

Distribution and neurochemical characterisation of neurons containing neuregulin 1 in the enteric nervous system within the porcine small intestine

Łukasz Puchała¹, Sławomir Gonkowski², Liliana Rytel³,
 Joanna Wojtkiewicz⁴, Waldemar Jarosław Grzegorzewski⁵✉

¹Pharma sp. z o.o., 00-844 Warszawa, Poland

²Department of Clinical Physiology, ³Department of Internal Diseases with Clinic,
 Faculty of Veterinary Medicine, University of Warmia and Mazury, 10-719 Olsztyn, Poland

⁴Department of Physiology and Pathophysiology, School of Medicine,
 University of Warmia and Mazury, 10-082 Olsztyn, Poland

⁵Institute of Biology, College of Natural Sciences, University of Rzeszów, 35-310 Rzeszów, Poland
 wgrzegorzewski@ur.edu.pl

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Abstract

Introduction: The enteric nervous system (ENS) in the wall of the gastrointestinal tract is complex and comprises many neurons, which are differentiated in terms of structure, function and neurochemistry. Neuregulin 1 (NRG 1) is one of the neuronal factors synthesised in the ENS about the distribution and functions of which relatively little is known. The present study is the first description of the distribution of NRG 1 in the ENS in various segments of the porcine small intestine. **Material and Methods:** Fragments were excised from the duodenum, jejunum and ileum of five euthanised Piétrain × Duroc sows, 18–20 kg in weight and eight weeks of age. Paraformaldehyde-fixed and dehydrated tissue was sectioned and double-labelling immunofluorescence was performed using Alexa Fluor-conjugated secondary antibodies to visualise neuregulin 1 and its colocalisation with vasoactive intestinal polypeptide (VIP), galanin (GAL), and the neuronal isoform of nitric oxide synthase (nNOS) in the myenteric and inner and outer submucosal plexuses, with PGP 9.5 serving as a pan-neuronal marker. **Results:** Neuregulin 1 was observed in all enteric plexuses in each segment of the small intestine. The percentage of NRG 1-positive neurons ranged from $8.38 \pm 0.55\%$ of all neurons in the jejunal inner submucous plexus to $21.52 \pm 0.98\%$ in the duodenal myenteric plexus. Cells which were NRG 1-positive also contained VIP, GAL and nNOS in all segments of the small intestine to a degree which varied by small intestine segment and enteric plexus type. **Conclusion:** The results indicate that NRG 1-positive neurons are present in the ENS of the porcine small intestine and differ significantly neurochemically, which may suggest a multifaceted role for NRG-1 in the controlling of the small intestine activity.

Keywords: neuregulin 1, enteric neurons, domestic pig.

Introduction

The enteric nervous system (ENS) is a complex anatomical structure made up of millions of cells, which is located in the wall of the gastrointestinal (GI) tract and is involved in the regulation of the functions of the oesophagus, stomach and intestine (7). Previous studies have shown that the anatomical structure of the ENS is specific to the mammal species. In small mammals (*e.g.* in rodents), in all segments of the GI tract the ENS is comprised of two types of intramural ganglia: myenteric ganglia located between circular and longitudinal muscle fibres, and submucous ganglia (SG)

located in the submucous layer near the mucosa (17). In large mammals (for example in domestic pigs), in the stomach the ENS has the same form as it has in rodents, but in the intestine the SG is divided into two kinds of ganglia: the inner submucous ganglion located in the same place as the SG in rodents, and the outer submucous ganglion located in the submucous layer, directly at the inner edge of the muscle layer (9, 18). Ganglia of each type are interconnected by a dense network of nerve fibres forming enteric plexuses: the myenteric plexus (MP), outer submucous plexus (OSP) and inner submucous plexus (ISP) (9, 18).

Enteric neurons are very diverse in term of morphology, functions and neurochemical coding. The latter term refers to the ability of the enteric neurons to produce and secrete active neuronal factors, which may function as neurotransmitters and/or neuromodulators (7, 14). It is known from previous studies that the enteric neurons can produce several dozen active substances, and that which of them are synthesised depends on the mammal species, the segment of the GI tract, the type of enteric ganglia in question, and the physiological and pathological factors acting on the animal, including its diet, inflammatory processes or toxic substances in its food (7). It should be pointed out that new active neuronal factors are still being discovered in the ENS, and knowledge of the distribution and functions of many of them is extremely limited.

One such substance is neuregulin 1 (NRG 1), which was described for the first time in 1992, and is a glycoprotein with a molecular weight of 44 kD and the best-known representative of the growth factors of the neuregulin family (15, 20). In the previous studies, the importance of NRG 1 was demonstrated in the biology of nerve cells, Schwann cell formation and cholinergic receptors (6, 28). Neuregulin 1 activates the epidermal growth factor receptor (and other receptors belonging to the ErbB protein family) because its sequence contains regions similar to it (13). It is known that NRG 1 is present in various parts of the central (16) and peripheral nervous systems (10), where it plays important roles in developmental processes and cell survivability (2). It is known that in the central nervous system, NRG 1 is involved in the regulation of functions of oligodendrocytes and higher nervous functions, and in the peripheral nervous system, it affects the development of Schwann cells and is involved in transmembrane transport and neuroprotective reactions (6, 11). However, information about the distribution and functions of NRG 1 within the ENS is extremely scant. This substance has been described in the enteric nervous structures of some mammal species, including humans, but its exact roles in the regulation of the GI tract functions are still unknown (2, 18, 25).

The similarities in the organisation and neurochemical characterisation of the enteric nervous structures between the human and domestic pig are well known (27). Therefore, the domestic pig is an animal model which well reflects processes occurring in the human ENS. As regards NRG 1, it has been described in the porcine large intestine, where it colocalises with some other neuronal factors including substance P, vasoactive intestinal polypeptide (VIP), galanin (GAL) and the neuronal isoform of nitric oxide synthase (nNOS) (18, 25). However, the distribution of NRG 1 in the ENS in the porcine small intestine has remained undocumented.

Therefore, the aim of the present study was to investigate the distribution of the enteric neurons containing NRG 1 in the particular types of enteric

ganglia located in the porcine duodenum, jejunum and ileum. Additionally, to expand knowledge of neuronal factor functions through elucidating their colocalisation with other neuronal factors, the presence of NRG 1 with GAL, nNOS and VIP in the same enteric neuronal cells was also investigated during this study. The present study is the first neurochemical characterisation and description of the distribution of NRG 1-positive neurons in the ENS located in the small intestine of the domestic pig, and the results obtained during the investigation will contribute to a better understanding of the role of NRG 1 in the regulation of porcine GI tract functions. Also, in light of the similarities in the organisation of the ENS between humans and domestic pigs, this study may be the first step toward understanding NRG 1 functions in the human small intestine.

