

## Changes in the tissue concentrations of several neuropeptides in porcine intestines and intestine-innervating ganglia in the course of porcine proliferative enteropathy

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**ABSTRACT:** Inflammatory processes are associated with changes in the interplay of different pro- and anti-inflammatory factors, including neuropeptides, in tissue. This study was performed to investigate the influence of proliferative enteropathy on the concentration of several neuropeptides known to be involved in the regulation of the inflammatory process in porcine intestine and intestine-innervating ganglia. The concentration of galanin, vasoactive intestinal polypeptide, somatostatin, neuropeptide Y, substance P and calcitonin gene-related peptide were assayed with ELISA in the coeliac-superior mesenteric ganglion, inferior mesenteric ganglion, selected dorsal root ganglia, ileum and the descending colon in healthy and sick pigs. The concentrations of the studied neuropeptides were higher in sick animals. Statistically significant differences were found for coeliac-superior mesenteric ganglion (galanin, vasoactive intestinal polypeptide, somatostatin and neuropeptide Y), inferior mesenteric ganglion (galanin, somatostatin and neuropeptide Y), dorsal root ganglia (galanin, somatostatin, neuropeptide Y and calcitonin gene-related peptide), ileum (galanin and somatostatin) and the descending colon (galanin, somatostatin and neuropeptide Y). The data clearly show the influence of the inflammatory process on the concentration of some of the studied neuropeptides present in inflamed tissues and ganglia innervating the inflamed gut. These changes must be associated with the role the studied neuropeptides play in the inflammatory process.

**Keywords:** adenomatosis; inflammation; gut; neuropeptides; pig

### List of abbreviations

**CGRP** = calcitonin gene-related peptide, **CSMG** = coeliac-superior mesenteric ganglion, **DRG** = dorsal root ganglion, **IMG** = inferior mesenteric ganglion, **NPY** = neuropeptide Y, **SOM** = somatostatin, **SP** = substance P, **VIP** = vasoactive intestinal polypeptide

Enteritis induced by infective or invasive agents is a common problem in human and veterinary medicine. These agents evoke inflammation, which disturbs normal processes in the gut thereby affecting, among others, the functions of the enteric nervous system. Enteritis can sometimes be induced by neu-

rogenic mechanisms, which may be illustrated by the numerous cases of appendicitis without typical symptoms of bacterial infection (Di Sebastiano et al. 1999). The role of the nervous system in precipitating inflammation was first discovered during studies on capsaicin-induced inflammation. This process is

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driven by C-class afferent nerve fibres equipped with a specific vanilloid receptor 1, releasing tachykinins involved in the emergence of the inflammatory state (Steinhoff et al. 2014). Tachykinins induce, among other effects, plasma extravasation and blood vessel dilatation (Bhatia 2010). Further studies have led to the elucidation of the anti-inflammatory effect of other neuropeptides, like vasoactive intestinal polypeptide (VIP) (Yu et al. 2011).

Neuropeptides, which are also synthesised and released by non-neuronal cells, affect the synthesis and action of interleukins, thereby modulating the inflammatory reaction. Interleukins are produced by activated enterocytes, immune cells in the mucosa and enteric glia (Sharkey and Mawe 2002) in the muscular layers of the gut. Tachykinins (Kiss et al. 1999) and VIP (Yu et al. 2011) affect the production of tumour necrosis factor  $\alpha$  and other interleukins. Reciprocally, cytokines influence the activity of the enteric neurons (Khan and Collins 1994).

The domestic pig, a typical omnivore, is widely regarded as a very good model animal for studies of the alimentary tract, especially as regards the innervation of the stomach and gut (Swindle et al. 2012). However, it has been seldom used in studies of gastrointestinal tract inflammation. Further, it may be advantageous to study the involvement of the peripheral nervous system in gastroenteritis in this species. Bacteria are not often used for enteritis induction in laboratory animals. One reason may be a shortage of specific pathogens able to evoke enteritis in laboratory animals, while infections with mixed microflora could be difficult to control and reproduce. However, in the pig, several naturally-occurring bacterial diseases are associated with enteritis. One such disease is proliferative enteropathy caused by the intracellular bacterium *Lawsonia intracellularis*. The characteristic symptoms of this disease include proliferative changes in the intestines, especially in the enteric epithelium. This study is devoted to an investigation of the tissue concentrations of several neuropeptides, both in the wall of the gut and in the nerve ganglia responsible for the innervation of the intestine. The studied neuropeptides are involved in the development of the inflammatory process (SP = substance P, CGRP = calcitonin gene-related peptide, and SOM = somatostatin) and associated with the plastic response of the neurons to noxious stimuli (galanin, VIP = vasoactive intestinal polypeptide, and NPY = neuropeptide Y).

