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Neuropeptide-immunoreactive nerve structures in the ileum and large intestine of pigs undergoing dysentery

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Summary

The aim of the study was to investigate the expression of biologically active substances in intramural neurons supplying the ileum and large intestines (caecum, spiral colon and descending colon) in normal (control) pigs and in pigs suffering from dysentery. Higher numbers of galanin (GAL)-, vasoactive intestinal polypeptide (VIP)- and calcitonin gene-related peptide (CGRP)-immunoreactive (-IR) neuronal somata were found in the myenteric (MP), and outer (OSP) and inner submucus (ISP) intestinal nerve plexuses in dysenteric pigs as compared to control animals. Additionally, the density of substance P (SP)- and VIP-IR nerve fibres in the studied tissues was higher in dysenteric than in controls animals, whereas the number of CGRP-IR nerve fibres remained unchanged, or even was lower in the experimental pigs. The number of SP-IR nerve cell bodies in the MP of all intestinal segments studied was comparable in dysenteric and control pigs. An increased number of SP-IR perikarya were observed in OSP and ISP of the ileum, cecum and centripetal turns; whereas the number of SP-IR somata was lower in the plexuses of centrifugal turns and the descending colon. The number of nerve fibres found in all layers of the intestinal wall was lower in dysenteric pigs. Each of the intramural plexuses in all the intestinal segments studied contained less than 1% of neuropeptide Y (NPY)-IR neurones and this characteristic was similar both in dysenteric and control pigs. The number of NPY-IR nerve fibres increased slightly in the plexuses as well as in both muscular layers and mucosa.

Keywords: pig, dysentery, intestines, inflammation

Intestines are innervated by two types of neurones, extrinsic neurones located in autonomic ganglia (8, 20, 22, 25) and intrinsic neurones found within the intestinal wall. In contrast to many other organs, the innervation of the gut is accomplished mainly by intrinsic neurones located in intramural plexuses. Intrinsic innervation of the intestines was studied in wide range of animals such as mouse, rat, guinea pig, large domestic animals and also in human. This system in the pig consists of three plexuses – -inner submucous (Meissner's), outer submucous (Schabadasch's) and myenteric – (Auerbach's). Their neurochemistry and function is relatively well studied, but most of the data were obtained in healthy animals (2, 3, 31, 32). In porcine intestines immunoreactivity for GAL, VIP, CGRP, SP, neuromedin U, enkephalin, SOM and NPY was found in varicose and non-varicose nerve fibres of both submucosal ganglionic plexuses, albeit with a distinct distributional pattern (32). Intrinsic and extrinsic innervation of the intestines was studied also in animals affected by different diseases, such as schistosomatosis (5, 6), cryptosporidiosis (1), dysentery, proliferative enteropathy (PE) (15-17) and chemically induced inflammation (26). Limited studies have shown that in intestinal schistosomosis, when the enteric nervous tissue becomes inflamed, disrupted and destroyed by granulomas, the content of peptides and amines is altered (4, 6). In inflamed areas, the VIP-IR was reduced in all plexuses whereas that of SP was increased in the enteric nerve plexuses. The alterations of the levels of VIP and SP were correlated with severity of inflammation (6). Growing body of evidence indicates that PE (proliferative inflammatory disease evoked by *Lawsonia intracellularis* bacterium) exerts strong influence on the intrinsic (15-17) intestinal innervation. PE has strong influence on intrinsic nerve structures of the descending colon. Increased number of NPY-, SP- and CGRP-positive neurones was observed in PE-influenced descending colon (15, 17). In the course of PE number of VIP-IR neurones increased rapidly also in all three plexuses studied (16). Changes in the immunohistochemical properties of the

intestinal plexuses were studied in pigs suffering from dysentery (29). This paper revealed the influence of inflammatory process evoked by bacteria on SOM-IR nerve structures in the ileum and large intestines.