Material and Methods

This study was carried out on five young sows of the Piétrain × Duroc breed, weighing 18–20 kg and aged 8 weeks. The animals were kept under typical experimental conditions at the animal house of the Faculty of Veterinary Medicine of the University of Warmia and Mazury in Olsztyn (Poland) in pens suitable for the species and age of the animals. All procedures carried out during the experiment were approved by the Local Ethical Commission for Animal Experiments in Olsztyn (Poland) (approval No. 28/13).

After a five-day period of adaptation, all animals were premedicated using azaperone administered intramuscularly at 75 µL/kg body weight (Stresnil; Elanco/Janssen Animal Health, Beerse, Belgium). After 20 min the pigs were euthanised by intravenous injection of sodium thiopental into the marginal vein of the ear (Thiopental; Sandoz, Kundl, Austria). Immediately after euthanasia, the following fragments of the intestine were excised as approximately 2-cm-long samples: the duodenum (the part located approximately 3 cm behind the pylorus of the stomach); the jejunum (the part located about 1 m from the stomach pylorus); and the ileum (the part located approximately 3 cm before the ileocaecal valve). Immediately after collection, the intestinal fragments were fixed in 4% buffered paraformaldehyde solution at pH 7.2 for 1 h at room temperature. Then, the tissues were washed out in phosphate buffer solution for 72 h with a daily exchange of buffer. After these three days, the fragments were put in 18% buffered sucrose solution and stored at 4°C for at least 3 weeks in order to dehydrate them. They were subsequently frozen at –22°C and cut perpendicularly to the intestinal lumen into 14-µm-thick sections with a cryostat (HM 525; Microm International, Walldorf, Germany). Sections were mounted on microscopic slides and stored at –20°C until further research was undertaken.

Table 1. The list of antisera and reagents used in immunohistochemical investigations

Primary antibodies				
Antigen	Code	Species	Working dilution	Supplier
GAL	T-5036	Guinea pig	1:2,000	Peninsula Labs, San Carlos, CA, USA
nNOS	N218	Mouse	1:1,000	Sigma-Aldrich, Saint Louis, MO, USA
NRG 1	AA 198–229	Rabbit	1:1,000	Antibodies-online, Aachen, Germany
PGP 9.5	7863-2004	Mouse	1:1,000	BioRad, Hercules, CA, USA
VIP	9535-0504	Mouse	1:1,000	BioRad
Secondary antibodies				
			Working dilution	Supplier
Alexa Fluor 488 donkey anti-rabbit IgG			1:1,000	ThermoFisher Scientific Waltham, MA, USA
Alexa Fluor 546 donkey anti-mouse IgG			1:1,000	ThermoFisher Scientific
Alexa Fluor 546 donkey anti-guinea pig IgG			1:1,000	ThermoFisher Scientific

GAL – galanin; nNOS – neuronal isoform of nitric oxide synthase; NRG 1 – neuregulin 1; PGP – protein gene product; VIP – vasoactive intestinal polypeptide

Intestinal sections were subjected to the double immunofluorescence method previously described by Szymanska *et al.* (25). Before immunofluorescent staining, the slides with intestinal fragments were left to dry for 30 min. Then they were put into a humidity chamber, in which the next stages of labelling were performed. First, intestinal fragments were rinsed with phosphate-buffered saline (PBS) at pH 7.4 three times for 15 min. Next, tissues were incubated for 1 h with a blocking solution consisting of 10% normal goat serum, 0.1% bovine serum albumin, 0.01% NaN₃, 0.25% Triton x-100 and 0.05% thimerosal in PBS to block possible non-specific staining. The next stage consisted in the overnight incubation of intestinal fragments with the mixture of two primary antibodies obtained in guinea pigs, mice and rabbits and directed against various neuronal factors. In the determination of the percentage of NRG 1-positive neurons, one of the antibodies was directed against the pan-neuronal marker protein gene product 9.5 (PGP 9.5), and the second was directed against NRG 1. In the neurochemical characterisation of NRG 1-positive cells, one of the antibodies was directed against NRG 1, and the second was directed against one of VIP, GAL or nNOS. The exact specification of the antibodies is given in Table 1. The next day, tissues were incubated for 1 h with the mixture of secondary antibodies conjugated with appropriate fluorochromes (Table 1) to visualise antigen–primary antibody complexes. The final preparation step was treating slides with the intestinal fragments with buffered glycerol and covering them with coverslips. All stages of this method were performed at room temperature, and between each stage and the next tissues were rinsed in PBS three times for 10 min.

The specificity of labelling was checked using routine specificity tests, including pre-absorption of primary antibodies with appropriate antigens, as well as an omission and replacement test. When specificity tests were used, intestinal fragments were not positively stained.

Labelled intestinal fragments were evaluated under a BX51 microscope (Olympus Life Science, Tokyo, Japan) using epifluorescence and appropriate filter sets. To define the percentage of NRG 1-positive neurons, at least 500 cells containing the pan-neuronal marker PGP 9.5 located in each type of enteric ganglion of each segment of the small intestine of each animal were evaluated for the presence of NRG 1. In this case, the number of PGP 9.5-positive cells included in the study was established as the 100% value. In turn, to neurochemically characterise NRG 1-positive cells, at least 300 cells containing NRG 1 also located in each type of enteric ganglion of each segment of the small intestine of each animal were evaluated for the presence of each of VIP, GAL and nNOS. In this case the number of NRG 1-positive cells included in the study was established as the 100% value. Only well-stained and visible neurons with clearly visible nuclear cells were included. To avoid possible double counting of cells, the intestinal slices which were evaluated were located at least 500 µm apart.

The obtained data were pooled and presented as means ± standard error of measurement. The statistical analysis was performed with a one-way analysis of variance, with Bonferroni's multiple comparison post hoc test using Statistica 12 software (StatSoft, Tulsa, OK, USA). Differences were considered significant at P-value < 0.05.

Results

The percentage of enteric neurons containing NRG 1.

