Praca oryginalna **Original paper**

Immunoreactivity to galanin in the small intestine of wild boars

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Summary

Galanin is a 29/30-amino acid peptide with a wide array of biological activities. At a central level, galanin mediates feeding behavior, nociception, learning and co-transmission. The presence of galanin has been found in the enteric nervous system (ENS) of the stomach, small intestine and large intestine. Although in the ENS of humans, domestic animals and laboratory animals, the presence of galanin-ergic neurons and their chemical coding have been studied, the occurrence and biochemical profile of galanin-ergic neurons in ENS of wildlife animals is still obscure. The aim of the present study was using immunohistochemical methods to evaluate whether and to what extend galanin is expressed in enteric neurons and nerve fibers supplying distinct regions of the wild boar small intestine (duodenum, jejunum, ileum). Double immunohistochemical stainings were applied in order to study the co-localization of galanin with vasoactive intestinal polypeptide (VIP), neuropeptide Y (NPY) and nitric oxide synthase (NOS) in enteric neurons and nerve fibers innervating the wild boar small intestine. Depending on the region of the wild boar small intestine, the expression of galanin was found in 5-10% of myenteric neurons and in 45-65% of submucous neurons. In all three segments of the small intestine, moderately numerous/numerous galanin-immunoreactive (IR) nerve fibers were found between neurons of myenteric and submucous ganglia, smooth muscle circular layer and between gland and villi of the mucosa. In the longitudinal smooth muscle layer of the jejunum and ileum (but not duodenum) the presence of single galanin-IR nerve fibers were found. The presence of single galanin-expressing nerve fibers was localized around duodenal Brunner's glands, blood vessels of the jejunal submucous layer, as well as in lamina muscularis mucosae of all regions of wild boars small intestines. In all regions of the small intestine, quite numerous galanin-IR submucous neurons (but not myenteric) as well as the smooth muscles-, submucosa- and mucosa-supplying galanin-IR nerve fibers co-expressed VIP. In galanin-IR nervous structures of the wild boar duodenum, jejunum and ileum no co-localizations with NOS and NPY have been found.

Keywords: immunohistochemisty, galanin, neuropeptides, small intestine, wild boar

Discovered in 1983 by Tatemoto et al. (48), galanin is a 29- or 30-amino acids long and C-terminally amidated multipotent neuropeptide. Galanin is relatively phylogenetically old, and in mammals the N-terminus of this neuropeptide has a highly conserved sequence of the first 15 amino acids (39). The galanin precursor molecule of 123-amino acid length, preprogalanin, is composed of a signal molecule, mature peptide and galanin message associated peptide (26). The signal peptide is removed from the preprogalanin by an enzymatic cleavage between the 23 and 24 amino acids (40). Immunohistochemisty and in situ hybrydization revealed that galanin and galanin mRNA are widely distributed in the central (29, 38, 42) and peripheral nervous system, including the sensory dorsal root ganglia (DRG) of pigs (31) and other mammals (37, 55). At the central level galanin acts as potent orexigenic peptide and regulates food intake, increases body weight and plays a key role in energy expenditure (for a review see 12). Combined immunohistochemical and retrograde tracing studies showed that galanin-immunoreactive (IR) sensory neurons of DRG supply numerous internal organs of the gastro-intestinal tract (8, 22, 35, 41) and urogenital (2, 23, 28, 32). Expression patterns of galanin in the gut has been studied in most laboratory (10, 14) and domestic mammals (7, 33, 35, 51, 53), as well as humans (15, 20). However, over the past three decades studies on the galanin-ergic component of the enteric nervous system (ENS) of wildlife animals have attracted relatively low attention. Therefore in the present study, the expression pattern of galanin was immunohistochemically studied in cryostat sections and whole mount preparations of wild boar small intestines. In order to elucidate whether in the duodenum, jejunum and ileum of wild boars galanin is co-expressed with neuropeptide Y (NPY), vasoactive intestinal peptide (VIP) and nitric oxide synthase (NOS) double immunohistochemical stainings were applied.

