mount specimens were collected from each segment of the large intestine and 30 areas of 1 mm^2 were analysed. Measurements and digital photographs were taken with a Nikon Digital Sight SD-LI camera (Japan). The non-parametric Kruskal-Wallis test was applied for the analysis of the morphometric data, as a Shapiro-Wilk test revealed a violation of the assumption of normality of the distributions (at $P<0.05$). If the Kruskal-Wallis test revealed significant ($P<0.05$) differences among groups, a Mann-Whitney test with Bonferroni correction was done. All calculations were made using the PAST software ver. 2.14 (HAMMER *et al*. 2001).

Two animals were used for immunocytochemical (ICC) studies. The antibodies VAChT and DßH were used. DßH (dopamine ß-hydroxylase) is an enzyme involved in noradrenaline synthesis by converting dopamine to noradrenaline, while VAChT (vesicular acetylcholine transporter) is a protein characteristic for cholinergic nerves, where it transports ACh into synaptic vesicles (CAŁKA *et al*. 2008; ANLAUF *et al*. 2003). Anaesthetized animals used for ICC were transcardially perfused with 0.4 l of 4% ice-cold buffered paraformaldehyde (pH 7.4) and tissues were collected as described above. The collected tissues were postfixed by immersion in the same fixative for 2 hours, rinsed with phosphate buffer (pH 7.4) and transferred to and stored in 30% buffered sucrose solution (pH 7.4) until further processing. The tissues were washed 3x10 min. in PB, incubated for 45 min. in 10% normal horse serum (NHS, Cappel, Warsaw, Poland) or normal goat serum (NGS, Cappel, Warsaw, Poland) in PBS containing 0.25% Triton X-100 (Sigma, USA) and then incubated overnight at room temperature (RT) with antibodies diluted in PB containing 0.25% Triton X-100. After incubation with primary antiserum, the tissues were washed 3x10 min. in PB and further incubated with secondary antisera for 1h in RT. After incubation the tissues were washed 3x10 min. in PB, cover slipped with buffered glycerol and examined under a confocal microscope (Zeiss LSM 700). For antibody details and concentrations see below:

Results

The myenteric plexus was situated between the circular and longitudinal muscle layers in all segments of the chinchilla's large intestine. It was located both within and between the taenia. The studied plexus formed a characteristic network with polygonal meshes. Macromorphological observations distinguished two parts of the investigated structures. The first, primary network, with a peripheral location, was visibly thicker, while the second, located internally, was thinner and more delicate. The network nodes were formed by autonomic ganglia containing from several to tens of neurons. The cell agglomerations were connected by nerve strands of various thicknesses (Figs 1, 2).

A low-density network (about 5 nodes per mm^2) was found in the cecum. The mesh length averaged 1580 μm and the width 581 μm. The network meshes were elongated and quadrangular in shape. The cell clusters varied in size: 134 μm long and 80 μm wide, while the average thickness of the connecting strands was about 29 μm (Figs 3, 10, Table 1).

The myenteric plexus of the ascending colon in chinchilla was significantly denser than in the cecum, with 9.93 nodes per mm^2. The length of the meshes was 709 μm and the width 293 μm. The meshes of the network varied in shape, and included oval, rectangular and pentagonal structures. The average size of the nodes was smaller than in the cecum and was 63.5 x 53.8 μm, and the connecting strands here were the thickest, at 42.8 μm (Figs 1, 10, Table 1).

The myenteric plexus of the transverse colon in the investigated species was formed by a large number of elongated meshes and a small number of nodes. The initial segments of the connecting nerve bundles were 36 μm wide and contained the nerve cells. The density of the network averaged 10.3 nodes per mm^2. The meshes were 690 μm long and 261 μm wide. The cell agglomerations were 136.5 x 94 μm and were larger than in the ascending colon (Fig. 10, Table 1).

