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Effect of different glyphosate doses on the chemical coding of neurons of the enteric nervous system of the porcine descending colon

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ABSTRACT

Background: Neurons of the enteric nervous system are characterised by high neuronal plasticity, with their number likely to change in response to various endogenous and exogenous substances.

Materials and methods: Fifteen sexually immature gilts divided into 3 groups were used: control — animals receiving empty gelatin capsules; G1 — animals receiving a low dose of glyphosate — 0.05 mg/kg bw/day; G2 — animals receiving a higher dose of glyphosate—0.5 mg/kg/day in gelatin capsules orally for 28 days. Frozen sections were then subjected to the procedure of double immunofluorescent staining.

Results: With low-dose supplementation, no effect on the SP- and CART-positive neuron population was observed. However, a reduction in the number of VAChT-positive neurons in the internal submucosal plexus was described, while the number of CGRP-positive neurons increased in all enteric plexuses. In response to a high glyphosate dose, the quantitative variability of the neurons was significantly more pronounced than that for a low dose. There was an increase in the number of SP- and CGRP-positive neurons and a decrease in the number of VAChT-positive neurons in both the myenteric plexus and the submucosal

plexuses. The response of CART-positive neurons was the weakest, as a high dose of glyphosate led to an increase in the number of neurons only in the myenteric plexus. **Conclusions:** The above data show that glyphosate is an exogenous substance that affects neuronal populations of the enteric nervous system, in this case, the descending colon.
Keywords: glyphosate, enteric neurons, pig, descending colon, immunofluorescence

INTRODUCTION

The descending colon is part of the large intestine. This is where stool is finally formed, and defecation begins due to neural mechanisms [2]. This part of the gastrointestinal tract is also the site of various pathological processes of neoplastic, inflammatory or bacterial origin [18, 17, 27,28]. The proper functioning of the entire gastrointestinal system, including the descending colon, is controlled at different levels of the nervous system. As regards the gastrointestinal tract, a key role is served by the enteric nervous system, a structure specific to the gastrointestinal tract, first described more than 150 years ago [15]. Within the porcine descending colon, the enteric nervous system is made up of the myenteric plexus located between the circular and the longitudinal layer of the muscle and the submucosal plexus within which two components can be distinguished. The outer submucosal plexus and the inner submucosal plexus are located within the submucosal membrane. These plexuses primarily control the normal motor activity of this part of the intestine and are the first element of the pathway conducting sensory stimuli from the mucosal and submucosal membrane regions [16,49].

The above-mentioned functions are largely controlled via biologically active substances referred to as neurotransmitters, which can also alter the nerve signal accordingly, in which case they are referred to as neuromodulators. The main neuropeptide involved in the sensory and pain impulse transmission processes is calcitonin gene-related peptide (CGRP). Its expression has been described in all sections of the gastrointestinal tract and in all components of the enteric nervous system. In addition to its sensory function, it is responsible for regulating peristalsis, gastric juice secretion, and mesenteric blood flow [45,42]. Another substance of a sensory nature found in the nervous system, including the enteric nervous system, is substance P (SP). Similar to CGRP, this peptide performs other functions in the gastrointestinal tract, which are largely determined by the type of receptor via which SP functions [43,8]. One of the substances most commonly found in the gastrointestinal tract is acetylcholine. To identify acetylcholine in neurons, researchers use the vesicular acetylcholine

transporter (VAChT), which transports acetylcholine into synaptic vesicles. Acetylcholine released from nerve endings is the main excitatory transmitter within the gastrointestinal tract [2,26]. Cocaine- and amphetamine-regulated transcript (CART) is a peptide with a relatively poorly recognised role within the gastrointestinal tract despite its widespread occurrence in various sections of this tract. According to the studies conducted to date, CART functions primarily by modifying the secretion of other substances, such as VIP or nNOS [31,47,32].