Material and methods

All the procedures involving the use of animals are in accordance with the ethical principles approved by the local ethical committee (8/2012). Five (n = 5) two-year-old free ranging wild boars of both sexes were used. Samples of 2.5 cm duodenum, jejunum and ileum were dissected out immediately after the animals' death. Samples were washed in cold saline, pinned serosa side up onto a piece of balsa wood and immersed in Stefanini's fixative for 2 days. Next, the material was cryoprotected with sucrose containing Tyrode's solution (1 change per day). Serial frozen sections of 10 μm thickness were made. With the use of a dissecting microscope and fine pointed forceps whole mount preparations were made as follows. Mucosa and submucosa were dissected out. In order to expose the myenteric plexus, the circular layer was carefully peeled off fiber by fiber from the longitudinal smooth muscle layer. Finally, the preparations were mounted on adhesion glass slides (SuperFrost Plus, Menzel GmbH&CoKG, Germany) and frozen. Both cryostat sections and whole mounts were stored at –20°C for further immunohistochemical analyses.

Both cryostat sections and whole mounts were incubated (3 × 15 minutes) in 0.01 M phosphate-buffered saline (PBS; pH = 7.4) containing 10% normal goat serum, 0.25% Triton X-100 and 0.25% bovine serum albumin (Sigma-Aldrich, Germany) to prevent non-specific binding. Sections were then incubated overnight in a humid chamber at room temperature (RT) with a mixture of primary antibodies. The following combinations have been used. In order to visualize galanin in enteric neurons mouse anti-Hu C/D antibodies (1 : 800, Molecular Probes, OR, USA, code A-21271) combined with rabbit anti-galanin sera (1 : 250; AdB Serotec, GB, code 4600-5004) were applied. Rabbit anti-galanin sera were also mixed with anti-rat NPY antibodies (1 : 500, Enzo Life Sciences, PA, USA, code NZ-1115). In order to study the co-expression patterns of galanin with VIP or NOS, guinea-pig anti-galanin antibodies (1 : 300, Peninsula Laboratories Inc., CA, USA, code T-5036.0050) were combined with rabbit sera raised against VIP (1 : 1200, Abcam, Cambridge, GB, code ab22736) or NOS (1 : 5000, Euro-Diagnostica AB, Malmö, Sweden, code 9223). After several rinses with PBS, sections were incubated for 1 h (RT) with a mixture of the corresponding species-specific secondary antibodies used as follows: FITC-conjugated anti-mouse goat IgG (dilution 1 : 400; MP Biomedicals); FITC-conjugated anti-guinea-pig goat IgG (dilution 1 : 400; MP Biomedicals), Texas Red-conjugated anti-rabbit goat IgG (1 : 400; MP Biomedicals, OH, USA) and Texas Red-conjugated anti-rat goat IgG (1 : 400; MP Biomedicals, OH, USA). Negative control experiments were performed using non-immune serum instead of primary antibodies or incubation with serum preabsorped with an antigen excess. No reaction was observed in control sections. After final washing in PBS, both whole mounts and cryostat sections were cover-slipped with phosphate-buffered glycerol (pH = 8.2) and viewed with a spinning disk confocal microscope (BX-DSU Olympus, Nagano, Japan) equipped with a set of interference filters appropriate for detection of Texas Red (545-580 nm) and FITC (470-490 nm). The proportion of myenteric/submucous neurons exhibiting the presence of galanin was expressed as the percentage of total Hu C/D--immunoreactive (IR) myenteric/submucous neurons. Random samples of minimum 300 myenteric and submucous neurons were used in double immunohistochemical studies. The proportions of galanin-IR enteric neurons co-expressing (or not) VIP, NOS or NPY were presented as percentages of the total number of galanin labeled perikarya. The densities of galanin-IR nerve fibers were assessed arbitrarily according to the following semi-quantitative scale: absent, single, moderate, numerous and very numerous.

All values are represented as means ± SEM. Comparisons among multiple groups involved one-way ANOVA followed by the Bonferroni's post hoc test. All differences were considered significant at $P < 0.05$.