Antibody details & concentrations

Primary antisera						
Antigen	Host	Type	Dilution	Cat. No.	Lot/Batch	Supplier
DßH	mouse	monoclonal	1:500	MAB308	0606031688	Milipore
VACHT	rabbit	polyclonal	1:5000	V5387	095K4751	Sigma

Secondary antisera					
Host	fluorochrom	Dilution	Code	Lot	Supplier
Goat-anti-mouse IgG (H+L)	Alexa Flour 488	1:500	A11008	51385A	Invitrogen
Goat-anti-rabbit IgG (H+L)	Alexa Flour 568	1:500	A11011	623962	Invitrogen

Table 1

Measurements taken on myenteric plexus in large intestine. Abbreviation: P, significance of difference (Kruskall-Wallis test)

Section		Number of nodes	Mesh length (μm)	Mesh width (μm)	Internodal fibre width (μm)	Ganglion length (μm)	Ganglion width (μm)
Cecum	Range	3-7	410.0-2540.0	250.0-1560.0	20.0-47.2	70.0-258.8	40.0-169.6
	Mean±S.D.	4.73±1.33	1580.63±581.35	581.33±335.68	29.16±6.60	134.11±50.50	80.88±31.99
Ascending colon	Range	5-16	450.0-1260.0	130.0-590.0	19.0-80.3	35.4-104.6	28.2-102.9
	Mean±S.D.	9.93±2.79	709.00±206.49	293.67±97.40	42.80±15.22	63.65±21.22	53.81±22.70
Transverse colon	Range	8-13	360.0-1190.0	80.0-420.0	17.7-54.0	74.3-213.2	60.9-169.1
	Mean±S.D.	10.30±1.60	690.67±185.95	261.67±84.77	36.00±9.95	136.59±35.68	94.08±24.85
Descending colon	Range	9-23	330.0-710.0	70.0-350.0	10.9-54.0	69.4-241.1	39.1-158.0
	Mean±S.D.	15.66±3.57	494.00±101.22	212.00±87.79	31.36±12.48	137.64±44.59	87.47±26.70
Rectum	Range	13-21	170.0-750.0	170.0-390.0	11.4-33.9	72.3-216.5	38.7-102.2
	Mean±S.D.	17.37±1.85	492.00±140.80	282.00±62.55	22.57±5.85	133.07±42.85	61.85±19.28
P		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Legend: a – autonomic ganglion, b – 1st order cholinergic nerve fibres, c – 2nd order cholinergic nerve fibres, d – adrenergic nerve fibres, e – blood vessel.

A higher density of network nodes (about 15.6) in the descending colon was observed. The mesh size in this part of the colon was the smallest, at 494 μm long and 212 μm wide. The nodes were large and irregular (137.6 x 87.4 μm). The connecting fibres (about 31.3 μm in width) were significantly thinner compared to those in the ascending and transverse segments of the colon (Figs 2, 10, Table 1).