The nervous system at all its levels is capable of adaptation and variability depending on the endogenous or exogenous factors that affect it. This property has already been observed in phylogenetically primitive species and is called neuronal plasticity [24]. The neuron synthesises and releases a biologically active substance that enables it to perform its function despite changing micro-environments [9]. Many potentially toxic substances are found in the external environment, which, when entering the animal or human body, can adversely affect neural functioning, a consequence of which can be the activation of the above-described neuronal plasticity mechanism. One of these compounds is glyphosate, a herbicide with a weed-killing activity, currently the most commonly used compound with the above-mentioned effect in agriculture [10]. The often unjustified and uncontrolled use of this compound creates the potential for accumulation in the soil and in edible plants used by animals and in the agri-food industry. Research on the toxic effects of glyphosate has been carried out for many years, with the obtained results being inconclusive and still incomplete. The literature offers no data on the effect of glyphosate on the neurons of the enteric nervous system. This is all the more important, due to its poor fat solubility, glyphosate is only minimally absorbed in the gastrointestinal tract but can reach high concentrations in food content of this tract, and thus affect components of the gastrointestinal wall, including neurons of the enteric nervous system [38,11,13].

Therefore, this study was aimed at determining the effect of glyphosate administered orally at two doses on the populations of intramural neurons of the descending colon, immunoreactive against SP, CGRP, VAChT, and CART. This study used the pig as an experimental model – an animal whose anatomy, and primarily physiology of the gastrointestinal tract, is much more similar to the processes occurring in the human body than in the gastrointestinal tract of rodents [20,51]. The obtained results can serve as a reference to the animal body but can also be extrapolated to the human body.

MATERIALS AND METHODS

This study was conducted on 15 sexually immature gilts with a body weight of 20 kg at the beginning of the experiment. All procedures related to the experimental part using animals were approved by the local committee for animal experiments in Olsztyn according to current Polish and European Union regulations (Approval No. 62/2020). The animals were kept in an animal house located at the Faculty of Veterinary Medicine in Olsztyn. In terms of welfare, the rooms were adapted to the animal species used in the experiment concerned. Before the start of the experimental part, the pigs were randomly divided into three experimental groups, each comprising five individuals. The experiment involved one control group and two experimental groups. Animals in the experimental groups received glyphosate administered orally in gelatine capsules. In the first experimental group, the animals received glyphosate at a dose of 0.05 mg per kg of body weight, while the animals in the second experimental group received a ten times higher dose (0.5 mg per kg of body weight). Glyphosate was administered once daily during the morning feeding for 28 days. At the same time, animals in the control group received empty gelatine capsules. Animals in all the groups were fed standard feed adapted to the animal species, had continuous access to water, and had the same light cycle (12 h L/12 h D). Once the experimental period was over, all the gilts were euthanised. To this end, the animals were premedicated by intramuscular administration of azaperone followed by an intravenous lethal dose of sodium pentobarbital. Immediately after the euthanasia, the gastrointestinal tract was collected from the animals. This experiment used the descending colon for further testing. Two-centimetre-long segments containing a cross-section through all the wall layers were collected from this section of the large intestine and further prepared for immunofluorescence testing.

Immediately after collection, the descending colon segments were immersion-fixed in a 4% buffered paraformaldehyde solution for 1 hour. In order to remove the unbound fixative, the tissues were then rinsed in a phosphate buffer solution. The tissues were rinsed three times, with the buffer being replaced every 24 hours. After this period, the tissues were transferred to a 30% sucrose solution, and kept at 4^0C until the tissues were saturated. The tissues prepared in this way were used to make freezing blocks from which 12 µm sections were obtained and placed on microscope slides. The sections were obtained using a freezing microtome. The samples were then subjected to a double immunofluorescence staining procedure (as described previously by Palus et al. [41]). On the first day, the slides were dried at ambient temperature for 1 h. Afterwards, the tissues were rinsed three times in a phosphate buffer solution. The next stage involved an hour-long blocking using a blocking solution. At the final stage, on the first day of the staining procedure, the tissues were incubated with

