The aim of this study was to investigate the changes of galanin (GAL) like immunoreactivity in the porcine descending colon during proliferative enteropathy (PE). Accordingly, the distribution pattern of GAL – like immunoreactive (GAL-LI) nerve structures was studied by the immunofluorescence technique in the circular muscle layer, myenteric (MP), outer submucous (OSP) and inner submucous plexuses (ISP), as well as in the mucosal layer of the porcine descending colon under physiological conditions and during PE. In control animals GAL-LI perikarya have been shown to constitute 4.03 ± 0.1%, 6.67 ± 0.3% and 11.20 ± 0.5% in MP, OSP and ISP, respectively. PE caused changes in the GAL – like immunoreactivity, which differed in particular parts of the studied bowel segment. During PE the number of GAL-LI perikarya amounted to 2.90 ± 0.5%, 8.42 ± 1.0% and 21.72 ± 1.4% within the MP, OSP and ISP, respectively. Moreover PE caused an increase in the number of GAL-LI nerve fibers in the colonic circular muscle and mucosal layers, as well as in all intramural plexuses, especially in ISP, where nearly every ganglion contained a very dense meshwork of the GAL-positive nerve fibers under the studied pathological factor.

This study for the first time reports on changes in GAL-LI nerve structures of the porcine descending colon during Lawsonia intracellularis infection.

Key words: enteric nervous system, proliferative enteropathy, galanin

INTRODUCTION

The porcine enteric nervous system (ENS) is organized into three layers called plexuses: myenteric plexus (MP) – located between the longitudinal and circular muscle layers, outer submucous plexus (OSP) – near the circular muscle layer and inner submucous plexus (ISP) located between the muscularis mucosa and lamina propria and it is similar in many aspects to the human ENS (Brown and Timmermans, 2004). Gut intramural plexuses contain about 20 functional classes of neurons which form circuits capable of autonomous activity. The ENS consists
of millions of neurons and due to its size, complexity and similarities to the central nervous system it is called a "second" or "gut" brain (Furness, 2000).

Previous immunohistochemical investigations on the ENS have shown that neurons in the gastrointestinal (GI) tract express several neurotransmitters according to their localization and functions (Furness, 2000). One of these neuromediators is galanin (GAL). Human GAL is composed of twenty-nine or thirty amino-acid residues, was isolated for the first time in 1983 from the porcine small intestine (Tatemoto et al., 1983). Consequently, GAL has been observed in the perikarya and nerve fibers within different fragments of the digestive tract in several species including humans (Tatemoto et al., 1983; Bauer et al., 1988; Hoyle and Burnstock 1989) and it has been suggested to play a crucial functions within the ENS (Matkowskyj et al., 2004; Sarnelli et al., 2004; Arciszewski and Ekblad, 2005; Piqueras et al., 2005; Pidsudko et al., 2008; Lang et al., 2007).

It is also well known that neurons of the ENS are able to change their structural, functional or chemical phenotype as a result of adaptative responses to both physiological (Philips and Powley, 2007) and pathological stimuli, such as intestinal and extra-intestinal diseases (Ekblad et al., 1999; Vasina et al., 2006). Apparently, most data on the plasticity of the ENS during inflammatory processes have been acquired on experimental models, where different pathological factors have been applied into the lumen of the gut or injected into the colonic wall (Elsön et al., 1995). On the other hand, the knowledge about neurochemical changes in the ENS during "natural" inflammation associated with diseases affecting the alimentary system is rather limited (Romanska et al., 1993; Balemba et al., 2001; Pidsudko et al., 2008).

One of such intestinal diseases, that can induce significant changes in the chemical coding of neurons within porcine ENS is proliferative entheropathy (PE) (Gonkowski et al., 2004; Pidsudko et al., 2008). Although the disease was described in 1931 (McOrist et al., 1993), its aethiological agent – the intracellular bacterium Lawsonia intracellularis was identified formally in the 1990's (Lawson et al., 1993). Contrary to other inflammatory diseases of the alimentary tract, PE is characterized by proliferative changes, such as hyperplasia of immature epithelial cells in different parts of the intestinal tract, most frequently within the ileum and colon (Lawson and Gebhart, 2000; Smith and Lawson, 2001). Moreover during PE sub-serosal oedema with small flecks of necrotic material on the surface of the thickened mucosa was observed within the inflamed gut (Lawson and Gebhart, 2000).

Infection of L. intracellularis induced changes in chemical coding of intramural neurons of the porcine ileum were described by Pidsudko et al. (2008). Our previous study (Gonkowski et al., 2004) has confirmed the PE induced changes in the immunoreactivity of some neuromediators in the ENS of the porcine large intestine and these changes varied from that, observed by Pidsudko et al. (2008). Up to now, the influence of PE on GAL-like immunoreactivity in the colonic wall has not been reported. Considering the fact that GAL is broadly expressed and affects many aspects of the large intestine physiology and pathology (Lang et al., 2007), the purpose of our study was examination of the PE-
induced changes of the GAL-like immunoreactivity in the ENS of the porcine distal colon.