Neuregulin 1 was found in all examined segments of the porcine small intestine in all types of enteric plexus (Table 2, Fig. 1). In the MP, the largest population of neurons containing NRG 1 was observed in the duodenum. This substance was noted in $21.52 \pm 0.98\%$ of all cells immunoreactive to the anti-PGP 9.5 antibody. In the jejunum and ileum the percentages of NRG 1-positive neurons were clearly and statistically significantly lower and amounted to 16.16 ± 0.88 and 18.48 ± 0.66 of all

PGP 9.5-positive cells, respectively. There were no statistically significant differences between the percentage of NRG 1-positive neurons in the jejunum and the percentage in the ileum. A similar percentage was noted in the OSP. In the OSP the largest percentage of NRG 1-positive cells was also noted in the duodenum, where it was 19.98 ± 0.69 of all cells immunoreactive to the anti-PGP 9.5 antibody. This value was statistically significantly higher than the values noted in the jejunum and ileum, where the percentages of NRG 1-immunoreactive neurons were 16.26 ± 0.84 and 15.2 ± 0.63 , respectively. There were no statistically significant differences between the percentage of NRG 1-positive neurons in the OSP in the jejunum and the percentage in the ileum. In the ISP the percentage of neurons containing NRG 1 was clearly lower than that in the MP and OSP. In the duodenal ISP, similarly to how it was in the MP and OSP, the percentage of neurons containing NRG 1 was the largest and was 16.92 ± 1.19 of all PGP 9.5-positive cells. In the jejunum and ileum, the percentages of NRG 1-positive neurons were statistically significantly lower and did not exceed 8.38 ± 0.55 and 10.26 ± 0.70 , respectively. There were no statistically significant differences between the percentage of NRG 1-positive neurons in the ISP in the jejunum and the percentage in the ileum. Data on the percentage of NRG 1-immunoreactive neurons in particular segments of the porcine small intestine are summarised in Table 2.

Colocalisation of NRG 1 and VIP. The presence of VIP was noted in NRG 1-immunoreactive neurons in all types of enteric plexus in all segments of the small intestine studied (Table 3, Fig. 2). Generally, the degree of colocalisation of NRG 1 and VIP was the highest in the duodenum.

In the MP, VIP was noted in $32.39 \pm 1.82\%$, $25.50 \pm 1.69\%$ and $29.17 \pm 1.45\%$ of all NRG 1-positive cells located in the duodenum, jejunum and ileum, respectively. The value noted in the jejunum was statistically significantly lower than values observed in other parts of the intestine. The differences between the percentages in the duodenum and ileum were not statistically significant.

In the OSP, the percentage of NRG1-positive neurons which also contained VIP amounted to 41.91 ± 1.90 , $24.96 \pm 2.24\%$ and 36.62 ± 1.43 in the duodenum, jejunum and ileum, respectively. Statistically significant differences were observed between each segment of the intestine and each other one.

In the ISP, the degree of colocalisation of NRG1 with VIP was lower than it was in the MP and OSP. In this type of enteric plexus, VIP was noted in $25.16 \pm 0.94\%$, $20.46 \pm 1.23\%$ and $23.55 \pm 0.87\%$ of all NRG 1-positive cells located in the duodenum, jejunum and ileum, respectively. The colocalisation noted in the jejunum was statistically significantly lower than that noted in other segments of the intestine, but there were no statistically significant differences between the values for colocalisation noted in the duodenum and ileum.

Data on the colocalisation of NRG 1 and VIP in the ENS of the porcine small intestine are summarised in Table 3.

Colocalisation of NRG 1 and GAL. Colocalisation of NRG 1 and GAL was noted in all types of enteric plexus in each segment of the small intestine (Table 4, Fig. 3), just as it was of NRG 1 and VIP. Generally, the highest degree of colocalisation of NRG 1 with GAL was noted in the jejunum.

In the MP, GAL was noted in $15.67 \pm 0.94\%$, $28.73 \pm 0.81\%$ and $26.38 \pm 1.37\%$ of all NRG 1-positive cells located in the duodenum, jejunum and ileum, respectively. The values noted in the duodenum were statistically significantly lower than those noted in other segments of the intestine. There were no statistically significant differences between colocalisation of NRG 1 and GAL in jejunum and this colocalisation in the ileum.

In the OSP, the highest degree of colocalisation of NRG1 and GAL was observed in the jejunum, where GAL was noted in $30.53 \pm 1.29\%$ of NRG1-positive cells. In the ileum, the degree of colocalisation of NRG1 and GAL amounted to $26.21 \pm 0.84\%$ and in the duodenum it was lower at $17.41 \pm 1.00\%$. Statistically significant differences were observed between the colocalisation percentages in the three segments of the intestine.

In the ISP, the degree of colocalisation of NRG 1 and GAL was lower than in other enteric plexuses. Galanin was noted in $11.47 \pm 1.21\%$, $26.01 \pm 1.10\%$ and $15.17 \pm 1.124\%$ of all NRG 1-positive cells located in the duodenum, jejunum and ileum, respectively. The values noted in the jejunum were statistically significantly higher than those noted in the duodenum and ileum. There were no statistically significant differences between the value for the duodenum and the value for the ileum.

Data on the colocalisation of NRG 1 and GAL in the ENS of the porcine small intestine are summarised in Table 4.

Colocalisation of NRG 1 and nNOS. The colocalisation of NRG 1 with nNOS, as was the case with the colocalisation of NRG 1 with VIP and of NRG 1 with GAL, was noted in all types of enteric plexus in each segment of the small intestine (Table 5, Fig. 4). Generally, the degree of colocalisation of NRG1 and nNOS was lower in the ISP than in the other types of enteric plexus.

In the MP, the degrees of colocalisation of NRG1 and nNOS in the jejunum and ileum were alike at $31.04 \pm 1.06\%$ and $31.77 \pm 1.14\%$, respectively. In the duodenal MP, the percentage of NRG1/nNOS-positive cells was statistically significantly lower, and was 17.03 ± 1.35 .

In the OSP of the jejunum and ileum, the percentages of cells containing NRG 1 and nNOS were similar to those noted in the MP and amounted to 31.43 ± 1.22 and 29.52 ± 0.93 , respectively. In the duodenal OSP, nNOS was observed in $26.25 \pm 1.46\%$ of all NRG 1-positive

cells, and this value was statistically significantly lower than those noted in the OSP of other intestinal segments.

In the ISP, nNOS was noted in $18.82 \pm 1.28\%$, $15.96 \pm 1.02\%$ and $16.96 \pm 1.19\%$ of all NRG 1-positive cells located in the duodenum, jejunum and ileum,

respectively. There were no statistically significant differences between these values. Data on the colocalisation of NRG 1 and nNOS in the ENS of the porcine small intestine are summarised in Table 5.