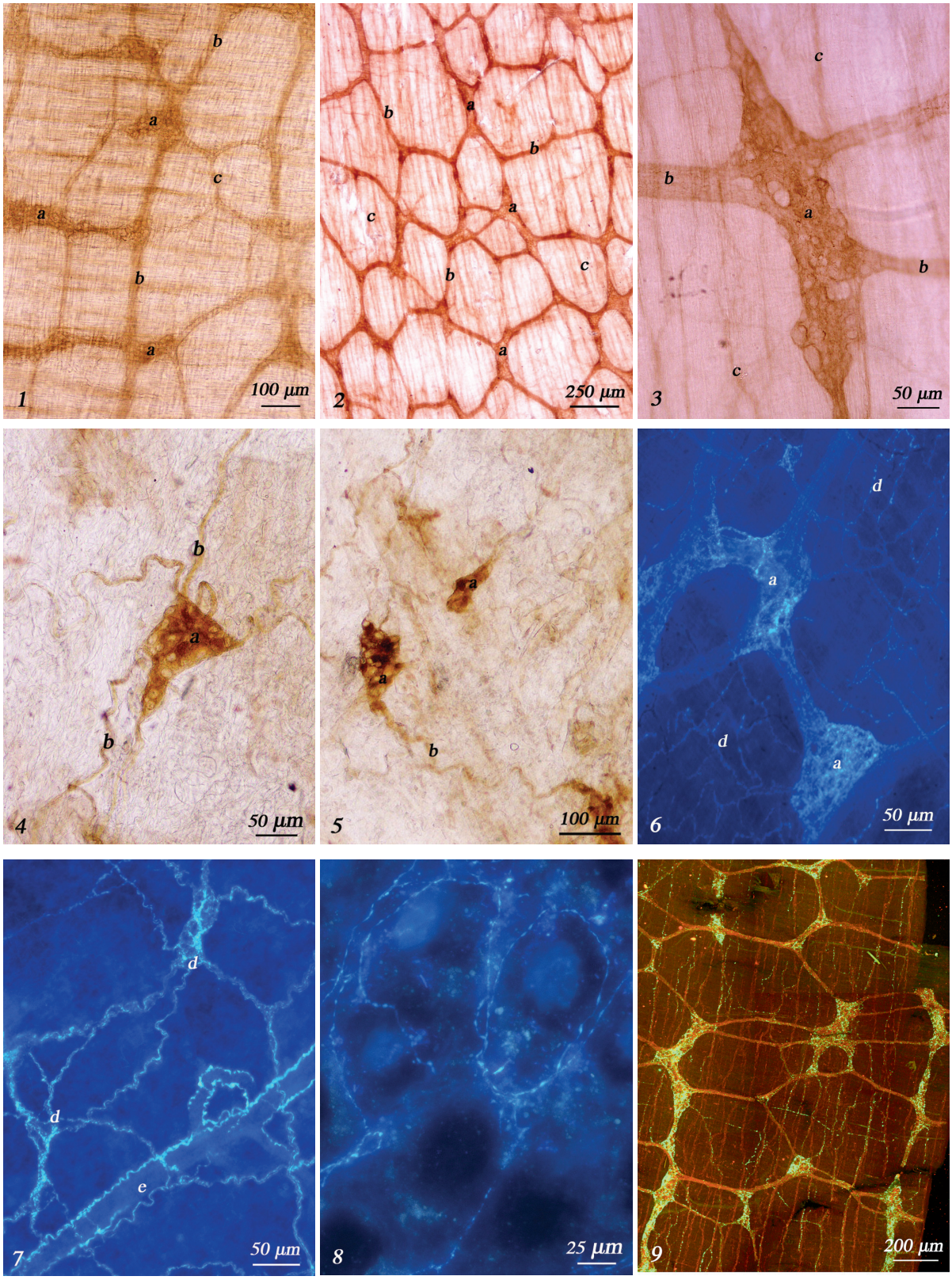
The myenteric plexus of the rectum was the densest in comparison with the same structure in the cecum and colon. There were 17 network nodes per mm². The meshes were 492 μm long and 282 μm wide. The agglomerations of neurocytes measured 133 x 61.8 μm. The connecting strands were the thinnest out of all segments of the large intestine, having a thickness of 22.5 μm (Fig. 10, Table 1).

Macromorphological investigations of the large intestine submucosus plexus revealed a characteristic delicate network with small agglomerations of neurons. This structure had an irregular pattern in comparison with the myenteric plexus and was distributed at several levels. The connecting strands were thinner, the nodes smaller and it seemed to be more dispersed. The density of this plexus was uniform along its entire length (Figs 4, 5).

Histochemical investigations revealed high activity of cholinesterase in the myenteric and submucosus plexus in all segments of the chinchilla's large intestine. The autonomic ganglia of the submucous plexus revealed 10-15 ganglionic neurocytes (Figs 4, 5). AChE positive fibres were observed in the muscularis mucose layer and in the vicinity of the intestinal crypts.