As clearly arise from presented above information there is still growing body of evidence dealing with influence of inflammatory processes on the neuronal structures of the intestines in the pig, but this data regard parasitic diseases, proliferative enteropathy and chemically induced inflammation. Data regarding influence of dysentery on the intestinal innervation are very limited, and this problem needs to be elucidated, especially due to similarities between organisation of intraintestinal nervous system between pig and human. Studying of influence of diarrheic diseases on innervation of intestines can be useful in preparing of new methods of their treatments, based on using of neuropeptides, their analogs or antagonists. Taking all those facts under consideration it was decided to study localization and changes in immunoreactivity to GAL, NPY, VIP, SP and CGRP in neuronal system of the ileum and large intestines in pigs undergoing dysentery.

Material and methods

The study was performed on 9 four-five months old pigs of the Large White Polish bred divided into two groups. Control group (n = 3) consisted of clinically healthy animals. Experimental animals (n = 6) were infected *per os* with *Brachyspira hyodysenteriae* bacterium cultured in anaerobic conditions on culturing media (agar with sheep blood in Petri dish). Cultures of the microbes were obtained from National Veterinary Institute in Puławy (Poland). First symptoms of infection appeared in animals approximately one week after. Diarrheic pigs were dehydrated, profoundly weakened, gaunt, and emaciated. Animals being in this stage of illness were sacrificed with pentobarbital overdose. The animals were perfused transcardially with 4% paraformaldehyde solution in 0.1 M phospate buffer, pH 7.4 (PB). Then, the samples of tissues (ileum, cecum, centripetal and centrifugal turns of the spiral colon, as well as the descending colon) were removed and post-fixed by immersion for 2 h in the same fixative. Tissues were rinsed in PB and transferred into 30% sucrose solution in PB (4°C for 72 h). They were cut with a cryostat. Serial sections 12 µm thick were put on chrome alum-coated slides and stored in a freezer (–30°C) until further processing. After washing with PB (3 × 10 min.), the sections were processed for double-labelling immunohistochemistry using antisera against PGP 9,5 (host – mouse, code – 13C4, dilution – 1 : 2000, supplier – Biogenesis UK), GAL (host – rabbit, code – 4600-5004, dilution – 1 : 1400, supplier – Biogenesis UK), VIP (host – rabbit, code – 20077, dilution – 1 : 300, supplier – Incstar), SP (host – rat, code – 8450-6505, dilution – 1 : 200, supplier – Biogenesis UK), CGRP (host – rabbit, code – RPN 1842, dilution – 1 : 1600, supplier – Affiniti UK) and NPY (host – rabbit, code – NA 1233, dilution – 1 : 800, supplier – Affiniti UK). The sections were incubated in a blocking mixture containing 1% normal goat serum (NGS), 1% bovine serum albumin (BSA) and 0.5% Triton X100 in PB. Then, they were incubated with the primary antiserum for 24 h. After rinsing in PB (3 × 10 min.), the sections were incubated with a secondary reagents (FITC-conjugated goat anti-mouse IgG, dilution – 1 : 400, supplier – Jackson Immunores Lab USA; biotinylated goat anti-rabbit IgG, dilution – 1 : 400, supplier – Dako, Denmark; biotinylated goat anti-rat IgG, dilution – 1 : 400, supplier – Amersham; streptavidin-conjugated CY3, dilution – 1 : 200, supplier – Dianova, Hamburg, GER). Rinsed, coverslipped sections were analysed using confocal microscope Bio-Rad MRA-2. Then, the percentage of neuropeptide-immunoreactive perikarya was calculated as a fraction of total number of neurons labelled with PGP 9,5.

All the animals were housed and treated in accordance with the rules approved by the Local Ethical Commission (conforming to Principles of Laboratory Animals Care, NIH publication no. 86-23, revised 1985).