Results and discussion

In general, in all regions of the wild boar small intestine myenteric neurons grouped in ganglia were well developed and regularly seen. In the submucous plexus of the duodenum and ileum submucous ganglia were arranged as a single layer, whereas in the jejunum clear secondary subdivision into outer submucous plexus (OSP) and inner submucous plexus (ISP) was observed. In the small intestine of wild boar the expression of galanin was found in nerve fibres as well as neuronal cell bodies. In the duodenum and jejunum as many as 9.2 ± 0.8% and 9.4 ± 1.0% (respectively) Hu-IR/galanin-IR myenteric neurons were found (Fig. 1), whereas a statistically lower ($P < 0.05$) subpopulation was detected in the ileum (4.9 ± 0.5%). When compared to myenteric neurons, significantly larger ($P < 0.05$) subpopulations of galanin expressing Hu-IR submucous neurons were found in the duodenum (49.9 ± 3.7%; Fig. 2), jejunum (8.8 ± 1.5% in OSP and 55.3 ± 5.8% in ISP) and ileum (45.9 ± 4.3%). In both myenteric and submucous ganglia of the small intestine very numerous galanin-IR nerve fibers nearly completely encircling enteric perikarya were seen. Numerous to very numerous galanin-IR nerve terminals were observed in the circular smooth muscle layer of all regions of the small intestine (Fig. 3). Singular galanin-containing nerve fibers run in the longitudinal smooth muscle layer of the jejunum and ileum (but not duodenum). In close vicinity to duodenal Brunner's glands fine galanin-IR nerve fibers were incidentally found. In the submucous layer of the jejunum (but not duodenum and ileum), single galanin-expressing nerve fibers supplying small blood vessels were detected (Fig. 4). In lamina muscularis mucosae of the duodenum, jejunum and ileum single galanin-IR nerve

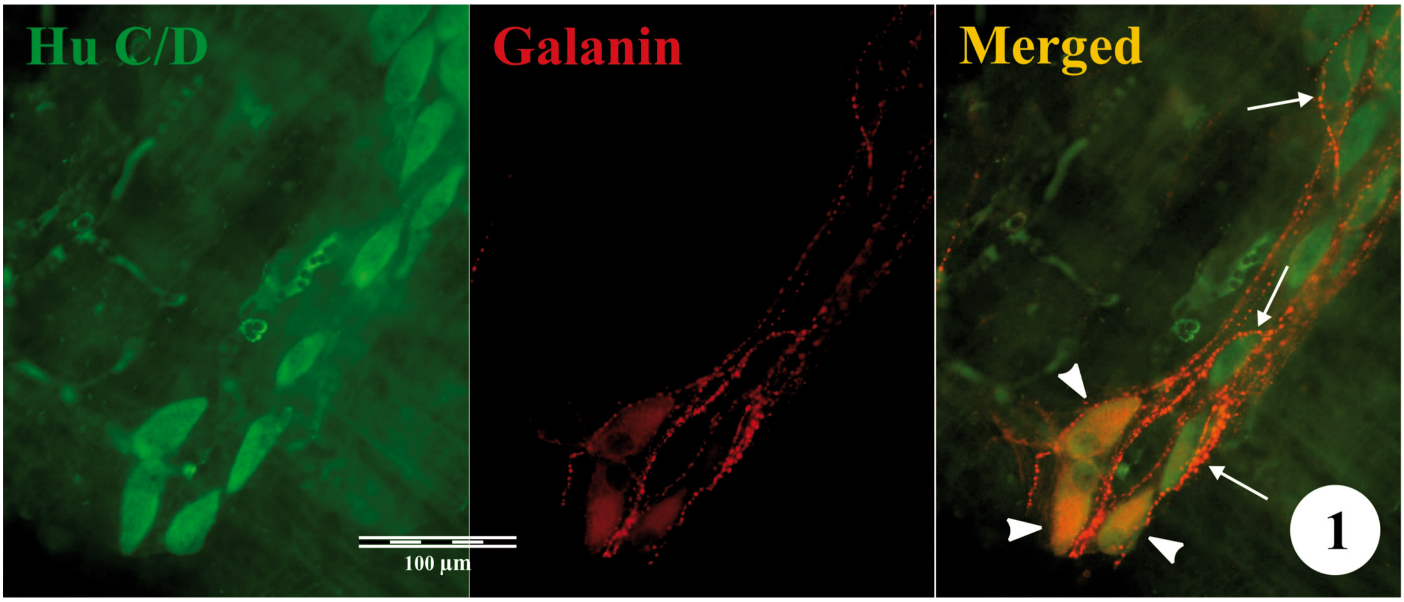

Fig. 1. Expression of galanin in the small intestine of wild boars. In whole mount preparations from the jejunum both galanin--expressing (arrowheads) and galanin-negative myenteric neurons are seen. Galanin-IR nerve fibers encircling myenteric neurons are marked with arrows