Whole-mount specimens enabled observation of the varicose adrenergic fibres located within the myenteric and submucous plexus of the large intestine. They were located peripherally in relation to the AChE positive connecting fibres and sometimes surrounded ganglionic neurocytes (Fig. 6). The adrenergic fibres of the submucous plexus were more delicate than in the myenteric plexus. The adrenergic components of both investigated plexuses penetrated each other in different layers of the large intestine wall where they formed perivascular plexuses observed in the vicinity of the intestinal crypts (Figs 7, 8). These results indicate that it is difficult to morphologically distinguish between the adrenergic structures of the myenteric and submucous plexus.

The immunocytochemical observations revealed the cholinergic and adrenergic character of the investigated plexuses. VAChT positive (red) fibres were observed in the Auerbach plexus. Moreover numerous VAChT positive and DßH (green) positive nerve fibres were found in both plexuses (Fig. 9).



Figs 1-9. Fig. 1. Organization of the myenteric plexus of the ascending colon – whole-mount specimen (Koelle-Friedenwald method). Fig. 2. Organization of the myenteric plexus of the descending colon – whole-mount specimen (Karnovsky-Roots method). Fig. 3. Autonomic ganglion in cecum – whole-mount specimen (Koelle-Friedenwald method). Fig. 4. The submucous plexus in rectum (Karnovsky-Roots method). Fig. 5. Autonomic ganglia of the submucous plexus in rectum (Karnovsky-Roots method). Fig. 6. Adrenergic fibres of the myenteric plexus in ascending colon (SPG method). Fig. 7. Adrenergic fibres of the submucous plexus in transverse colon (SPG method). Fig. 8. Adrenergic plexus of the submucous plexus in ascending colon (SPG method). Fig. 9. VACHT positive (red colour) and DßH positive (green colour) structures of the myenteric plexus in descending colon.