appropriately diluted primary antibodies; Hu C/D proteins (mouse, 1:1000; Thermo Fisher Scientific, Waltham, MA USA; code A-21271); SP (rat, 1:150; AbD Serotec, Raleigh, NC, USA; code 8450-0505); CGRP (rabbit, 1:3000; Millipore, Burlington, MA, USA; code MAB 317); VAChT (rabbit, 1: 2000; Phoenix Pharmaceuticals, Burlingame, CA, USA; code H-V007); CART (rabbit, 1: 8000; Phoenix Pharmaceuticals, Burlingame, CA, USA; code H-003-61). The incubation time was 18 hours, and the entire procedure was conducted at ambient temperature in a so-called humid chamber. On the second day, the sections were rinsed three times in a phosphate buffer, after which the tissues were incubated with a mixture of secondary antibodies conjugated with the appropriate fluorochrome to visualise the antibodies under fluorescence microscopy; Alexa Fluor 488 anti mouse (donkey, 1:1000; Thermo Fisher Scientific. Waltham, MA, USA; code A21202); Alexa flour 594 anti-rat (donkey, 1:1000; Thermo Fisher Scientific. Waltham, MA, USA; code A21208); Alexa flour 594 anti rabbit (goat, 1:1000; Thermo Fisher Scientific. Waltham, MA, USA; code A11010) . After 2 hours of incubation, the sections were again rinsed three times and sealed with a coverslip using a buffered glycerol solution. The prepared tissues were then analysed using a fluorescence microscope.

A fluorescence microscope with appropriate filters, a digital camera, and software were used for analysing the staining and photographic documentation. The final result of the population of neurons immunoreactive against the studied neuropeptides was presented as a percentage of neurons in relation to neurons immunoreactive against the Hu C/D neuronal marker. In this study, the percentage of neurons immunoreactive against the substances studied was obtained by counting at least 700 Hu C/D-positive neurons. In addition, to avoid counting populations of the same neurons, neurons located at least 100 µm apart from each other were subjected to analysis. The obtained results were then analysed statistically using the Statistica 13 program (Stat Soft Inc., Tulsa, OK, USA) using a one-way analysis of variance (ANOVA) with Dunnett's test and expressed as a mean ± standard error of the mean (SEM) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

RESULTS

The experiments conducted in the present study showed the expression of the studied substances in all studied plexuses of the enteric nervous system of the descending colon. The number of neurons expressing the studied substances varied depending on the studied substance, the plexus, and the glyphosate dose (Table 1).

SP in the control group did not exceed 10% of the Hu C/D-positive neuron population in any of the plexuses studied (Table 1). In the myenteric plexus, the number of SP-positive neurons was 6.87 ± 0.33% (Fig. 1A). Similar numbers of neurons were noted in the outer submucosal plexus [(4.98 ± 0.25%) (Fig. 1D)] and inner submucosal plexus [(8.89 ± 0.77%) (Fig. 1G)]. A low glyphosate dose supplementation caused no statistically significant changes in the SP-positive neuron population in any of the studied plexuses. Only the application of glyphosate at a high dose resulted in a statistically significant increase in the SP-positive neuron population (Table 1). This increase was apparent in all the plexuses. In the myenteric plexus, the number of SP-positive neurons increased to 15.00 ± 1.03% (Fig. 1C); in the outer submucosal plexus to 12.34 ± 0.98% (Fig. 1F); and in the inner submucosal plexus to 17.56 ± 1.88% (Fig. 1I).

The second neuron population under study was that of CGRP-positive neurons. In the control group, in the myenteric plexus, their number amounted to 15.08 ± 0.8 % (Fig. 2A); in the outer submucosal plexus, to 19.87 ± 1.90% (Fig. 2D); and in the inner submucosal plexus, to 21.31 ± 0.97% (Fig. 2G). In the group of experimental animals receiving glyphosate at a low dose, there was an increase in the CGRP-positive neuron population in all the plexuses studied (Table 1). In the myenteric plexus, the number of CGRP-positive neurons increased to 19.97 ± 1.45% (Fig. 2B); in the outer submucosal plexus, to 22.90 ± 1.62% (Fig. 2E); and in the inner submucosal plexus, to 24.97 ± 1.83% (Fig. 2H). An even more statistically greater increase was noted in the group of animals receiving glyphosate at a high dose (Table 1). In the myenteric plexus, the number of CGRP-positive neurons increased to 28.53 ± 2.99% (Fig. 2C); in the outer submucosal plexus, to 28.90 ± 1.96% (Fig. 2F); and in the inner submucosal plexus, to 32.04% ±2.74 (Fig. 2I).