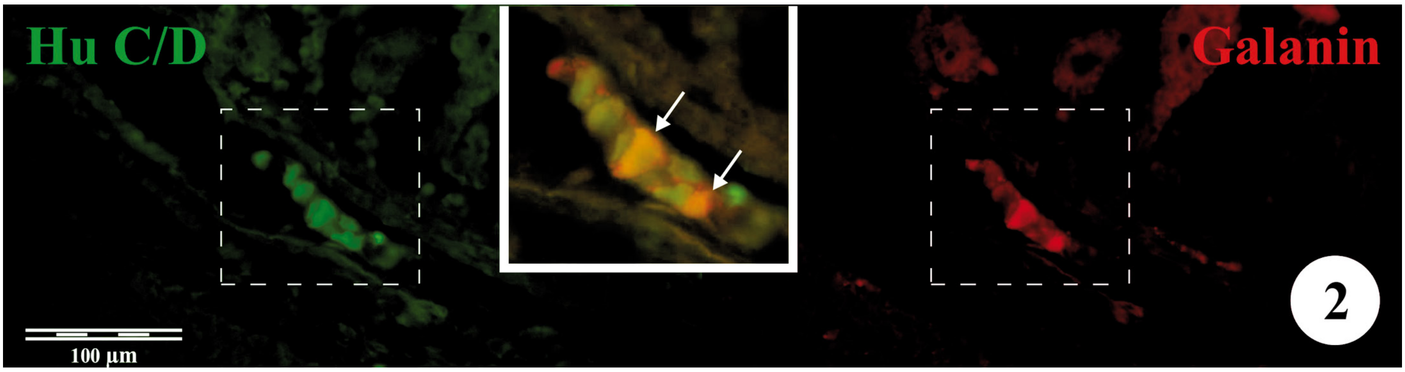

Fig. 2. In transverse section through the duodenum galanin-immunoreactive submucous neurons are marked with arrows

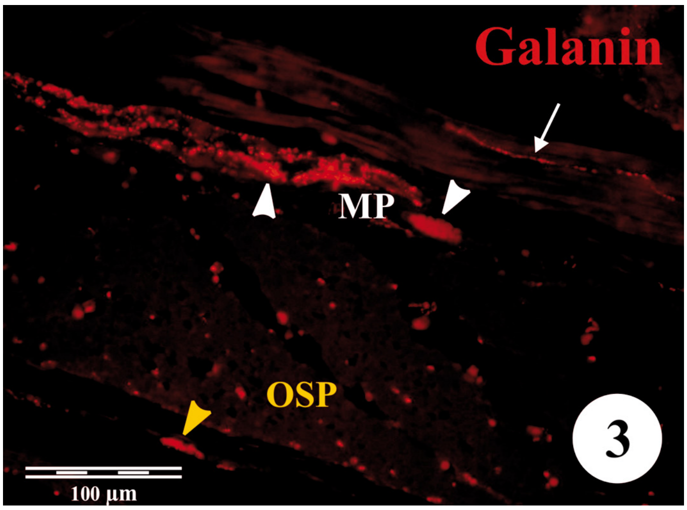

Fig. 3. In the transverse section through the jejunum arrow indicates galanin-IR nerve fibers in the longitudinal smooth muscle layer; note the presence of galanin-IR neurons in myenteric plexus (MP; white arrowheads) and outer submucous plexus (OSP; yellow arrowhead)

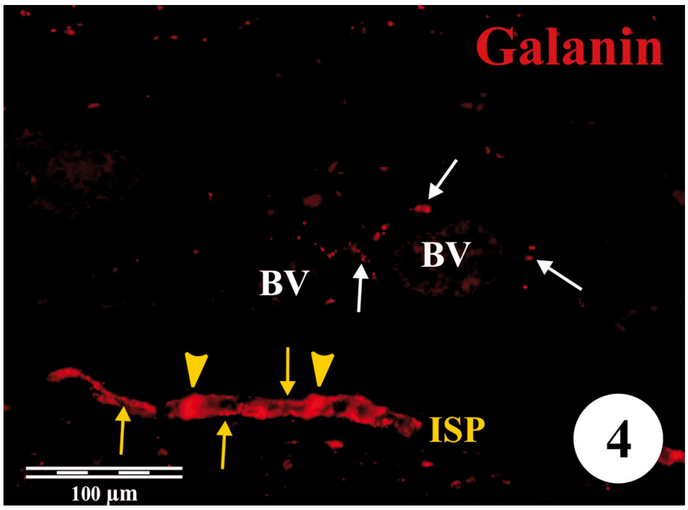

Fig. 4. Submucous blood vessels (BV) of the jejunum are supplied with galanin-IR nerve fibers (white arrows); note that in inner submucous plexus (ISP) both neurons (yellow arrowheads) as well as nerve fibers (yellow arrows) exhibit the presence of galanin