Table 2. The percentage of neuregulin 1-positive neurons in the porcine small intestine

Enteric plexus	Duodenum	Jejunum	Ileum
MP	$21.52 \pm 0.98\%^a$	$16.16 \pm 0.88\%^b$	$18.48 \pm 0.66\%^b$
OSP	$19.98 \pm 0.69\%^a$	$16.26 \pm 0.84\%^b$	$15.20 \pm 0.63\%^b$
ISP	$16.92 \pm 1.19\%^a$	$8.38 \pm 0.55\%^b$	$10.26 \pm 0.70\%^b$

MP – myenteric plexus; OSP – outer submucosal plexus; ISP – inner submucosal plexus. Protein gene product 9.5-positive neurons were considered to represent 100%. Data are presented as the percentage of PGP 9.5-positive enteric neurons which contained neuregulin 1 (means $\pm$ standard error of the mean). ^{a, b} – different superscript letters for any pair of values indicate significant difference (P-value < 0.05)

Table 3. The degree of colocalisation of neuregulin 1 (NRG 1) and vasoactive intestinal polypeptide (VIP) in the porcine small intestine

Enteric plexus	Duodenum	Jejunum	Ileum
MP	$32.39 \pm 1.82\%^a$	$25.50 \pm 1.69\%^b$	$29.17 \pm 1.45\%^a$
OSP	$41.91 \pm 1.90\%^a$	$24.96 \pm 2.24\%^b$	$36.62 \pm 1.43\%^c$
ISP	$25.16 \pm 0.94\%^a$	$20.46 \pm 1.23\%^b$	$23.55 \pm 0.87\%^a$

MP – myenteric plexus; OSP – outer submucous plexus; ISP – inner submucous plexus. NRG 1-positive neurons were considered to represent 100%. Data are presented as the percentage of cells (means $\pm$ standard error of the mean) of NRG 1-positive enteric neurons which contained VIP. ^{a, b} – different superscript letters for any pair of values indicate significant difference (P-value < 0.05)

Table 4. The degree of colocalisation of neuregulin 1 (NRG 1) and galanin (GAL) in the porcine small intestine

Enteric plexus	Duodenum	Jejunum	Ileum
MP	$15.67 \pm 0.94\%^a$	$28.73 \pm 0.81\%^b$	$26.38 \pm 1.37\%^b$
OSP	$17.41 \pm 1.00\%^a$	$30.53 \pm 1.29\%^b$	$26.21 \pm 0.84\%^c$
ISP	$11.47 \pm 1.21\%^a$	$26.01 \pm 1.10\%^b$	$15.17 \pm 1.24\%^a$

MP – myenteric plexus; OSP – outer submucous plexus; ISP – inner submucous plexus. NRG 1-positive neurons were considered to represent 100%. Data are presented as the percentage of cells (means $\pm$ standard error of the mean) of NRG 1-positive enteric neurons which contained GAL. ^{a, b} – different superscript letters for any pair of values indicate significant difference (P-value < 0.05)

Table 5. The degree of colocalisation of neuregulin 1 (NRG 1) and the neuronal isoform of nitric oxide synthase (nNOS) in the porcine small intestine

Enteric plexus	Duodenum	Jejunum	Ileum
MP	$17.03 \pm 1.35\%^a$	$31.04 \pm 1.06\%^b$	$31.77 \pm 1.14\%^b$
OSP	$26.25 \pm 1.46\%^a$	$31.43 \pm 1.22\%^b$	$29.52 \pm 0.93\%^b$
ISP	$18.82 \pm 1.28\%^a$	$15.96 \pm 1.02\%^a$	$16.96 \pm 1.19\%^a$

MP – myenteric plexus; OSP – outer submucous plexus; ISP – inner submucous plexus. NRG 1-positive neurons were considered to represent 100%. Data are presented as the percentage of cells (means $\pm$ standard error of the mean) of NRG 1-positive enteric neurons which contained nNOS. ^{a, b} – different superscript letters for any pair of values indicate significant difference (P-value < 0.05)

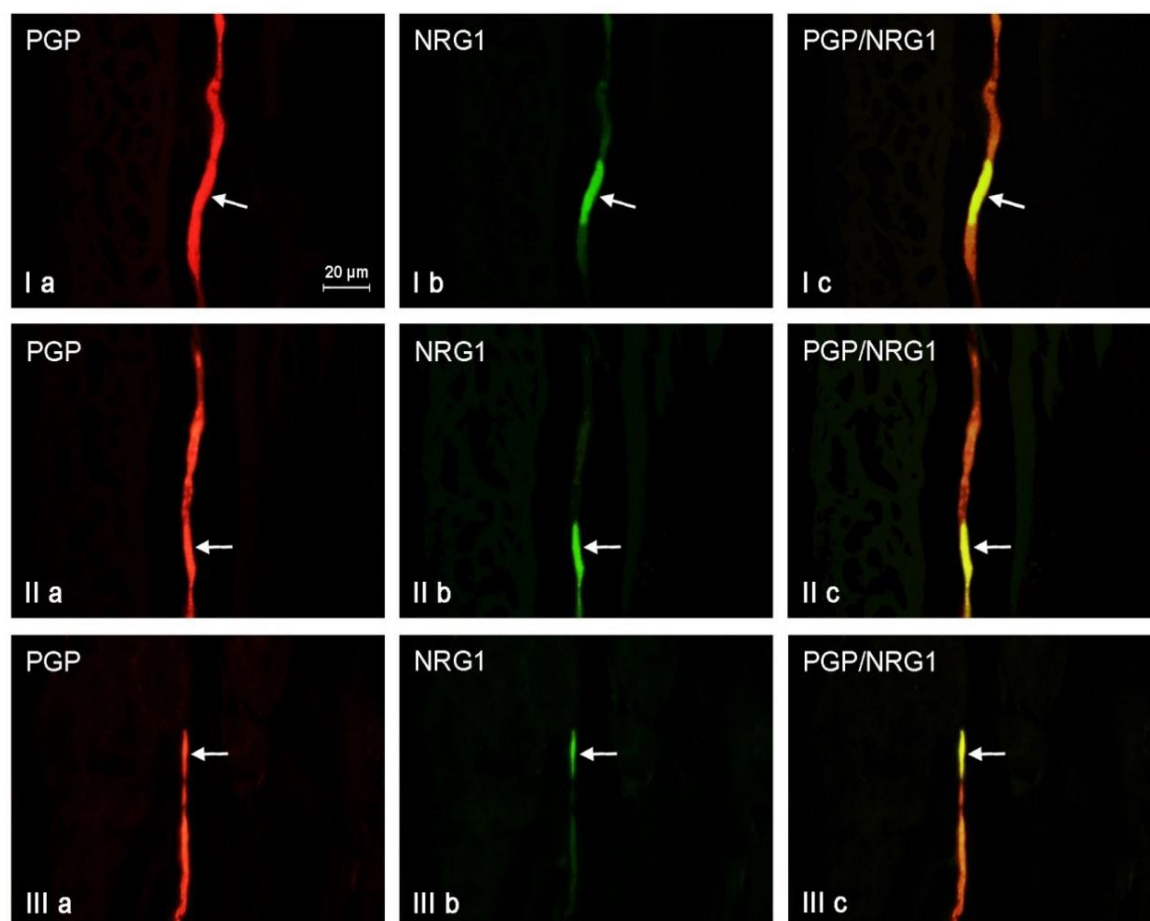


Fig. 1. Example micrographs of neuregulin 1 (NRG 1)-positive neurons in the duodenal myenteric plexus (I), jejunal outer submucous plexus (II) and ileal inner submucous plexus (III). Arrows – neurons immunoreactive to both the anti–protein gene product (PGP) 9.5 and anti-NRG 1 antibodies; scale bar – 20 µm