The population of VAChT-positive neurons in the control group was slightly larger than the population of CGRP-positive neurons. At the same time, VAChT-expressing neurons represented the most numerous neuron population among the substances under this study (Table 1). In the myenteric plexus, this population amounted to 20.92 ± 1.44% (Fig. 3A); in the submucosal plexuses, to 22.00 ± 1.82% (Fig. 3D) and 23.06 ± 1.39% (Fig 3F) in the outer and the inner plexus, respectively. In the group of experimental animals receiving a low glyphosate dose, a reduction in the number of VAChT-positive neurons to a level of 18.53 ± 0.76% was noted in the inner submucosal plexus (Fig. 3H). No statistically significant change in the number of VAChT-positive neurons was noted in the two other plexuses studied (Table 1). However, glyphosate supplemented at a high dose reduced the number of VAChT-positive neurons in all of the plexuses studied (Table 1). In the myenteric plexus, this population

decreased to a level of 16.36 ± 1.80 % (Fig. 3C), while in the outer submucosal plexus, a level of 17.09 ± 0.45% (Fig. 3F) was noted, and in the inner submucosal plexus, a level of 15.77 ± 0.70% (Fig. 3I) of VAChT-positive neurons was noted.

The final group of neurons being examined were those that tested positive for CART. In the myenteric plexus, neurons with this phenotype represented 8.99 ± 0.90% (Fig. 4A) of all neurons. In the outer submucosal plexus, their proportion was 3.89 ± 0.09% (Fig. 4D), while in the inner submucosal plexus, it was even lower, at 2.98 ± 0.12% (Fig. 4G; Table 1). A low glyphosate dose supplementation caused no statistically significant changes in any of the plexuses studied. A high glyphosate dose only resulted in a statistically significant increase in the CART-positive neuron population within the myenteric plexus region [(14.94 ± 1.04%) (Fig. 4C)], without causing statistically significant changes within the submucosal plexuses (Table 1).

DISCUSSION

As glyphosate is currently the most commonly used herbicide worldwide, this compound is frequently tested for toxicity [11]. Nevertheless, the available literature provides no precise data on the effect of glyphosate on the enteric nervous system of animals or humans. Considering that the gastrointestinal tract is one of the main pathways for glyphosate to enter the body, and, in addition, in view of the low-fat solubility of this compound, which translates into its low absorbability, the gastrointestinal tract is particularly vulnerable to the direct action of glyphosate. In this study, the quantitative interchangeability of intramural neurons in the porcine descending colon that are immunoreactive against sensory neurotransmitters such as SP or CGRP, acetylcholine, and CART was analysed. SP, a peptide with a broad spectrum of function within the gastrointestinal tract [42, 43, 44], was present in this study in each studied plexus of the descending colon. Under physiological conditions, its number did not exceed 10% of the population of neurons of a particular plexus in any of the plexuses studied. A low glyphosate dose supplementation caused no changes in the number of SP-positive neurons, as only the application of a high dose resulted in a statistically significant increase in the number of SP-immunoreactive neurons in the myenteric plexus and both submucosal plexuses. The relatively low number of SP-positive neurons in the control animal group is characteristic of this section of the porcine gastrointestinal tract [42, 48]. At the same time, it is lower than the number of SP in other sections of the gastrointestinal tract, which confirms the variation in the amount of a particular substance depending on the gastrointestinal tract section. Moreover, other studies have shown that various pathological

factors increase substance P expression in enteric neurons [8, 39,7,35,3,37]. Axotomy of endogenous neurons innervating the porcine descending colon resulted, similar to the current study, in an increase in the SP-positive neuron population in the myenteric plexus and both submucosal plexuses. In addition, a chemically induced inflammatory condition increased the number of SP-positive neurons [43,8]. A toxicological study using such substances as acrylamide and bisphenol A and S also increased SP-positive neurons [31]. It should be noted that glyphosate, an exogenous substance, was found to increase the population of intramural neurons in the descending colon in the current study. SP is one of the main substances responsible for the transmission of sensory stimuli from the gastrointestinal tract to the brain. An increase in its amount due to an increase in the number of neurons producing it may indicate an irritant effect of glyphosate on the gastrointestinal wall. In addition, SP, a substance with anti-inflammatory activity, is involved in suppressing this process, and an increase in the number of neurons producing it may indicate that glyphosate leads to the development of colitis despite the absence of evident clinical lesions [3,37,12].