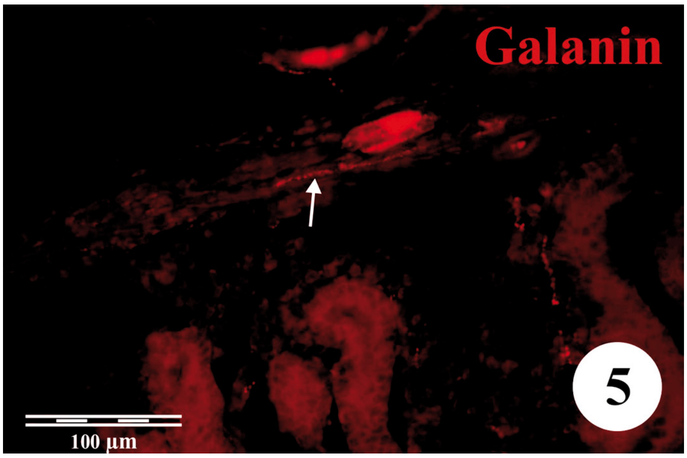

Fig. 5. In wild boars, galanin-expressing nerve fibers (arrow) are also present in lamina muscularis mucosae of the jejunum

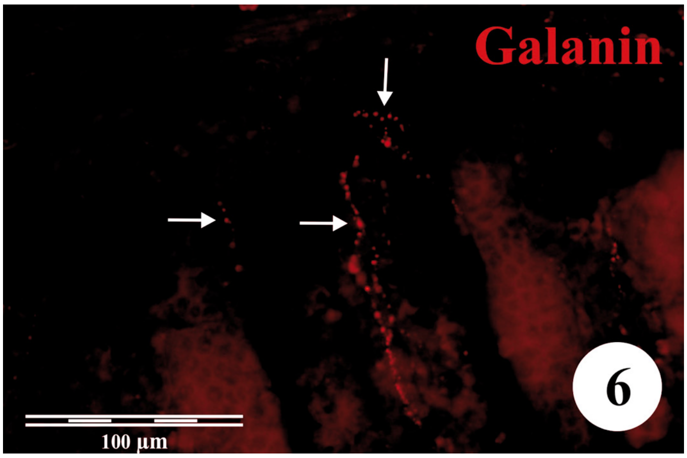

Fig. 6. In transverse section through wild boar jejunum galanin-IR nerve fibers (arrows) supplying the mucosa are seen