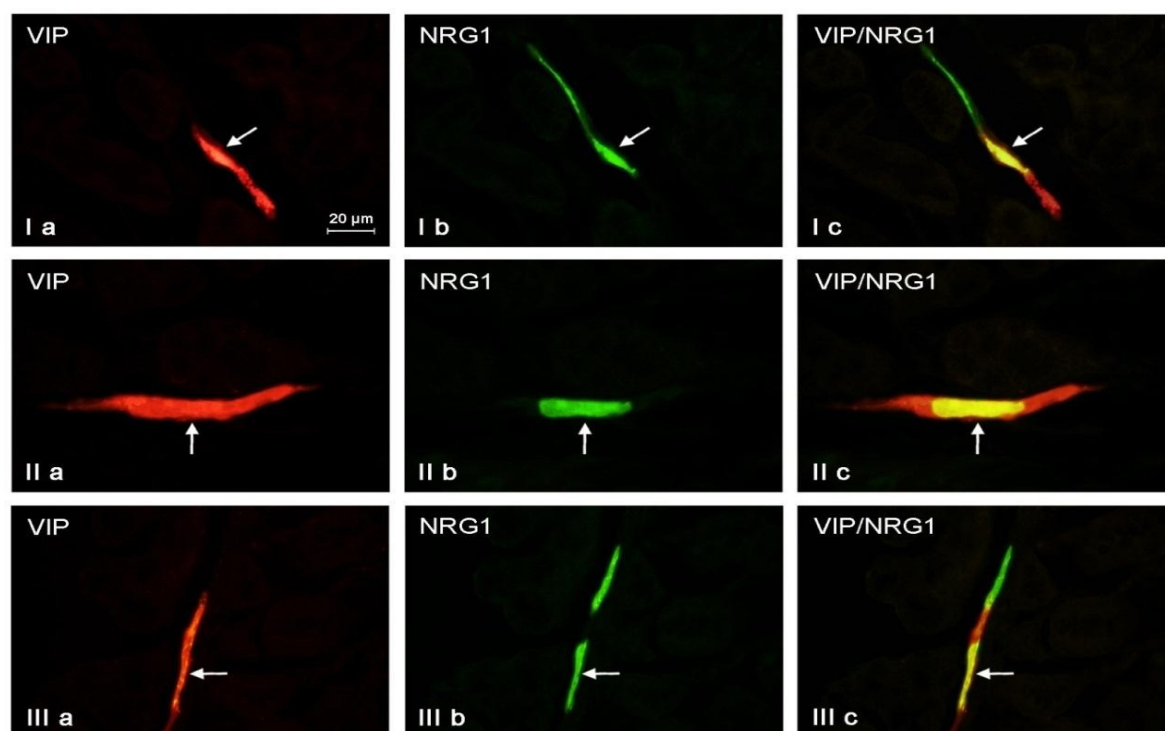


Fig. 2. Example micrographs of the colocalisation of vasoactive intestinal polypeptide (VIP) and neuregulin 1 (NRG 1) in neurons located in the duodenal outer submucous plexus (I), jejunal inner submucous plexus (II) and ileal myenteric plexus (III). Arrows – neurons immunoreactive to both the anti-VIP and anti-NRG1 antibodies; scale bar – 20 µm

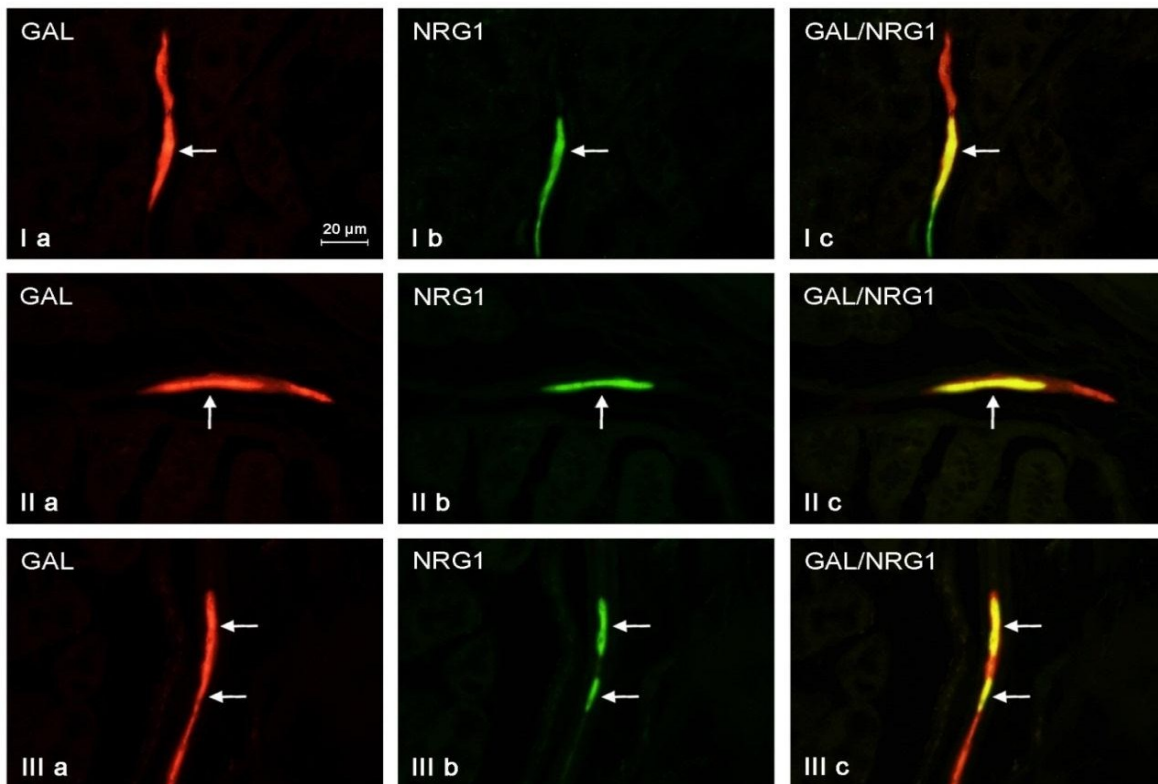


Fig. 3 Example micrographs of the colocalisation of galanin (GAL) and neuregulin 1 (NRG 1) in neurons located in the duodenal myenteric plexus (I), jejunal inner submucous plexus (II) and ileal outer submucous plexus (III). Arrows – neurons immunoreactive to both the anti-GAL and anti-NRG1 antibodies; scale bar – 20 μm