Results and discussion

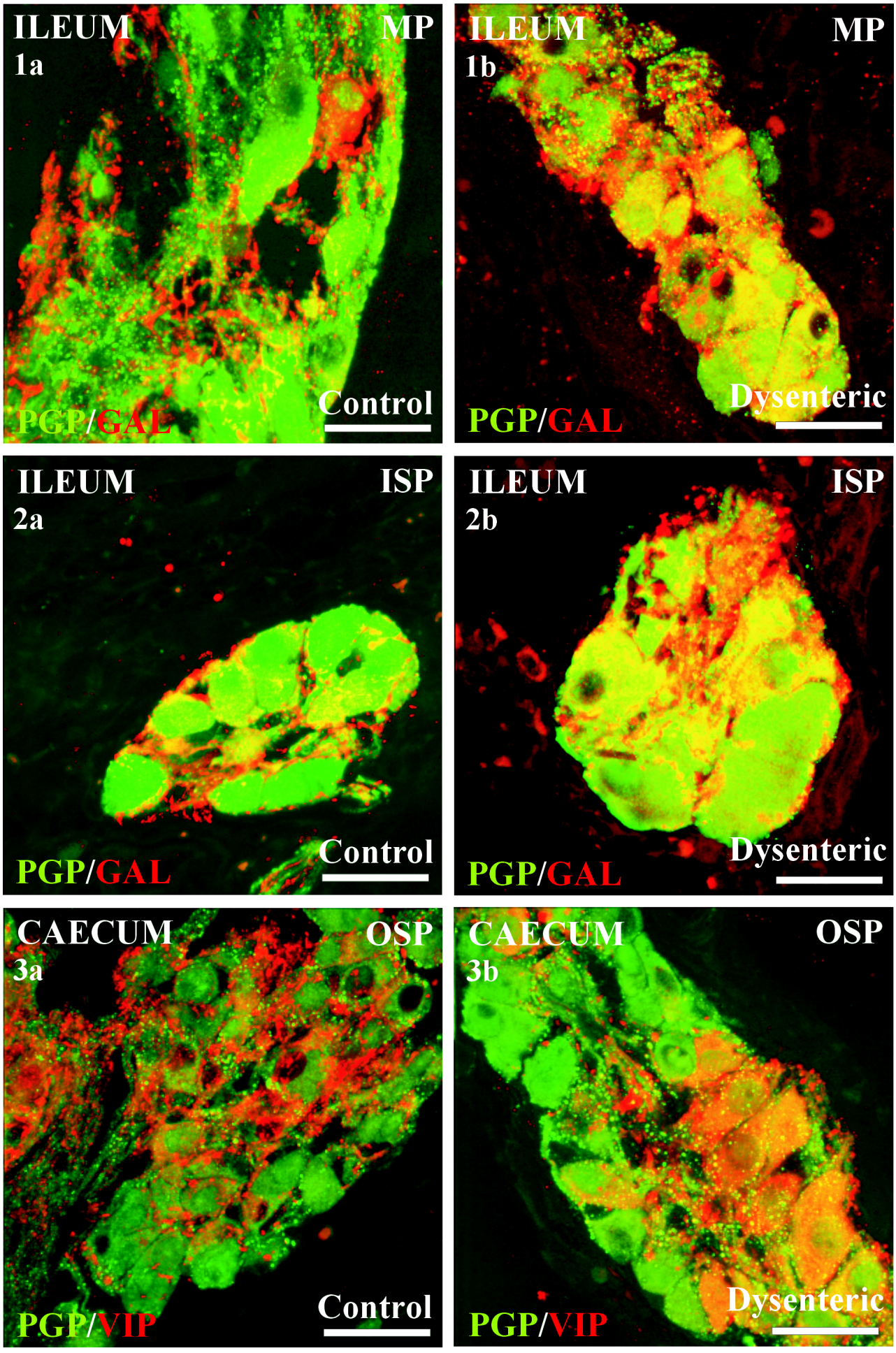
In dysenteric pigs as compared to controls the percentage of to GAL-IR neurones increased in all three plexuses in all intestinal segments studied, namely in ileum, cecum, centripetal and centrifugal turns of spiral colon and descending colon. Strongest increase was observed in ISP (fig. 2a, b), whereas in MP (fig. 1a, b) and OSP observed changes were slightly weaker. Some changes in the number of GAL-IR nerve fibres, both intraganglionic and these in the muscular coat and mucous layer of intestines were observed. Detailed data regarding the GAL-immunoreactive structures in the studied intestines are shown in tab. 1. These data are in accordance with results of our quantitative study (21) where increasing levels of GAL were found in ileum and colon of pigs suffering from dysentery. Within the gastrointestinal tract GAL is best known for its ability to alter smooth muscle contractility and regulate intestinal motility. It exerts a contractile

Fig. 1. a, b. The ileum myenteric plexus in control (fig. 1a) and dysenteric (fig. 1b) pigs. Note the higher number of GAL-positive neurones in the dysenteric animals. Bar = 50 µm.