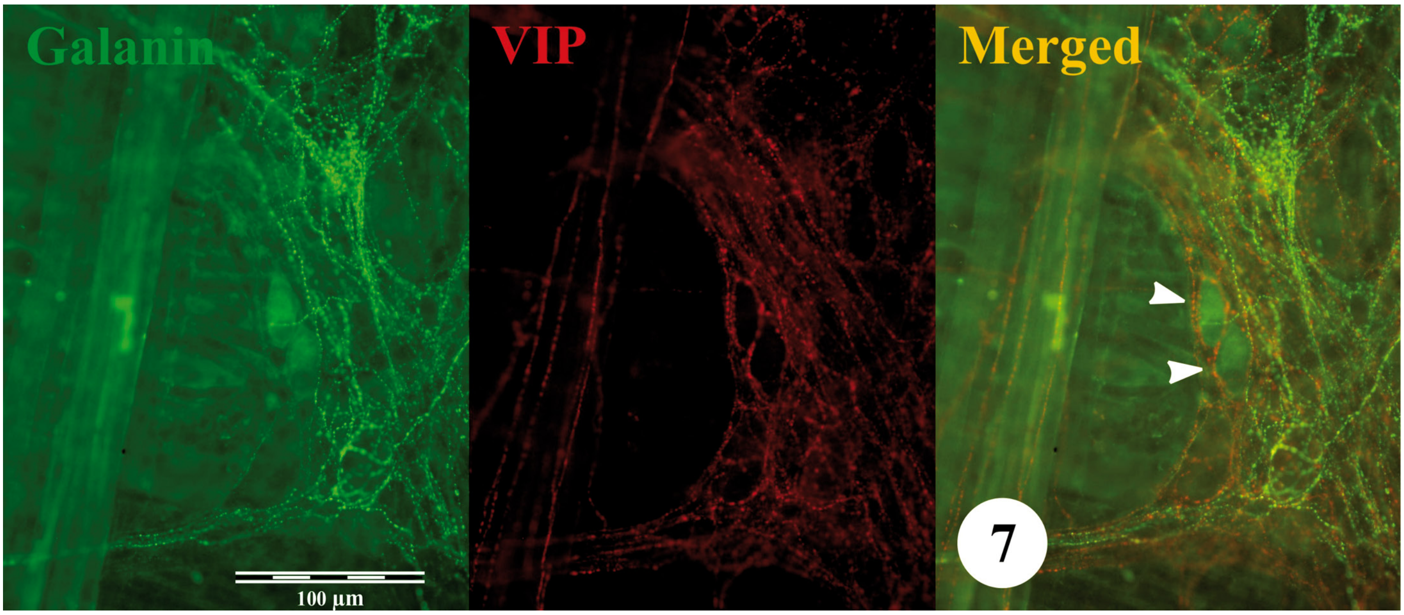

Fig. 7. Whole mount preparation from wild boar jejunum illustrates no co-localization between galanin and VIP in the myenteric plexus; arrowheads indicate VIP-IR myenteric neurons

fibers were regularly seen (Fig. 5). In all regions of the small intestine, moderate to numerous varicose galanin-expressing nerve fibers were commonly observed in the core of the intestinal villi (Fig. 6) as well as between mucosal glands.

In the myenteric plexus of the duodenum, jejunum and ileum neither galanin-IR neurons nor nerve fibers showed simultaneous expression of VIP; however, galanin-IR myenteric neurons were regularly encircled by VIP-IR nerve fibers (Fig. 7). In the duodenum as many as 73.6 ± 5.2% of galanin-IR submucous neurons were additionally VIP-positive (Fig. 8). Statistically similar ($P < 0.05$) proportions of galanin-IR/VIP-IR neurons were detected in the ISP of the jejunum (71.0 ± 4.1%) and ileum (69.5 ± 6.9%). In the jejunal OSP, virtually all galanin-IR neurons exhibited the presence of VIP. Additionally, the vast majority of galanin-IR nerve fibers innervating blood vessels of the jejunum and projecting to Brunner's gland of the duodenum were VIP-positive. In the circular smooth muscle layer of all regions of the small intestine, moderate numbers of galanin-IR nerve fibers additionally co-expressed VIP. Virtually all galanin-IR nerve fibers in the longitudinal smooth muscle layer of the jejunum and ileum showed immunoreactivity to VIP. Expression of VIP in galanin-IR nerve fibers supplying lamina muscularis mucosae (all regions) was moderately seen. In the duodenum, jejunum and ileum, numerous galanin-IR/VIP-IR nerve fibers were noted between mucosal glands as well as in the center of intestinal villi (Fig. 9). In none of the galanin-IR nervous structures of the small intestine of wild boars was the presence of NPY detected. In the myenteric plexus of the duodenum, jejunum and ileum NPY-IR nerve fibers frequently run in close vicinity to bundles of galanin-IR nerve fibers and encircled galanin-IR myenteric neurons. Additionally, in all portions of the small intestine galanin-IR nerve fibers run in close vicinity to NPY-IR submucous neurons to form "basket-like" formations. Numerous NPY-IR nerve fibers running next to galanin-IR nerve