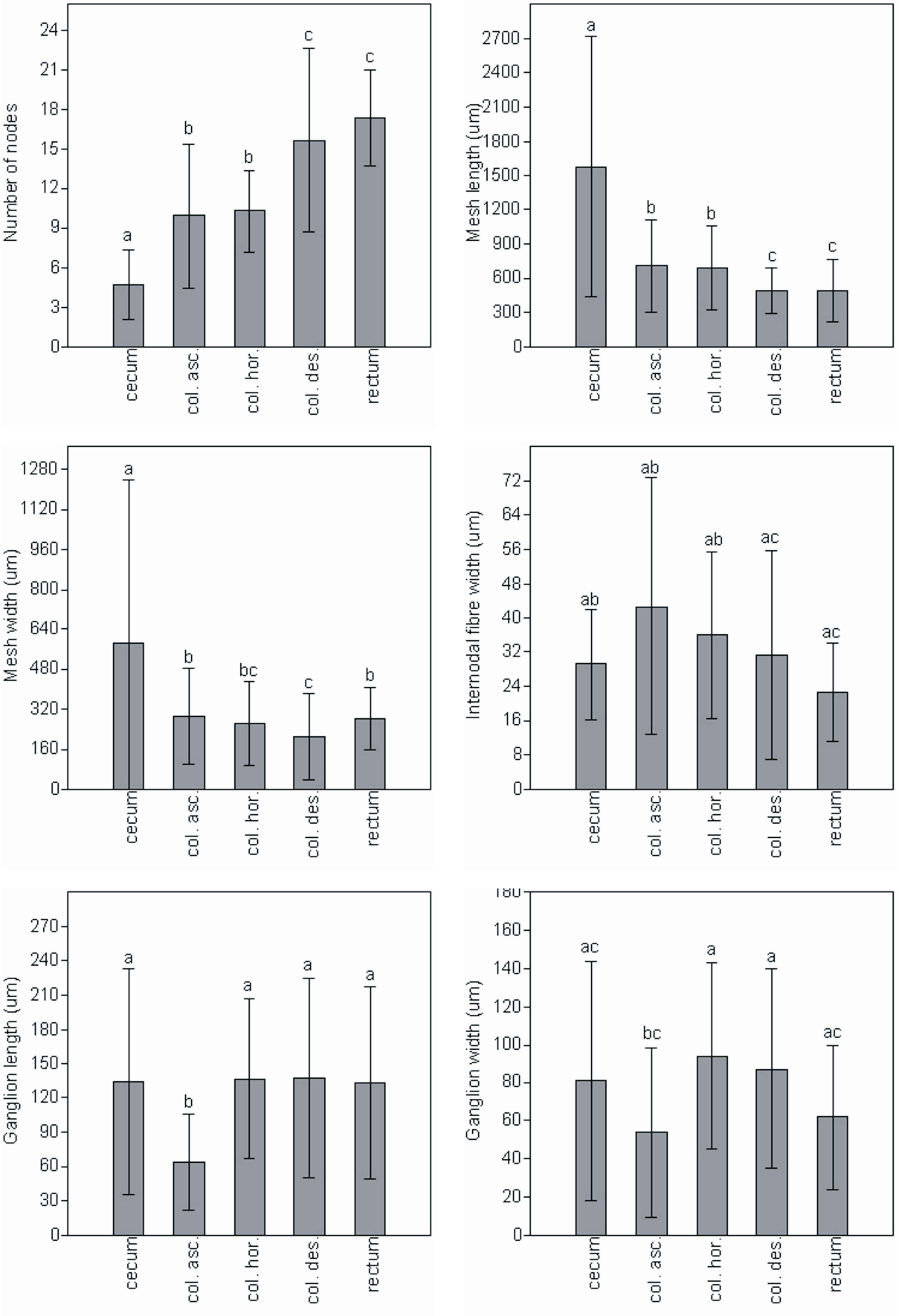


Fig. 10. Arithmetic mean and its SD of the measurements taken on the myenteric plexus of the chinchilla large intestine (N=30).

Discussion

Investigations carried out on the large intestines of vertebrates indicate significant topographical similarity to the intramural plexuses found in the small intestine (SCHABADASCH 1930; GUN 1968; COSTA & GABELLA 1971; GABELLA 1979). Morphological investigation of the gut myenteric plexus in the hen revealed the presence of cholinergic and adrenergic structures in all parts of the intestine (ALI & MCLELLAND 1978). This autonomic plexus was most developed in the rectum. A characteristic primary (1^{st}-order) thicker network and secondary (2^{nd}-order) thinner network connected with the longitudinal layer of the muscle were observed, differing in pattern and density. The longer, interstitial parts of the primary network were arranged along the long axis of the large intestine, whereas the secondary fibres formed a delicate cholinergic plexus around the blood vessels. The clearly cholinergic ganglionic neurocytes were spaced in the nodes of the network and inside the initial segments of the postganglionic connecting nerve fibres. Moreover, numerous varicose adrenergic nerve fibres were observed among the nerve cells (ALI & MCLELLAND 1978).

Studies on the morphology of the large intestine myenteric plexus in mammals revealed a higher density of cholinergic than adrenergic innervation of this structure. A clear cholinergic plexus formed with primary and secondary networks and differently sized agglomerations of neurons were observed in mice (MAIFRINO *et al*. 1997, 2007). A similarly developed primary network with small clusters of nerve cells connected by thicker nerve bundles and a secondary network with more delicate fibres were found in the myenteric plexus of the large intestine in chinchilla.

Comparative anatomical analysis performed in mouse, guinea pig, rabbit and sheep showed differences in the development of this plexus in the various parts of the large intestine (GABELLA & TRIGG 1984). Similarly, a gradual increase in the number of the network nodes from the caecum to the rectum was observed in chinchilla. The autonomic ganglia differed in shape and contained from several to tens of neurocytes (GUN 1968). In the proximal part of the mouse colon the cell agglomerations were polygonal and ring-shaped, while they were slightly elongated in the middle part, and thin and significantly extended in the distal part (MAIFRINO *et al*. 1999). The large intestine of the chinchilla showed oval, elongated and irregularly shaped aggregations of neurocytes. Single neurons were also present inside the connecting fibres. A significant increase in the number of nerve cells in the Auerbach plexus of the colon compared to the small intestine was observed in the guinea pig. Detailed analysis of the neuron agglomerations in successive parts of the large intestine in rats showed that they occupied 100-2000 μm^2 of the cecum, and in mice the small neurons occupied about 200 μm^2, medium ones 400 μm^2, and large ones 600 μm^2 (GABELLA 1979; MAIFRINO *et al*. 1999).