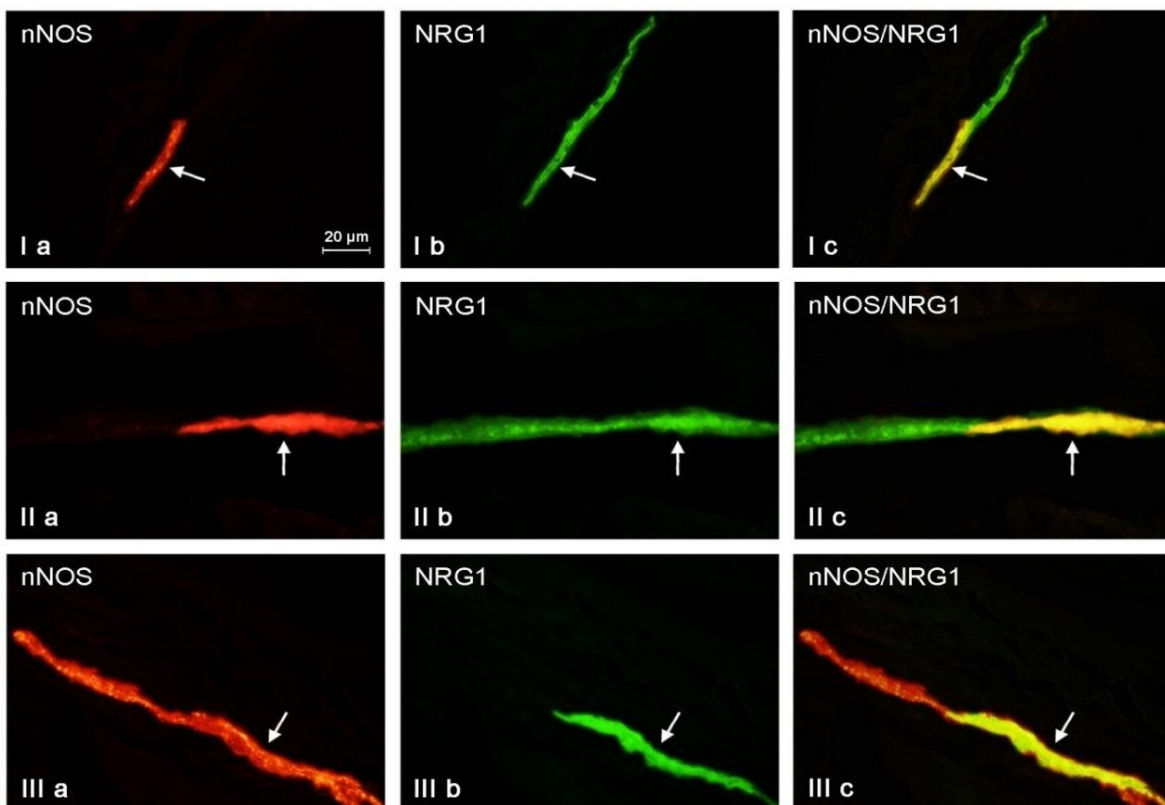


Fig. 4. Example micrographs of the colocalisation of the neuronal isoform of nitric oxide synthase (nNOS) and neuregulin 1 (NRG 1) in neurons located in the duodenal outer submucous plexus (I), jejunal inner submucous plexus (II) and ileal myenteric plexus (III). Arrows – neurons immunoreactive to both the anti-nNOS and anti-NRG 1 antibodies; scale bar – 20 μm

Discussion

The results obtained in the present study indicate for the first time that NRG 1 is present in the enteric neurons within the porcine small intestine. It should be pointed out that current knowledge on the distribution and functions of NRG 1 in the GI tract is relatively scant and fragmentary. Previous studies have described the presence of NRG 1 in various structures located in the wall of the GI tract, including the chief and parietal cells of gastric glands, lamina propria and enteroendocrine cells in the small intestine, stratified squamous epithelial cells of the oesophagus and the mucosal and muscular layers of the colon (2, 30). It has also been found in the tumour cells located in the stomach (29). Even less is known about distribution of NRG 1 in the ENS. To the best of the authors' knowledge, all previous studies on NRG 1 in the enteric nervous structures concerned the large intestine and described the presence of this substance in the enteric ganglia (both in neurons and glial cells) and nerve fibres of the colon and caecum (18, 25).

The present study's findings confirmed previous observations that NRG 1 was a substance commonly found in the GI tract. Comparing the present results with those of previous investigations, significant differences in the distribution of NRG 1 could be seen in the enteric nervous structures both between various mammal species and between particular segments of the GI tract (25). The most interesting is how the present results compared with the observations made in the previous study conducted on the porcine large intestine (25). It showed that in the small and large intestine of the domestic pig, NRG 1 was present in all types of enteric plexus, but in the ascending and descending colon the percentage of NRG 1-positive neurons was clearly higher than it was in the small intestine. This strongly suggested that the roles of NRG 1 in the ENS depended on the intestinal segment, and that the participation of NRG 1 in the regulation of colonic functions was greater here than in the small intestine. The distribution of NRG 1 in all types of enteric plexus noted in the porcine GI tract both in the present and previous studies (25) indicated that NRG 1 was involved in regulation of a wide range of intestinal functions, including motility (regulated mainly by neurons located in the MP and OSP), secretory activity and processes in the mucosal layer (regulated mainly by neurons in the ISP).