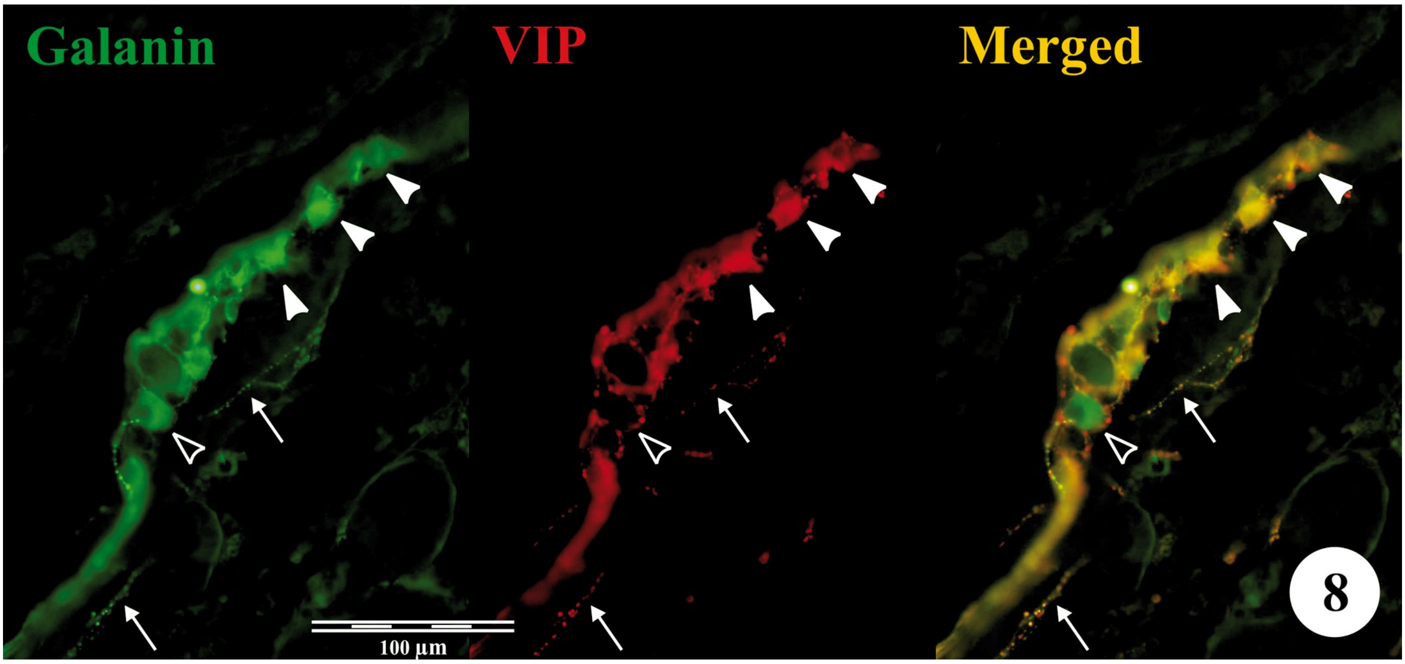

Fig. 8. In the transverse section through the duodenum co-localization of galanin and VIP is seen both in submucous neurons (arrowheads) and in nerve fibers of the lamina muscularis mucosae (arrows); hollow arrowhead indicates a galanin-positive/VIP-negative neuron

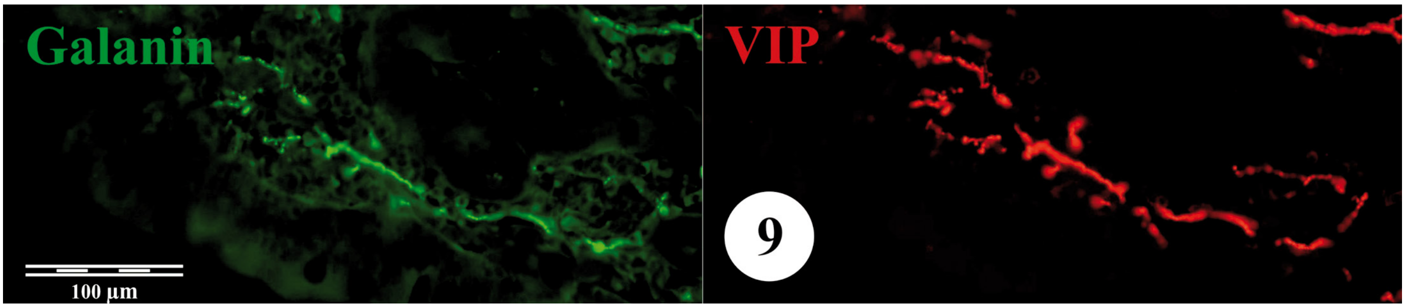

Fig. 9. In the core of a jejunal villus galanin-IR/VIP-IR nerve fibers are seen

fibers were frequently observed in intestinal villi. No co-expression of NOS and galanin was found in the duodenum, jejunum and ileum. In all regions of the small intestine, galanin-IR frequently encircled NOS--positive myenteric neurons.