We hypothesised that the proliferative inflammation induced by infection with *L. intracellularis* induces changes in the tissue concentration of neuropeptides involved in promoting and modulating the inflammatory process, and, that these changes differ from those present in other types of inflammation associated with degenerative changes, e.g., necrosis.

The aim of the study was to assess changes in the concentration of selected neuropeptides (SP, CGRP, SOM, galanin, VIP and NPY) in the wall of the porcine colon, as well as in sensory and autonomic ganglia responsible for its innervation, in pigs suffering from the proliferative enteritis evoked by *L. intracellularis* infection.

## MATERIAL AND METHODS

The study was performed on ten female pigs, between four and five months old, of the Large white Polish breed. The test group ( $n = 5$ ) consisted of animals displaying clinical symptoms of adenomatosis (mucoïd diarrhoea) in which infection with *L. intracellularis* was confirmed with PCR. The method of *L. intracellularis* detection with PCR has been described elsewhere (Pejsak et al. 2001). The control group ( $n = 5$ ) consisted of clinically healthy animals which tested negative for *L. intracellularis*. The test and control animals were sacrificed with an overdose of sodium pentobarbital. First, they were premedicated with propionylpromazine (Combelen, Bayer, Germany) at a dose of 0.4 mg/kg of body weight, (*i.m.*). After 30 minutes the animals were killed with an overdose of pentobarbital sodium (Vetbutal, Biowet, Poland, *i.v.*).

The animals were housed and treated in accordance with the rules of the local Ethics Commission (affiliated to the National Ethics Commission for Animal Experimentation, Polish Ministry of Science and Higher Education) – Permission No. 48/2009 dated June 24, 2009.

From all the animals, both control and test ones, samples of the ileum and descending colon, as well as the ganglia (IMG, CSMG and DRG) were collected, weighed and snap-frozen in liquid nitrogen for storage. The DRG ganglia collected were the lumbar ganglia  $L_1$ - $L_3$  and the sacral ganglion  $S_3$  where the majority of primary afferent neurons innervating the ileum and colon are located (Bossowska et al. 2003). Samples were then taken

Table 1. Enzyme immunoassay kits used (Peninsula Laboratories Inc.)

Substance	Code	Lot No.
Neuropeptide Y	S-1220 (EIAH-7172)	015669
VIP	S-1183 (EIAH-7161)	016535
Galanin	S-1210 (EIAH-7100)	016537
Somatostatin	S-1179 (EIAH-8001)	016538
Substance P	S-1180 (EIAH-7451)	016536
$\beta$ -CGRP	S-1200 (EIAH-6012)	016533

VIP = vasoactive intestinal polypeptide,  $\beta$ -CGRP =  $\beta$ -calcitonin gene-related peptide

out of the liquid N<sub>2</sub> and homogenised with a homogeniser (UltraTurrax, Germany) in 0.5 M acetic acid at 4 °C and placed in a boiling water bath for 10 min. After cooling on ice, the homogenates were centrifuged for 20 min at 10 000 × g, the clear supernatant was collected and the pellet was re-extracted twice. The supernatants were pooled and lyophilised. The dried extracts were dissolved in 2 ml of water and stored frozen at –70 °C. Tissue concentrations of NPY, VIP, galanin, SOM, SP and CGRP were determined with ELISA using commercial kits (Peninsula Laboratories, USA) according to the manufacturer instructions. Details of the ELISA kits are listed in Table 1. ELISA plates were read with a Dynex MRX (Dynex Technologies, USA) immunoplate reader equipped with a 450-nm filter. A ten-point standard curve was prepared and

absorbencies were converted to peptide concentrations. The results were re-calculated for 1 g of wet tissue. The data were statistically analysed with Student's *t*-test using GraphPad PRISM 3.0 software. Differences were considered to be statistically significant at *P* < 0.05.

## RESULTS

The tissue concentrations of the studied neuropeptides in the tissues under investigation are shown in Table 2.