MATERIALS AND METHODS

The study was carried out on six immature female pigs of the large White Polish breed (18 kg body weight, approximately 9 weeks old). The animals were kept in standard laboratory conditions with admission to species-specific fodder and water ad libitum. All surgical operations were performed in compliance with the instructions of Local Ethical Committee in Olsztyn (Poland), with special attention paid to minimising of stress reactions.

The pigs were divided into two experimental groups: control group (C) consisting of three clinically healthy animals and experimental group consisting of three animals with a diagnosed *L. intracellularis* infection (PE). The diagnosis of PE was confirmed with polymerase chain reaction (PCR) based test performed at State Veterinary Research Institute in Pulawy, Poland. Animals were euthanized by an overdose of sodium thiopental (Thiopental, Sandoz, Kundl-Rakúsko, Austria; 20 mg/kg of body weight given intravenously) and then perfused transcardially with 4% buffered paraformaldehyde (pH 7.4) prepared *ex tempore*.

Samples of distal colon (ca. 3 cm long) were collected from all studied animals, fixed by immersion in the same fixative for several hours and, finally, stored in 18% sucrose. Cryostat sections (10 μm) were subject to routine double-labelling immunofluorescence as described previously by Pidsudko et al. (2001), using a combination of antisera raised in different species and directed towards protein gene-product 9.5 (PGP 9.5; mouse monoclonal, Biogenesis, UK, working dilution 1:2000, used here as a pan-neuronal marker) and GAL (rabbit monoclonal, Peninsula, UK, 1: 24000). Primary antisera were visualized by species specific secondary antisera conjugated to FITC or biotin (all from Jackson Immunochemicals, USA, in working dilution 1:800). The latter antibodies were then visualized by a streptavidin-CY3 complex (Jackson, 1:8000). Double-labelled perikarya were evaluated under Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each ganglionic plexus (i.e. the myenteric – MP, outer submucous – OSP, and inner submucous – ISP plexus), found in the studied sections (4 sections per animal; only neurons with a clearly visible nucleus were included), pooled and presented as mean ± SEM. To prevent double counting of the same perikarya the sections were located at least 100 μm apart from each other. Negative controls employed in the immunofluorescence procedure included pre-absorption of the neuropeptides with an appropriate antigen, omission and replacement control.

For a semi-quantitative evaluation of the density of intraganglionic GAL-LI nerve fibers, an arbitrary scale was used, where (-) means the absence of fibers and (+ + + +) depicts a very dense meshwork of studied fibers.

A semi-quantitative evaluation of the density of the GAL-LI nerve fibers within the muscular or mucosal layers was based on a count of all the profiles immunoreactive to a given antigen per observation field. Nerve profiles were
counted in 4 sections per animal (in 5 fields per section) and the data obtained were pooled and presented as a mean.

All pictures were captured by a digital camera connected to a PC, analyzed with AnalySIS software (version 3.02, Soft Imaging System, FRG) and printed on a wax printer (Phaser 8200, Xerox, USA).

RESULTS

In control animals the GAL-LI structures have been observed within the myenteric (MP), outer submucous (OSP) and inner submucous plexus (ISP) (Fig. 1 I) of the porcine descending colon. The number of GAL – positive perikarya have differed from over 4% within MP to over 11% within ISP (Tab. 1). Furthermore, GAL-immunoreactive nerve fibers were found in all layers of the porcine descending colon, including the mucosa (Fig. 2, Ib) and the circular muscle layer (Fig. 2, Ia ), as well as in the myenteric, outer and inner submucous plexuses (Fig. 1 I).

Table 1. GAL-like immunoreactivity in various parts of the porcine descending colon under physiological conditions (C group) and during proliferative entheropathy (PE group)

<table>
<thead>
<tr>
<th>Bowel part</th>
<th>C group</th>
<th>PE group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CML1</td>
<td>21.7±1.2</td>
<td>33.0±2.1</td>
</tr>
<tr>
<td>MP</td>
<td>CB2</td>
<td>4.03±0.1</td>
</tr>
<tr>
<td></td>
<td>NF3</td>
<td>++</td>
</tr>
<tr>
<td>OSP</td>
<td>CB2</td>
<td>6.67±0.3</td>
</tr>
<tr>
<td></td>
<td>NF3</td>
<td>++</td>
</tr>
<tr>
<td>ISP</td>
<td>CB2</td>
<td>11.20±0.3</td>
</tr>
<tr>
<td></td>
<td>NF3</td>
<td>++</td>
</tr>
<tr>
<td>S/ML1</td>
<td>38.3±1.2</td>
<td>48.3±2.4</td>
</tr>
</tbody>
</table>

CML – circular muscle layer; MP – myenteric plexus; OSP – outer submucous plexus; ISP – inner submucous plexus; S/ML – submucosal/mucosal layer; CB – cell bodies; NF – nerve fibers.