The multidirectional activity of NRG 1 in the enteric neurons suggested by the presence of this substance in all types of enteric ganglia has also been described in previous studies. In spite of the roles of NRG 1 in the ENS still eluding full explanation, it is known that this substance may have regulated intestinal motility (2) and that it affected the development and differentiation of enteric neurons during ontogenesis (2, 22). Previous studies also described an NRG 1 influence on the development of Schwann cells and synaptogenesis (19). In experimental animals deprived of NRG 1 or ErbB receptors, declines in the number of the enteric ganglia

and the number of synapses between enteric neurons were also noted in the ENS (23). Some previous studies described changes in the expression of NRG 1 in the intestine during pathological processes, such as diverticular disease and Hirschsprung's disease, and under the impact of food-borne toxins (25, 26). These observations strongly suggested that NRG 1 regulates intestinal functions not only in physiological conditions, but also in pathological ones. In disease states, it is involved in reactions triggered by factors damaging the GI tract, which may be connected with adaptive and/or protective processes.

Nevertheless, the exact functions of NRG 1 in the ENS of particular segments of the GI tract still remain unknown. One of the methods to gain a better understanding of this issue are studies on the colocalisation of NRG 1 with other better-known neuronal factors in the same enteric structures. This is because the active substances produced by the same neuronal cells usually played similar roles and took part in similar processes (4).

In the present study, the colocalisation of NRG 1 with all the other neuronal factors investigated was noted in the porcine small intestine. Similarly to the presence of NRG 1 in all types of enteric plexus, this observation suggested multiple roles of this substance in the regulation of intestinal function. It is in agreement with a finding of a previous investigation on the porcine large intestine, where broad neurochemical diversity of NRG 1-positive neurons was also described (25).

The colocalisation of NRG 1 with VIP, GAL and nNOS in the same neuronal cells noted in the present study suggested that NRG 1 may play similar roles to these neuronal factors. The gaseous neurotransmitter nitric oxide is synthesised by neurons of which nNOS is the marker. It and VIP are main inhibitory factors in the ENS. They caused the hyperpolarisation of the smooth muscles in the wall of the GI tract and inhibited intestinal motility (14). Galanin is also a substance which may regulate the intestinal muscles' work, but its effects either relaxed or contracted them depending on the segment of the GI tract and the animal species (3). Therefore, the observed colocalisation of NRG 1 with VIP, GAL and/or nNOS strongly suggested that NRG 1 is one regulator of porcine small intestine motility. It is all the more likely given that previous studies described NRG 1 as a substance involved in muscular metabolism and regulation of neuro-muscular synapses (8).

Galanin, VIP and nitric oxide in the GI tract are not only involved in the regulation of intestinal motility. They also functioned in other ways, regulating the secretory activity of the GI tract and the blood flow in the intestinal wall and modulating the immune processes within the intestine (7, 12, 21). Therefore, the colocalisation of NRG 1 with these substances revealed in this study suggested that NRG 1 possibly also participates in these regulatory processes.

The colocalisation of NRG 1 with GAL, VIP and/or nNOS in the same neurons may have also confirmed that

NRG 1 acts in certain pathological processes connected with intestinal diseases and toxic substances which were described in previous studies (25, 26). This theory is lent support by GAL, VIP and nitric oxide being neuronal factors known to have been involved in the neuroprotective and adaptive processes within the nervous system which enhanced the survivability of neurons and stimulated them to produce many active substances (1, 5). Moreover, all these substances were involved in the inflammatory processes which often accompany gastrointestinal pathological states and are connected with the impact of toxic substances (24).

Conclusion

This is the first description of NRG 1 in the enteric neurons in the porcine small intestine. The results of the present study clearly showed that NRG 1 commonly occurred in the enteric neurons located in the wall of the porcine small intestine, which suggested that this substance was an important regulator of the functions of the investigated segments of the GI tract. Neurons containing NRG 1 were distributed in all types of enteric plexus, which may have indicated that it controls intestinal function in various mechanisms. Neuregulin 1-positive enteric neurons also contained various other neuronal factors, including GAL, VIP and nNOS, which was indicative of NRG 1's involvement in various regulatory processes in the small intestine. The colocalisation of NRG 1 with GAL, VIP and/or nNOS in the same neuronal cells suggested that these substances may play similar roles. However, many aspects of NRG 1's functions in the small intestine remain in the sphere of guesses, and detailed explanations of them require further comprehensive research.