### Changes in neuropeptide concentrations in the autonomic and sensory ganglia

In CSMG, proliferative enteropathy evoked dramatic increases in the concentration of four out of the six neuropeptides studied. Statistically significant differences were found in the cases of galanin, VIP, SOM and NPY. The concentration of galanin increased more than two-fold, the concentration of VIP increased more than three-fold, the concentration of SOM and NPY rose dramatically by seven-fold and almost five-fold, respectively. In IMG, statistically significant changes in the concentration of neuropeptides were found in the case of galanin, SOM and NPY. The concentration of galanin increased more than eight-fold,

Table 2. Results of the quantitative analysis of the content of neuropeptides in ganglia and guts of the control (control) and adenomatous (test) pigs. Results are expressed as ng/g of wet tissue ± SEM. Statistically significant differences are marked with an asterisk (*P* < 0.05).

	CSMG		IMG		DRG		ileum		colon	
	control	test	control	test	control	test	control	test	control	test
Galanin	14.9 ± 4.2	39.9 ± 12.4*	4.1 ± 1.2	34.3 ± 8.0*	4.4 ± 0.7	30.8 ± 2.2*	113.4 ± 44.6	262.0 ± 29.6*	16.0 ± 7.3	168.3 ± 21.1*
VIP	14.0 ± 3.1	50.9 ± 23.2*	51.3 ± 6.4	80.8 ± 18.1	4.5 ± 0.4	5.7 ± 0.4	17.0 ± 5.4	39.1 ± 14.9	8.9 ± 1.0	17.2 ± 11.8
SOM	7.2 ± 2.4	50.8 ± 8.6*	10.2 ± 1.7	48.4 ± 12.4*	1.7 ± 0.3	17.2 ± 6.1*	13.8 ± 2.7	55.8 ± 2.2*	3.3 ± 0.7	21.3 ± 9.4*
NPY	89.9 ± 41.2	463.3 ± 95.2*	432.4 ± 112.2	1498.0 ± 340.7*	6.5 ± 1.7	22.0 ± 7.8*	11.4 ± 3.5	32.9 ± 13.9	4.9 ± 1.7	43.1 ± 18.5*
CGRP	6.4 ± 1.0	7.0 ± 0.6	9.3 ± 1.3	14.0 ± 1.9	2.9 ± 0.3	5.0 ± 0.5*	8.2 ± 1.8	17.9 ± 6.1	3.7 ± 1.0	7.0 ± 1.4
SP	11.5 ± 3.6	12.4 ± 2.6	1.3 ± 0.2	1.9 ± 0.3	5.8 ± 1.5	6.6 ± 1.3	14.1 ± 1.9	17.8 ± 1.4	5.4 ± 0.9	6.2 ± 1.2

CGRP = calcitonin gene-related peptide, CSMG = coeliac-superior mesenteric ganglion, DRG = dorsal root ganglion, IMG = inferior mesenteric ganglion, NPY = neuropeptide Y, SOM = somatostatin, SP = substance P, VIP = vasoactive intestinal polypeptide

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that of SOM increased almost five-fold and that of NPY rose more than three-fold. Measurements of neuropeptides in DRG revealed that significant increases in galanin, SOM, NPY and CGRP concentrations occurred in the adenomatic animals. The level of galanin rose dramatically more than seven-fold, that of SOM rose ten-fold, that of NPY increased more than three-fold and the concentration of CGRP rose almost two-fold.

### Changes in the neuropeptide concentrations in the ileum and descending colon

In the ileum, concentrations of all the studied neuropeptides were elevated in the test animals. Only the changes in the concentrations of galanin and SOM were found to be statistically significant. The concentration of galanin rose more than two-fold and the concentration of SOM rose six-fold. Similar changes, albeit with different absolute values, were found in the tissues of the descending colon. Statistically significant differences were found in the cases of galanin, SOM and NPY. The concentration of galanin increased ten-fold, the concentration of SOM increased almost seven-fold and the concentration of NPY increased almost ten-fold.

## DISCUSSION

The presented results revealed dramatic changes regarding the concentrations of galanin, SOM, NPY and, often, VIP both in the wall of the studied regions of the gut and in the investigated nerve ganglia. Surprisingly, changes in the concentrations of CGRP and SP, although clearly visible, were modest (although more pronounced in the case of CGRP). The differences were not significant.