1 Average number of nerve profiles per area studied (mean±SEM).

2 Relative frequency of particular neuronal subclasses is presented as % (mean ± SEM) of all neurons counted within the ganglia stained for PGP 9.5.

3 The density of intraganglionic nerve fibers positive for GAL is presented in arbitrary units.

The _L. intracellularis_ infection produced changes in the distribution of GAL-like immunoreactivity within the ENS of the porcine colonic wall. The features of these changes depend on the particular region of the ENS studied. In PE animals a decrease of the number GAL-LI cell bodies within MP was described (Fig. 1, II MP, Tab. 1), while the number of such perikarya within OSP and ISP increased in comparison to the control group (Fig 1, II OSP ISP, Tab. 1). The most significant changes in the number of the GAL immunostained cell bodies were observed...
within ISP (Fig. 1 II ISP), where the number of the GAL-LI perikarya doubled following PE stimulation and was above 20% of all counted neurons. Also, the increase in the density of GAL – positive intraganglionic fibers was observed in all

![Figure 1. Distribution pattern of the GAL–like immunoreactive (GAL-LI) nervous structures in the intramural plexuses of the porcine descending colon under physiological conditions (C) and during proliferative enteropathy (PE) (II) immunostained for PGP 9.5 (PGP) and galanin (GAL); MP myenteric plexus, OSP - outer submucous plexus, ISP - inner submucous plexus. Colocalisation of both antigens in studied perikarya is indicated with arrows. M x 400.](image-url)
studied plexuses during PE (Fig. 1 II), and the most visible changes have been found in ISP, where nearly every ganglion contained a very dense meshwork of GAL-positive nerve fibers (Fig. 1, II ISP).

Moreover, during PE, the number of GAL-LI nerve fibers was higher in the circular muscle layer and in the mucosa compared with control animals (Fig. 2, Tab. 1). In the control animals GAL-LI nerves of the circular muscle layer were often delicate and thin (Fig. 2 I a), while during *L. intracellularis* infection they were thick and long and often observed within large nerve bundles (Fig. 2 II a).

**DISCUSSION**

During the present investigation GAL-LI structures have been observed within all studied subdivisions of the colonic ENS and these results are in agreement with previous investigations, where galanin has been observed in ENS
of human and other animal species (Tatemoto et al., 1983; Bauer et al., 1988; Hoyle and Burnstock 1989). Within the distal colon, as well as in the duodenum (Pidsudko et al., 2008), the most numerous population of GAL-IR cell bodies has been observed in ISP, but the number of GAL-LI neurons within colonic plexuses observed in the present study under physiological conditions is lower than previously reported in porcine ileum, where GAL – positive neurons have been shown to constitute about 20% of all neurons in all intramural plexuses (Pidsudko et al., 2008). Our results are in agreement with the results acquired in humans, where decreased galanin immunoreactivity in the colon than in the stomach or small intestine was observed (Bauer et al., 1988; Hoyle and Burnstock, 1989). This can confirm once again the well-known similarity between the human and porcine ENS (Brown and Timmermans, 2004).

The presence of GAL-LI nerve structures in all layers of the porcine colon confirms its multiple biological effects within the alimentary tract, described in previous studies. Within GI tract GAL has been involved in the regulation of blood flow, water and electrolytes transport and gut secretion (Furness et al., 1987; Fox-Threlkeld et al., 1991; Piqueras et al., 2005). Furthermore, GAL has affected gut motility both by action on the intestinal smooth muscle cells (Umer et al., 2005) and by indirect neuromodulatory mechanisms such as neurotransmitter release (Sarnelli et al., 2004).

Moreover, the effects of GAL on the alimentary system depend on the species and gastrointestinal tract fragments investigated. For instance, it is known that GAL induces the contraction of muscles in the rat, guinea-pig, rabbit and pig (Botella et al., 1992), while in canine pylorus and ileum (Fox-Threlkeld et al., 1991) and guinea-pig ileum (Sternini et al., 2004) it displays the relaxatory activity.

The present investigation revealed that the proliferative enteropathy can change GAL – like immunoreactivity within the porcine descending colon and these changes depend on the subdivision on the studied colonic ENS.