Fig. 2. a, b. The ileum inner submucous plexus in control (fig. 2a) and dysenteric (fig. 2b) pigs. Note the higher number of GAL-positive neurones in the dysenteric animals. Bar = 50 µm.

Fig. 3. a, b. The caecum outer submucous plexus in control (fig. 3a) and dysenteric (fig. 3b) pigs. Note the higher number of VIP-positive neurones in the dysenteric animals. Bar = 50 µm.

Explanations: Confocal laser scanning microscope images showing the distribution of PGP 9.5 (green; fluorescein isothiocyanate (FITC) visualisation) and neuropeptide- (red; Cy3 visualisation). Green and red channels were digitally superimposed. Double-labelled (PGP 9.5/neuropeptide-positive) neurones are yellow.


ILEUM
MP
1a
PGP/GAL
Control
ILEUM
MP
1b
PGP/GAL
Dysenteric
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ISP
2a
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Control
ILEUM
ISP
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PGP/GAL
Dysenteric
CAECUM
OSP
3a
PGP/VIP
Control
CAECUM
OSP
3b
PGP/VIP
Dysenteric

Tab. 1. Relative density of nerve fibres (NF) and percentage of GAL-IR neurones (NCB) in intestines studied

GALANIN			Muscular coat	Myenteric plexus	Outer submucous plexus	Inner submucous plexus	Mucous layer
Ileum	Control pigs	NF	++++	+++++	++++	+++	++++
		NCB		12,16 ± 0,5485	17,51 ± 0,6524	20,54 ± 0,3349	
	Dysenteric pigs	NF	+++	++++	+++	+++	++++
		NCB		23,36 ± 2,892	25,74 ± 1,421	41,63 ± 1,686	
Cecum	Control pigs	NF	+++	++++	++	+	++++
		NCB		12,16 ± 1,337	11,58 ± 0,3233	12,46 ± 0,2771	
	Dysenteric pigs	NF	++	+++	++	+++	+++++
		NCB		34,97 ± 1,000	29,21 ± 1,424	48,65 ± 0,4461	
Centripetal turns	Control pigs	NF	+++	++++	++	+++	++++
		NCB		15,79 ± 1,273	13,45 ± 0,6004	17,76 ± 0,1934	
	Dysenteric pigs	NF	++++	+++++	++++	++++	+++++
		NCB		25,63 ± 3,304	30,54 ± 0,8418	34,44 ± 1,681	
Centrifugal turns	Control pigs	NF	++++	++++	+++	+++	++++
		NCB		13,21 ± 3,476	18,38 ± 0,2973	19,36 ± 0,5802	
	Dysenteric pigs	NF	++++	+++++	++++	++++	+++++
		NCB		15,57 ± 5,405	32,52 ± 1,920	40,64 ± 4,633	
Descending colon	Control pigs	NF	++++	+++++	++++	++++	++++
		NCB		10,25 ± 0,6004	10,04 ± 0,7015	12,45 ± 0,3147	
	Dysenteric pigs	NF	++++	+++++	+++	++++	+++++
		NCB		21,18 ± 4,038	25,22 ± 1,178	37,60 ± 1,921	

Tab. 2. Relative density of nerve fibres (NF) and percentage of VIP-IR neurones (NCB) in intestines studied