CGRP is a 37-amino acid peptide commonly found in the gastrointestinal tract, including in neurons of the enteric nervous system in many mammal species [22,34,25]. In the current study, the population of CGRP-positive neurons in the control group was a more numerous population than that of SP-positive neurons, which is identical to the results obtained by other authors. It should be stressed that CGRP-positive neurons, in contrast to SP neurons, demonstrated a quantitative increase in the myenteric plexus and in the submucosal plexuses already at a low glyphosate dose. Obviously, the increase in the CGRP-positive neuron population was also visible for a high glyphosate dose. In a previously conducted study, the CGRP-positive neuron population exhibited a response similar to that obtained in the current study. CGRP neurons of the porcine descending colon responded with an increase in population to the supplementation with bisphenol A, zearalenone, and T2 toxin [18, 46, 33]. Moreover, the population of CGRP-positive neurons located in gastric intramural plexuses increased under the influence of orally administered acrylamide [39]. All of the above-mentioned substances have an irritant effect on the mucous membrane of the individual sections of the gastrointestinal tract, which results in the activation of endogenous primary afferent neurons. These neurons are located in both the myenteric plexus and the submucosal plexuses and, when activated, transmit excitation onto motoneurons which, in turn, trigger the activation of motor and secretory processes in a particular section of the gastrointestinal tract. This leads to the release of short intestinal reflexes. What is more, the primary sensory neurons may be involved in the transmission of nociceptive stimuli. The key role in the

above-mentioned process is served by CGRP, which is the main neurotransmitter of neurons of this class. An increase in the number of CGRP-positive neurons can intensify the process of the transmission of the above-described signal, and lead to the intensification of intramural intestinal reflexes [33,29,23,21] .

Acetylcholine is considered to be the most important excitatory neurotransmitter in the enteric nervous system [30]. In order to identify cholinergic neurons, this study used the vesicular acetylcholine transporter, i.e. the most commonly used marker of cholinergic neurons. This study also noted a reduction in the VAChT-positive neuron population in the experimental animal group, with a low glyphosate dose resulting in a decrease only in the inner submucosal plexus, while a high dose reduced the population of VAChT-positive neurons in all of the studied plexuses. A different responsiveness of VAChT-positive neurons was observed in the porcine gastric intramural neurons under the influence of acrylamide supplementation [40]. In addition, chronic hyperglycaemia led to an increase in the number of VAChT-positive neurons in the porcine small intestine region [30,40]. The different response of cholinergic neurons of the descending colon to glyphosate supplementation may be due to a different function of this gastrointestinal tract section. Specifically, in the descending colon, the resorption of nutrients and the secretion of digestive juices no longer occurs [2]. Its main function is proper motor activity to ensure the excretion of undigested food residues. In view of the prokinetic properties of cholinergic neurons, a decrease in their number in this part of the gastrointestinal tract may result in impaired motility, and lead to its reduction, which may also contribute to over-stimulation of the mucous membrane and release the previously described short reflex arcs [5].

CART neurons represented a small population of intramural neurons found in the descending colon, and were most numerous in the myenteric plexus. Glyphosate did not affect CART-positive neurons in low doses. High doses did not affect submucosal plexuses, but increased neurons in the myenteric plexus. A decrease in the number of CART-positive neurons was noted in the course of diabetes mellitus, where a decrease in their number was observed in the gastric enteric plexuses. However, in the descending colon, hyperglycaemia increased the number of CART-positive neurons [14]. Inflammation and damage to nerve processes within the descending colon slightly increased the number of CART-positive neurons [3,36]. The above data show that the variability of the CART-positive neuron population is significantly influenced by the gastrointestinal tract section and the type of pathological factor affecting the gastrointestinal tract. The main CART function within the descending colon is to influence the motility of this gastrointestinal tract section, for which

neurons of the myenteric plexus, among other things, are responsible. An increase in the CART-positive neuron population under the influence of a high glyphosate dose in this plexus may suggest the impact of this peptide on this process. It should be stressed that CART also has a modifying effect on the release of other neurotransmitters, including nitric oxide, which may secondarily lead to changes in nitrergic transmission [31,47,32,19,4,50].

CONCLUSIONS

The results obtained in this study show a high adaptive capacity of neurons of the enteric nervous system. For the first time, the effect of glyphosate on the populations of four classes of intramural neurons (immunoreactive against SP, CGRP, VAChT, and CART) of the porcine descending colon was demonstrated. Glyphosate, as a substance that is poorly absorbed from the food content, may directly affect the gastrointestinal tract by altering the tissue micro-environment within the structures found in the gastrointestinal wall. This, in turn, translates into neuronal adaptation to the new unfavourable conditions. It is important to note that during the experiment, both the amount of glyphosate administered and the length of time it was given were strictly limited. The results obtained under these conditions show that this herbicide is not a substance neutral to neurons. Obviously, the determination of the exact mechanism of glyphosate action on neurons of the enteric nervous system requires further research. In addition, modification of a glyphosate dose and the duration of exposure can also affect its potential toxic effect.