SP is regarded as one of the key factors involved in precipitating inflammation. The involvement of SP and sensory neurons in the induction of enteritis was clearly illustrated by the inhibitory effect of capsazepine, a vanilloid receptor-1 antagonist, on TNBS-induced colitis in the rat (Fujuno et al. 2004). In the present study, we did not find any clear-cut changes in the tissue concentration of SP in the ileum and descending colon. While this situation is not strange in the case of the ileum, where the number of SP-positive neurons did not change in PE (and their number even decreased in the outer myenteric

plexus) (Pidsudko et al. 2008), it is more puzzling in the descending colon, where the number of SP-positive neurons clearly increased (Gonkowski et al. 2004a). However, it is necessary to keep in mind that the methods used to measure neuropeptide concentrations in tissues do not localise the substance and assess only the overall presence of the peptide in the sample. It is possible that the apparent increase in the number of SP-positive neurons was due to the decreased release of SP from the neurons which, at the level of the tissue, appears as an unchanged SP concentration. It is also possible that, despite the expected high specificity of the ELISA kit, it may also have detected some related substances (Page 2005). No clear changes in the SP concentration in CSMG, IMG and DRG were found. While changes in SP expression in CSMG and IMG were not reported, the induction of preprotachykinin gene expression in DRG neurons in response to inflammation in laboratory animals is a well-known phenomenon (Bulling et al. 2001). An immunohistochemical study revealed that in porcine proliferative enteropathy the number of SP-positive neurons in DRGs is clearly higher than in control animals (Bossowska et al. 2004).

CGRP is usually co-localised with SP in the same nerve terminals and both peptides are released from sensory nerve fibres. CGRP, although it displays potent vasodilatory activity and may augment the action of SP, is regarded as being involved in tissue healing (Lundeberg 2013). CGRP tissue concentration was found to decrease in experimental colitis in animal models (Eysselein et al. 1992), but in porcine proliferative enteropathy the number of CGRP-positive neurons in the ileum (Pidsudko et al. 2008) was higher than in the control animals.

A dramatic rise in the concentration of SOM was detected in both the intestinal wall and the sympathetic and sensory ganglia studied. Morphological studies performed on the porcine ileum during proliferative enteropathy revealed a higher number of SOM-positive neurons in the intramural ganglia in sick animals (Pidsudko et al. 2008). SOM is suggested to be a peptide with anti-inflammatory activity which moderates the inflammatory reaction when released from sensory fibres during inflammation. In enteritis it blocks, among others, the release of pro-inflammatory cytokines from epithelial cells (Chowers et al. 2000). No reports are available on the changes in SOM expression in sympathetic neurons following inflammation in laboratory animals.

The rise in galanin concentration is in accordance with the morphological findings, as an elevated number of galanin-positive neurons was found in the ileum of pigs with adenomatosis (Pidsudko et al. 2008). Galanin plays a significant role in the development of enteritis. It is a secretagogue inducing release of chlorine ions into the lumen of the gut (Benya et al. 1998).

VIP is regarded as a potent anti-inflammatory factor (Goursaud et al. 2015). In the present study, the concentration of this neuropeptide was found to be significantly higher in all the studied tissues of adenomatous animals, except the spinal ganglia. During enteritis induced by *Schistosoma japonicum*, immunoreactivity to VIP was diminished in the inner and outer submucous plexuses and even absent in the most severely damaged regions of the intestine (Balemba et al. 1998). It is difficult to explain our results demonstrating the clear rise in the VIP concentration. The number of VIP-positive enteric neurons in the ileum (Pidsudko et al. 2008) of the animals suffering from proliferative enteropathy was clearly higher than in the control animals. In formalin-induced ileitis, the level of VIP in the porcine sensory ganglia was the same in the control and test animals (Pidsudko et al. 2003).

NPY is regarded as an important peptide for the regulation of the inflammatory process (Taylor et al. 2014). It is thought to be mainly a pro-inflammatory peptide (Lin et al. 2004). In the present study, a dramatic increase in the tissue concentration of NPY was found both in the tissues of the intestines (ileum and colon) and in the studied ganglia (CSMG, IMG, DRG). Immunohistochemical studies revealed that in the animals suffering from proliferative enteropathy the number of NPY-positive neurons increased in the nerve plexuses of the descending colon (Gonkowski et al. 2004b), while the number of such neurons in IMG slightly decreased.

In conclusion, the data presented here clearly show dramatic changes in the concentration of some neuropeptides in the colonic wall and the nerve ganglia responsible for colon innervation. Despite the changes in the innervation of the porcine ileum and descending colon, the changes observed during porcine proliferative enteropathy do not strictly follow those observed in laboratory animals suffering from enteritis. This study contributes to the knowledge of the pathophysiology of the gastrointestinal tract. It must be kept in mind that species-related differences are very often seen

and the question is whether the pig may be a better experimental model of human gastrointestinal pathological conditions than laboratory animals. This will need further studies.

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