VASOACTIVE INTESTINAL POLYPEPTIDE			Muscular coat	Myenteric plexus	Outer submucous plexus	Inner submucous plexus	Mucous layer
Ileum	Control pigs	NF	+++	+++	+++	++	+++
		NCB		8,253 ± 0,1802	7,290 ± 0,2228	17,95 ± 1,048	
	Dysenteric pigs	NF	+++	++++	+++	++++	++++
		NCB		14,32 ± 1,026	17,30 ± 0,6712	24,57 ± 0,5990	
Cecum	Control pigs	NF	++++	+++++	+++	+++	+++
		NCB		5,407 ± 0,1934	7,703 ± 0,4310	12,06 ± 0,7915	
	Dysenteric pigs	NF	++	+++	++	+++++	+++++
		NCB		24,51 ± 3,624	24,87 ± 0,2639	32,66 ± 2,445	
Centripetal turns	Control pigs	NF	+++	++++	+++	++++	+++
		NCB		8,427 ± 1,405	13,60 ± 1,811	12,41 ± 0,8291	
	Dysenteric pigs	NF	+++	++	++	+++++	+++++
		NCB		22,73 ± 0,2577	23,26 ± 0,5891	22,75 ± 0,5646	
Centrifugal turns	Control pigs	NF	++++	+++++	+++++	+++	++++
		NCB		7,797 ± 0,5720	13,52 ± 0,6168	14,80 ± 0,8286	
	Dysenteric pigs	NF	++++	++++	+++	++++	+++++
		NCB		11,78 ± 1,186	10,38 ± 1,068	24,84 ± 0,5834	
Descending colon	Control pigs	NF	+++	++++	++	+++	+++
		NCB		3,687 ± 0,2282	6,197 ± 0,5867	10,46 ± 0,8219	
	Dysenteric pigs	NF	+++	++	++	+++	+++++
		NCB		6,450 ± 0,6503	10,71 ± 0,5348	27,53 ± 0,8391	

effect on rat jejunal muscle while it relaxes guinea-pig ileum by inhibiting cholinergic transmission (7). Effect of GAL was studied on isolated smooth muscle cells obtained from the porcine ileum. This neuropeptide induced a concentration-dependent contraction of the smooth muscle cells (11). GAL acts as a secretagogue in epithelial cells lining the human colon, playing an important role in the diarrhoea associated with various inflammatory processes affecting the gastrointestinal tract (10). All above mentioned data indicate that upregulated number of GAL-IR structures in the porcine intestines during dysentery can be one of the mechanisms underlying one of the dysentery symptoms – diarrhoea. This symptom is probably evoked by inducing the contraction of the intestinal muscles and increasing level of fluid excretion through the intestinal epithelium.

Similarly to GAL, percentage of VIP-IR neurones increased in all three plexuses in all intestinal compartments studied, especially in the caecum (fig. 3a, b). Alterations in nerves density was also seen and regarded both intraganglionic nerves as well as muscular and mucosal nerve fibres. All data describing VIP-immunoreactivity in intestines of

Explanations for tables 1-4: Relative density of the NF: +++++ – very numerous nerve fibres, ++++ – numerous nerve fibre, +++ – moderate in number nerve fibres, ++ – small number of the nerve fibres, + – few nerve fibres, + – – single nerve fibres

the pigs suffering from dysentery are presented in tab. 2. The strongest increase of the number of VIP-IR structures was observed in caecum and centripetal turns of the spiral colon. Those results are in accordance with quantitative data of our previous studies (21) observed in the course of dysentery. During the experimental inflammation of the porcine intestines induced by *Schistosoma japonicum* invasion the immunoreactivity to VIP was diminished in the ISP and OSP (6). Also in the *Trichinella spiralis*-infested ferret the tissue concentration of VIP was found to be lower than in control animals (24). Similar results were found in human colon in the course of ulcerative colitis (28, 30). Those discrepancies between results obtained in the dysenteric pigs and the above mentioned studies can be explained as interspecies differences, but also can be evoked by specific reaction to different pathogens causing the inflammation – parasites and *Brachyspira hyodesynteriae.*

In MP of all intestines studied percentage of CGRP-IR nerve cell bodies was elevated in dysenteric animals, as compared to controls. In OSP of the ileum, cecum, centripetal turns of the spiral colon and descending colon of dysenteric animals, an increased number of CGRP-IR perikarya was found, as compared to controls, whereas in centrifugal turns of spiral colon a slightly decreased number of CGRP-IR neuronal somata was noticed.