Our results showed that galanin is relatively widely expressed in the duodenum, jejunum and ileum of wild boars. In general, the galanin distribution pattern only partially resembles those previously described in other mammals. To date, the presence of numerous galanin-IR nerve terminals was reported in the smooth musculature of rat, murine, swine, canine, feline as well as human small intestines (3, 10, 16, 29, 34, 53). Contrary to the latter, in the circular layer of the small intestine of guinea-pig galanin-IR nerve fibers were only incidentally found (29). In the small intestine of pigs, rats, rabbits and guinea-pigs, galanin induces smooth muscle contraction in a dose-dependent manner, whereas in dog intestinal smooth muscles relaxant properties of galanin were noted (5, 9, 17). Further studies indicated that in induced by galanin intestinal smooth muscle relaxation/contractions different receptors are involved (4). Experimental studies in which extrinsic as well as local denervations were made showed that in the rat small intestine the main source of galanin in the myenteric plexus are myenteric neurons, which suggests that galanin-IR myenteric neurons may act as interneurons as well as motor neurons (10, 46). In the small intestine of wild boars 5-10% of subpopulations of myenteric neurons express galanin. In previous studies in pigs, relatively low (up to 2%) subpopulations of galanin-IR myenteric neurons were found in the porcine jejunum (1) or rat ileum (11). Two distinct mechanisms of galanin action on myenteric neurons involving K^+ opening and membrane hyperpolarization as well as blocking of voltage gated Ca^{2+} channels have been reported (47). Relatively high percentages of galanin-IR submucous neurons were found in all regions of the wild boar small intestine which is in line with previous results in pigs (29, 36), guinea-pigs (13), rats (11), cows (51), dogs (16) and humans (3). Retrograde tracing experiments revealed that galanin-IR submucous neurons project to other submucous ganglia, submucosa, mucosa and even to intestinal smooth musculature, and thus act as interneurons or secretomotor/vasoconstrictor neurons

(6, 46, 49). Neuroprotective action of galanin has been recently reported in relation to sensory neurons subjected to axotomy or peripheral inflammation (27, 54). Galanin overexpression has been noted in enteric neurons of pigs with proliferative enteropathy (36). Under pathological conditions galanin stimulates neurite outgrowth, increases neuronal survival and shows anti-nociceptive properties (19, 45). Secretory activity of galanin in the small intestine has been also verified experimentally both *in vivo* (43) and *in vitro* (18).

Co-localization studies showed that in the small intestine of wild boars galanin-IR enteric neurons/nerve fibers exhibit the presence of VIP (but not NPY or NOS). As previously shown in the small intestine of other species galanin and VIP are widely co-expressed in submucous neurons (3, 14, 16, 18, 51). Autoradiographic studies indicated that in the small intestine of rats and humans both galanin and VIP have nearly identical binding sites (25), which may suggest the existence of functional co-operations between these neuropeptides. A hypothesis that VIP-IR/galanin-IR submucous neurons modulate secretory and motor activity of the porcine ileum has been postulated mainly due to the findings that during distension and chemical stimulation of the mucosa the increased release of both neuropeptides have been observed (18). Interestingly, in myenteric neurons from the porcine jejunum cultured in the presence of synthetic VIP the expression level of galanin dramatically increased, and in approx. 13% of neurons a co-localization of VIP and galanin was found (1). Previously, the presence of NOS in galanin-IR/VIP-IR submucous neurons has been demonstrated in the small intestine of cows, pigs and dogs (21, 51, 52). This is contrary to our studies, but it must be kept in mind that in the mentioned experiments colchicine was used. In the ENS of the mammalian small intestine, NOS plays similar role(s) to galanin and VIP and mainly acts as inhibitory neurotransmitter of motoneurons (24). Immunohistochemical studies revealed no co-localization of galanin and NPY in all regions of the wild boar small intestine. Generally, NPY-inhibits excitatory enteric neurons projecting to smooth musculature of the small intestine as well as, together with VIP, modulates secretomotor activity of the mucosa supplying neurons (50). Additionally, extrinsic NPY-IR nerve fibers regulate intestinal blood flow (44). Interestingly, in guinea-pig small intestines a subpopulation of galanin-IR/NPY-IR mucosa projecting submucous neurons has been detected (14).

In conclusion, the present study has for the first time revealed the presence of galanin in the small intestine of wild boars. It has been found that galanin-IR enteric neurons and nerve fibers frequently co-store VIP but not NPY or NOS. We believe that the obtained results provide a basis for further comparative studies on the role of neuropeptides in the ENS of wild-life animals.

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