In MP, the decreased number of GAL-LI neurons has been found. These results differ from previous studies on rodents, where different pathological factors such as axotomy, colchicine treatment or diabetes caused a numerical increase of GAL-positive neurons within this ganglion (Ekblad et al., 1998, 1999) and can suggest the differences in GAL functions within rodents and porcine ENS and/or in pathological mechanisms during PE and the mentioned above processes. Interestingly, our observations in GAL-like immunoreactivity in MP are also different from the investigations of Pidsudko et al. (2008), who noticed the significant increase in the number of GAL-LI neurons in porcine ileum MP during infection of L. intracellularis. These discrepancies can be the confirmation of well known differences in GAL functions according to the investigated GI tract segment (Lang et al., 2007). On the other hand the present results are congruent with the studies of human colon, where a significant decrease of the GAL-like immunoreactivity has been observed during ulcerative colitis (Kaminska et al., 2006).

Contrary to MP, the increase of GAL – positive cell bodies and intraganglionic fibers have been observed in OSP and ISP during PE, which is in agreement with previous studies in the pig (Pidsudko et al., 2008). Generally it is
accepted that the injury of neurons may result in the increase of expression of neurotransmitters, which promote the regeneration of injured cells. So, the obtained results, together with a well known neuroprotective function of GAL in the regeneration and survival of damaged neurons within the central nervous system (Elliot-Hunt et al., 2007), may indicate to the participation of GAL of the ENS in the same mechanisms in the mucosa and submucosal layer of porcine distal colon in spite of the fact that the neuroprotective roles of GAL in porcine GI tract are not clear. Namely, it is known that the addition of GAL to the cultures of myenteric neurons of the small intestine reduces their survival, contrary to other well known neuroprotective factors - VIP, hence causes the promotion of neuronal survival (Arciszewski and Ekblad, 2005).

The increased expression of GAL immunoreactivity in nerve structures within OSP, ISP and the mucosal layer can be also connected with the role of GAL in the regulation of pain, where it can function as both an analgesic and hyperalgesic mediator and it is congruent with previous studies, where the increase of GAL expression has been observed within the dorsal root ganglia (DRG) and spinal cord in pathological pain condition (Lu and Hokfelt, 2002). Moreover, our results may suggest a role of GAL within the mucosa and submucosal layer in PE, which is accordance with previous investigations in rodents, where galanin has been shown to be involved in colonic fluid secretion in infectious diarrhea due to different enteric pathogens (Matkowskyj et al., 2004) and it reduces the formation of gastric ulcers and demises the production of inflammatory response factors like tumor necrosis factor (Talero et al., 2007).

In summary, the results obtained in the present study suggest that GAL-LI structures of the porcine colonic ENS are involved in the pathological processes during infection of L. intracellularis. However the relevance of GAL in neural circuits controlling the functions of the porcine descending colon in physiological and under pathological conditions remains still unclear and should be further investigated in detail.

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PROLIFERATIVNA ENTEROPATIJA (PE) – INDUKOVANE PROMENE U GALANINU-SLIČNOJ IMUNOREAKTIVNOSTI ENTERIČNOG NERVNOG SISTEMA DISTALNOG KOLONA SVINJA

GONKOWSKI S, BURLINSKI P i CALKA J

SADRŽAJ

Cilj ove studije je bio da se ispituju promene u galaninu-sličnoj imunoreaktivnosti nervnih vlakana descedentnog kolona svinja tokom proliferativne enteropatije. Imajući u vidu karakteristike distribucije galaninu-slične imunoreaktivnosti (GAL-LI), nervne strukture su proučavane imunofluorescentnom tehnikom u: kružnom mišićnom sloju, mienteričnom (MP), spoljašnjem submukoznom (OSP) i unutrašnjem submukoznom pleksusu (ISP) kao i u mukoznom sloju descedentnog kolona pod fiziološkim uslovima i tokom PE. Kod kontrolnih životinja, GAL-LI perikarion je sačinjavao 4.03±0.1%, 6.67±0.3% i 11.20±0.5% u MP, OSP i ISP respectivno. PE je dovodila do promena u GAL – LI u zavisnosti od proučavanog segmenta creva. U toku PE, procenat perikariona sa GAL-LI je iznosio 2.90±0.5%, 8.42±1.0% i 21.72±1.4% u MP, OSP i ISP respectivno. Šta više, PE je prouzrokovala povećanje broja nervnih vlakana sa GAL-LI u cirkularnom sloju kolona i mukoznom sloju kao i u svim intramuralnim pleksusima. Ovo se posebno odnosilo na ISP, gde je skoro svaki ganglion sadržavao gustu mrežu GAL-pozitivnih nervnih vlakana. Ovi rezultati predstavljaju prvi izveštaj o promenama u GAL-LI nervnih struktura descedentnog kolona svinja tokom infekcije sa Lawsonia intracellularis.