Tab. 3. Relative density of nerve fibres (NF) and percentage of CGRP-IR neurones (NCB) in intestines studied

CALCITONINE GENE RELEATED PEPTIDE			Muscular coat	Myenteric plexus	Outer submucous plexus	Inner submucous plexus	Mucous layer
Ileum	Control pigs	NF	++	++++	+++	+++	++
		NCB		5,687 ± 0,3535	6,580 ± 1,661	7,533 ± 1,452	
	Dysenteric pigs	NF	++	++++	+++	+++	+
		NCB		21,14 ± 3,160	20,34 ± 1,552	27,36 ± 2,677	
Cecum	Control pigs	NF	+	+++	++	+	+–
		NCB		6,270 ± 1,074	6,757 ± 1,107	4,480 ± 0,8474	
	Dysenteric pigs	NF	+	++	++	+	+–
		NCB		32,49 ± 3,063	23,31 ± 2,127	29,31 ± 1,749	
Centripetal turns	Control pigs	NF	+	++++	+++	+	+–
		NCB		3,423 ± 0,09333	5,580 ± 0,2669	1,520 ± 0,6442	
	Dysenteric pigs	NF	+	++++	+++	+	+–
		NCB		3,470 ± 0,2100	8,593 ± 0,3659	3,380 ± 0,3233	
Centrifugal turns	Control pigs	NF	+–	++++	+++	+	+–
		NCB		4,663 ± 0,2252	7,793 ± 0,8576	3,627 ± 0,4112	
	Dysenteric pigs	NF	+–	+++	+++	+–	+–
		NCB		7,540 ± 0,2793	7,407 ± 0,6753	2,987 ± 0,8488	
Descending colon	Control pigs	NF	+–	++++	+	+	+–
		NCB		4,670 ± 0,4020	4,447 ± 0,6249	1,660 ± 0,02646	
	Dysenteric pigs	NF	+–	++++	+++	++	+–
		NCB		8,517 ± 0,3014	7,873 ± 0,09821	1,653 ± 0,08293	

Tab. 4. Relative density of nerve fibres (NF) and percentage of SP-IR neurones (NCB) in intestines studied

SUBSTANCE P			Muscular coat	Myenteric plexus	Outer submucous plexus	Inner submucous plexus	Mucous layer
Ileum	Control pigs	NF	+++	++++	+++	+++	++
		NCB		2,380 ± 0,3522	4,035 ± 0,1703	19,25 ± 1,412	
	Dysenteric pigs	NF	++	+++	+	+	+–
		NCB		2,527 ± 0,3105	6,807 ± 0,8247	18,33 ± 1,105	
Cecum	Control pigs	NF	+++	++++	++	++	+
		NCB		1,850 ± 0,1328	9,090 ± 1,184	20,54 ± 2,489	
	Dysenteric pigs	NF	++	+++	+	+	+–
		NCB		1,873 ± 0,1876	17,46 ± 1,865	41,40 ± 2,356	
Centripetal turns	Control pigs	NF	+++	++++	++	++	+
		NCB		2,090 ± 0,2234	2,967 ± 0,1732	11,55 ± 0,4053	
	Dysenteric pigs	NF	++	+++	++	+	+–
		NCB		2,963 ± 0,2338	8,563 ± 0,2948	25,37 ± 0,9449	
Centrifugal turns	Control pigs	NF	++++	+++++	++	++	+
		NCB		5,723 ± 0,2260	4,153 ± 0,1099	11,53 ± 0,7512	
	Dysenteric pigs	NF	++	+++	+	+–	+–
		NCB		6,280 ± 0,4081	3,813 ± 1,299	8,230 ± 0,8780	
Descending colon	Control pigs	NF	+++	++++	++	++	+
		NCB		8,537 ± 0,6293	6,237 ± 0,2206	13,72 ± 0,8937	
	Dysenteric pigs	NF	++	++	+	+–	+–
		NCB		8,343 ± 0,7448	5,763 ± 0,4157	7,583 ± 0,5874	

In ISP of the ileum, cecum and centripetal turns of the spiral colon, the percentage of CGRP-IR neurons increased, whereas in ISP of centrifugal turns and descending colon the number of CGRP-IR perikarya decreased or remained unchanged, respectively. Exact data regarding percentage of CGRP-IR neurones and density of nerve fibres are shown in tab. 3. In this study the number of CGRP-IR neuronal structures was higher, especially in ileum and caecum in dysenteric animals. Similar changes regarding the quantitative analysis were found in ileum and cecum of the pig suffering from dysentery in our earlier studies dealing with dysentery (21). Surprisingly, the immunoreactivity and content of CGRP decreased during the inflammation in the intestines of the rat and rabbit (13, 14, 23). However, those studies dealt with early stages of inflammation, so, it can be the reason of observed discrepancies between our results and those mentioned above. It is assumed that CGRP exerts a protective and healing-promoting function in the gut. Data from gastric ulcer models support the hypothesis that a main action of CGRP is regulation of mesenteric and mucosal blood flow resulting in enhanced protection and tissue healing (27). We can suspect that the peptide plays similar role in the porcine intestines during the inflammatory process evoked by *Brachyspira hyodesynteriae*.