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References

- Arciszewski M.B., Sand E., Ekblad E.: Vasoactive intestinal peptide rescues cultured rat myenteric neurons from lipopolysaccharide induced cell death. *Regul Pept* 2008, 146, 218–223, doi: 10.1016/j.regpep.2007.09.021.
- Barrenschée M., Lange C., Cossais F., Egberts J.-H., Becker T., Wedel T., Böttner M.: Expression and function of Neuregulin 1 and its signaling system ERBB2/3 in the enteric nervous system. *Front Cell Neurosci* 2015, 9, 360, doi: 10.3389/fncel.2015.00360.
- Botella A., Delvaux M., Frexinos J., Bueno L.: Comparative effects of galanin on isolated smooth muscle cells from ileum in five mammalian species. *Life Sci* 1992, 50, 1253–1261, doi: 10.1016/0024-3205(92)90325-J.
- Brehmer A.: Classification of human enteric neurons. *Histochem Cell Biol* 2021, 156, 95–108, doi: 10.1007/s00418-021-02002-y.
- Cordero-Llana O., Rinaldi F., Brennan P.A., Wynick D., Caldwell M.A.: Galanin promotes neuronal differentiation from neural progenitor cells in vitro and contributes to the generation of new olfactory neurons in the adult mouse brain. *Exp Neurol* 2014, 256, 93–104, doi: 10.1016/j.expneurol.2014.04.001.
- Fledrich R., Stassart R.M., Klink A., Rasch L.M., Prukop T., Haag L., Czesnik D., Kungl T., Abdelaal T.A.M., Keric N., Stadelmann C., Brück W., Nave K.A., Sereda M.W.: Soluble neuregulin-1 modulates disease pathogenesis in rodent models of Charcot-Marie-Tooth disease 1A. *Nat Med* 2014, 20, 1055–1061, doi: 10.1038/nm.3664.
- Furness J.B., Callaghan B.P., Rivera L.R., Cho H.J.: The enteric nervous system and gastrointestinal innervation: Integrated local and central control. *Adv Exp Med Biol* 2014, 817, 39–71, doi: 10.1007/978-1-4939-0897-4_3.
- Geuna S., Tos P., Raimondo S., Lee J.M., Gambaretta G., Nicolino S., Fornaro M., Papalia I., Perroteau I., Battiston B.: Functional, morphological and biomolecular assessment of posttraumatic neuro-muscular recovery in the rat forelimb model. *Acta Neurochir Suppl* 2007, 100, 173–177, doi: 10.1007/978-3-211-72958-8_36.
- Gonkowski S.: Substance P as a neuronal factor in the enteric nervous system of the porcine descending colon in physiological conditions and during selected pathogenic processes. *Biofactors* 2013, 39, 542–551, doi: 10.1002/biof.1097.
- Huang L.L., Liu Z.Y., Huang J.H., Luo Z.J.: Expression pattern of neuregulin-1 type III during the development of the peripheral nervous system. *Neural Regen Res* 2015, 10, 65–70, doi: 10.4103/1673-5374.150708.
- Inoue T., Ohkawara B., Bushra S., Kanbara S., Nakashima H., Koshimizu H., Tomita H., Ito M., Masuda A., Ishiguro N., Imagama S., Ohno K.: Zonisamide upregulates neuregulin-1 expression and enhances acetylcholine receptor clustering at the *in vitro* neuromuscular junction. *Neuropharmacology* 2021, 195, 108637, doi: 10.1016/j.neuropharm.2021.108637.
- Iwasaki M., Akiba Y., Kaunitz J.D.: Recent advances in vasoactive intestinal peptide physiology and pathophysiology: Focus on the gastrointestinal system. *F1000Research* 2019, 8, 1629, doi: 10.12688/f1000research.18039.1.
- Jorissen R.N., Walker F., Pouliot N., Garrett T.P.J., Ward C.W., Burgess A.W.: Epidermal growth factor receptor: Mechanisms of activation and signaling. *Exp Cell Res* 2003, 284, 31–53, doi: 10.1016/S0014-4827(02)00098-8.
- Kasperek M.S., Fatima J., Iqbal C.W., Duenes J.A., Sarr M.G.: Role of VIP and substance P in NANC innervation in the longitudinal smooth muscle of the rat jejunum-influence of extrinsic denervation. *J Surg Res* 2007, 141, 22–30, doi: 10.1016/j.jss.2007.01.021.
- Kataria H., Alizadeh A., Karimi-Abdolrezaee S.: Neuregulin-1/ErbB network: An emerging modulator of nervous system injury and repair. *Prog Neurobiol* 2019, 180, 101643, doi: 10.1016/j.pneurobio.2019.101643.
- Law A.J., Weickert C.S., Hyde T., Kleinman J., Harrison P.: Neuregulin-1 (NRG-1) mRNA and protein in the adult human brain. *Neuroscience* 2004, 127, 125–136, doi: 10.1016/j.neuroscience.2004.04.026.
- Makowska K., Lepiarczyk E., Gonkowski S.: The comparison of the influence of bisphenol A (BPA) and its analogue bisphenol S (BPS) on the enteric nervous system of the distal colon in mice. *Nutrients* 2022, 15, 200, doi: 10.3390/nu15010200.
- Makowska K., Szymańska K., Całka J., Gonkowski S.: The influence of bisphenol A (BPA) on the occurrence of selected active substances in neuregulin 1 (NRG1)-positive enteric neurons in the porcine large intestine. *Int J Mol Sci* 2021, 22, 10308, doi: 10.3390/ijms221910308.

19. Mei L., Nave K.A.: Neuregulin-ERBB Signaling in the Nervous System and Neuropsychiatric Diseases. *Neuron* 2014, 83, 27–49, doi: 10.1016/j.neuron.2014.06.007.
20. Peles E., Bacus S.S., Koski R.A., Lu H.S., Wen D., Ogden S.G., Levy R.B., Yarden Y.: Isolation of the Neu HER-2 stimulatory ligand: A 44 kd glycoprotein that induces differentiation of mammary tumor cells. *Cell* 1992, 69, 205–216, doi: 10.1016/0092-8674(92)90131-U.
21. Psichas A., Glass L.L., Sharp S.J., Reimann F., Gribble F.M.: Galanin inhibits GLP-1 and GIP secretion via the GAL1 receptor in enteroendocrine L and K cells. *Br J Pharmacol* 2016, 173, 888–898, doi: 10.1111/bph.13407.
22. Pu J., Tang S.T., Tong Q., Wang G., Jia H., Jia Q., Li K., Li D., Yang D., Yang J., Li H., Li S., Mei H.: Neuregulin 1 is involved in enteric nervous system development in zebrafish. *J Pediatr Surg* 2017, 52, 1182–1187, doi: 10.1016/j.jpedsurg.2017.01.005
23. Riethmacher D., Sonnenberg-Riethmacher E., Brinkmann V., Yamaai T., Lewin G.R., Birchmeier C.: Severe neuropathies in mice with targeted mutations in the ErbB3 receptor. *Nat Cell Biol* 1997, 389, 725–730, doi: 10.1038/39593.
24. Sharma J.N., Al-Omran A., Parvathy S.S.: Role of nitric oxide in inflammatory diseases. *Inflammopharmacology* 2017, 15, 252–259, doi: 10.1007/s10787-007-0013-x.
25. Szymanska K., Makowska K., Całka J., Gonkowski S.: The endocrine disruptor bisphenol A (BPA) affects the enteric neurons immunoreactive to neuregulin 1 (NRG1) in the enteric nervous system of the porcine large intestine. *Int J Mol Sci* 2020, 21, 8743, doi: 10.3390/ijms21228743.
26. Tang W., Li B., Xu X., Zhou Z., Wu W., Tang J., Qin J., Geng Q., Jiang W., Zhang J., Sha J., Xia Y., Wang X.: Aberrant high expression of NRG1 gene in Hirschsprung disease. *J Pediatr Surg* 2012, 47, 1694–1698, doi: 10.1016/j.jpedsurg.2012.03.061.
27. Verma N., Rettenmeier A.W., Schmitz-Spanke S.: Recent advances in the use of *Sus scrofa* (pig) as a model system for proteomic studies. *Proteomics* 2011, 11, 776–793, doi: 10.1002/pmic.201000320.
28. Viehover A., Miller R.H., Park S.K., Fischbach G., Vartanian T.: Neuregulin: an oligodendrocyte growth factor absent in active multiple sclerosis lesions. *Dev Neurosci* 2001, 23, 377–386, doi: 10.1159/000048721.
29. Yun S., Koh J., Nam S.K., Park J.O., Lee S.M., Lee K., Lee K.S., Ahn S.H., Park D.J., Kim H.H., Choe G., Kim W.H., Lee H.S.: Clinical significance of overexpression of NRG1 and its receptors, HER3 and HER4, in gastric cancer patients. *Gastric Cancer* 2018, 21, 225–236, doi: 10.1007/s10120-017-0732-7.
30. Zhao W.J.: The expression and localization of neuregulin-1 (Nrg1) in the gastrointestinal system of the rhesus monkey. *Folia Histochem Cytobiol* 2013, 51, 38–44, doi: 10.5603/FHC.2013.006.