The adrenergic innervation of the large intestine was observed as characteristic delicate, mainly varicose nerve fibres. Previous investigations revealed the presence of these fibres in hen (ALI & MCLELLAND 1978), guinea pig, rat and rabbit (COSTA & GABELLA 1971; GABELLA 1979). Analysis of the autonomic innervation of the appendix in cat and rabbit showed that the adrenergic fibres accompanied the blood vessels forming the perivascular plexus (SIROTAKOVA *et al*. 2000). Moreover nerve fibres of this type were observed around the arteries of the cat cecum (SIROTAKOVA *et al*. 2001). Similarly, numerous varicose adrenergic nerve fibres and perivascular plexuses were found in chinchilla.

The submucous plexus of the large intestine shows a great similarity to the analogous structures of the remaining part of the intestine. It formed a well-developed irregular network arranged in a single layer in the rectum of the hen (ALI & MCLELLAND 1978). Investigations of the submucous plexus of the large intestine in *Calomys callosus* indicated a significant dispersion of the cholinergic cell aggregation in comparison with the small intestine. Analysis of number of cells revealed about 3067 neurons per cm^2 in the cecum and 3817 neurons per cm^2 in the colon. Moreover a significant increase was observed in the neuron surface of the large intestine in comparison with the small intestine (SOUZA *et al*. 1998). Analysis of the size of cell aggregations of the Meissner plexus in some species of rodents showed that the autonomic ganglia in guinea pig were larger than in rabbit and rat (COSTA & GABELLA 1971). Investigation of the submucous plexus in man revealed two plexoganglionic networks: external (monolayer) and internal (multilayer; 2-3 layers). The external network had wide polygonal meshes, while the internal network had irregularly shaped meshes and was situated around the large blood vessels. The shape of the cellular agglomerations in the two plexuses differed noticeably. Individual neurons were observed in the initial segments of the connecting fibres of the external plexus, while oval, grape-shaped cell aggregations were characteristic of the internal plexus (BREHMER *et al*. 2010). The distribution of the nerve fibres and clusters of neurons in the investigated chinchilla was uniform.

A small number of investigations on the autonomic innervation of the large intestine have examined its immunocytochemical properties. Analysis of intramural neurons (mainly interneurons) in mice revealed only 25% non-cholinergic neuro-

cytes (SANG & YOUNG 1998). Moreover investigations of the intramural plexuses in the human colon revealed the presence of galanin LI fibres in all muscle layers of the distal segment of the sigmoid colon. Similar activity was also observed in the mucosa. The final branches of GAL LI supplied the epithelial cells and intestinal crypts. No connection with the blood vessels was found (HOYLE & BURNSTOCK 1989).

Immunohistochemical investigations of the colon submucous plexus in mouse and rat revealed significant similarity between the two species. Axons of the adrenergic neurons were immunoreactive to tyrosine hydrolase (TH) supplying the ganglia and the circular muscles. Neurons immunoreactive to somatostatin (SOM) were observed between the muscle layers and on the surface of the plexus. Moreover substance P (SP) was characteristic for the body of neurons, interganglionic fibres and axons supplying the smooth muscles (HEINICKE & KIERNAN 1990).

The results revealed morphological differentiation of the myenteric plexus in the individual parts of the large intestine in chinchilla. Innervation density increased gradually from the cecum to the rectum. The different density of myenteric plexus in the subsequent parts of the large intestine is associated with the disparate functions of this part of the gut. Because of the thin wall of the investigated organ, it is difficult to differentiate the inner and outer layers of the submucous plexus. These results may be useful for comparative anatomy and the analysis of the pathological changes of the intramural plexuses in the gut.

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