In the MP of all intestinal segments studied percentage of SP-IR nerve cell bodies was unchanged in dysenteric animals as compared to controls. In OSP of the ileum, cecum and spiral colon centripetal turns of dysenteric animals, an increased number of SP-IR perikarya was found as compared to controls, whereas in centrifugal turns of spiral colon and in descending colon slightly decreased number of SP-positive neuronal somata was noticed. In ISP of the ileum changes in the number of SP-IR perikarya were not observed, in ISP of the cecum and centripetal turns of the spiral colon, the percentage of SP-IR neurons increased, whereas in ISP of centrifugal turns of the spiral colon and descending colon number of SP-IR perikarya was lower. Exact data regarding the percentage of SP-IR neurones and density of nerve fibres are shown in tab. 4. SP-IR was found to be unchanged in MP of all studied parts of intestinal tract in dysenteric pigs, as compared to controls. The percentage of SP-IR neurones was higher in OSP and ISP of those parts of intestines which were strongly affected by inflammatory process, so it can by concluded that severity of the process has direct influence on the expression of the peptide during the inflammation. Results of quantitative studies (21) are not with accordance with this observation where only small, statistically insignificant increase of the SP level was observed. It is generally accepted that SP is the pro-inflammatory factor. The data from experimental study clearly indicate that the SP immunoreactivity increases in the porcine intestine during *Schistosoma japonicum* – induced enteritis (6). Also in the course of the porcine proliferative enteropathy the number of SP-positive neurons in the descending colon clearly increased (17). SP also has an excitatory effect on gut motility (9, 12) and electrolyte and fluid secretion (18), so we can suspect that the elevated number of SP-IR neuronal structures can induce stronger intestinal motility and disturb electrolyte and fluid secretion, what in result, leads to diarrhoea.

In control animals all three plexuses of all studied intestinal segments contained less than 1% of NPY-IR neurones. In dysenteric animals the percentage of those neuronal somata was the same. In diarrheic animals number of NPY-IR nerve fibres increased slightly in plexuses as well as in both muscular layer and mucosa as compared to controls. Contrary to these results, the increased number of NPY-IR neurons in all nerve plexuses of the porcine colon was found in animals undergoing PE (15). This discrepancy can be explained by the different influence of pathogens evoking these two diseases on the neuronal structures of the intestines. Quantitative studies (21) revealed the elevated level of NPY in intestines of the pig suffering from the dysentery. NPY is known to have an inhibitory actions on the gut motility, secretion and blood flow (for references see 19). Higher quantity of NPY (21) and numerous nerve fibres in the mucosa of the affected intestines can indicate that this peptide is involved in the regulation of secretory functions of intestines during the inflammatory process. It is well known that many biologically active substances in this number all neuropeptides investigated in this study, have effect on intestinal functions such as motility, secretion and fluid balance, ion transport and others. The present paper for the first time reports changes in the number of intestinal, intramural nerve structures of the pigs undergoing dysentery. It should be emphasized that the data presented here do not match to high extent the results obtained in laboratory animals suffering from enteritis. The question whether the gastrointestinal tract reacts in the pig in a manner different from that found in laboratory animals, or whether pathogenesis of *Brachyspira hyodesynteirae*-evoked enteritis differs from that of other types of experimental enteritis still remains open. Elucidation of these problem needs further studies. The changes observed in the chemical coding of the porcine intramural nerve structures indicate their important role for functioning of intestines undergoing inflammation. The exact function of neuropeptides in the enteritis is still not fully understood and needs further systematic studies. The present results seem to have not only basic scientific significance but also suggest that neuropeptides, their analogues and antagonists can be applied in therapy of gastrointestinal diseases, and that further studies dealing with this problem should be performed.

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