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Conflicts of interest:

None declared

REFERENCES

1. Arvidsson, U, Riedl, M, Elde, R, et al. Vesicular acetylcholine transporter (VAChT) protein: A novel and unique marker for cholinergic neurons in the central and peripheral nervous systems. J Comp Neurol. 1997; 378:454–467.
2. Azzouz LL, Sharma S. Physiology, Large Intestine. Treasure Island (FL) StatPearls Publishing, 2023.

3. Brunsson I, Fahrenkrug J, Jodal M, et al. Substance P effects on blood flow, fluid transport and vasoactive intestinal polypeptide release in the feline small intestine. J Physiol. 1995;15:727-34, doi: 10.1113/jphysiol.1995.sp020617.
4. Brzozowska M, Całka J. Acetylsalicylic Acid Supplementation Affects the Neurochemical Phenotyping of Porcine Duodenal Neurons. Int J Mol Sci. 2023;24(12):9871, doi: 10.3390/ijms24129871.
5. Bulc M, Całka J, Palus K. Effect of Streptozotocin-Inducted Diabetes on the Pathophysiology of Enteric Neurons in the Small Intestine Based on the Porcine Diabetes Model. Int J Mol Sci. 2020 Mar 17;21(6):2047, doi: 10.3390/ijms21062047.
6. Bulc M, Gonkowski S, Całka J. Expression of Cocaine and Amphetamine Regulated Transcript (CART) in the Porcine Intramural Neurons of Stomach in the Course of Experimentally Induced Diabetes Mellitus. J Mol Neurosci. 2015;57(3):376-85, doi: 10.3390/ijms21062047.
7. Bulc M, Palus K, Całka J, et al. Changes in Immunoreactivity of Sensory Substances within the Enteric Nervous System of the Porcine Stomach during Experimentally Induced Diabetes. J Diabetes Res. 2018;24;2018:4735659, doi: 10.1155/2018/4735659.
8. Calka J. Increased expression of CART, nNOS, VIP, PACAP, SP and GAL in enteric neurons of the porcine stomach prepyloric region following hydrochloric acid infusion. Folia Histochem Cytobiol. 2019;57(4):179-187, doi: 10.5603/FHC.a2019.0020.
9. Cathenaut L, Schlichter R, Hugel S. Short-term plasticity in the spinal nociceptive system. Pain. 2023:11, doi: 10.1097/j.pain.0000000000002999.
10. Duke SO. Perspectives on transgenic, herbicide-resistant crops in the United States almost 20 years after introduction. Pest Manag Sci. 2015;71(5):652-7, doi: 10.1002/ps.3863.
11. Duke SO. The history and current status of glyphosate. Pest Manag Sci. 2018;74(5):1027-1034, doi: 10.1002/ps.4652.
12. Fehér E, Altdorfer K. Neuroimmunomodulation in the mucosa of the alimentary tract. Orv Hetil. 2020;161(35):1436-1440, doi: 10.1556/650.2020.31795.
13. Ferreira NGC, da Silva KA, Guimarães ATB, et al. Hotspots of soil pollution: Possible glyphosate and aminomethylphosphonic acid risks on terrestrial ecosystems and human health. Environ Int. 2023;179:108135, doi: 10.1016/j.envint.2023.108135.

14. Furness J.B.. Types of neurons in the enteric nervous system. J. Auton. Nerv. Syst. 2000; 81: 87–96.
15. Furness JB, Stebbing MJ. The first brain: Species comparisons and evolutionary implications for the enteric and central nervous systems. Neurogastroenterol Motil. 2018;30(2), 10.1111/nmo.13234.
16. Furness JB. Integrated Neural and Endocrine Control of Gastrointestinal Function. Adv Exp Med Biol. 2016;891:159-73, doi: 10.1007/978-3-319-27592-5_16.
17. Godlewski J, Kmiec Z. Colorectal Cancer Invasion and Atrophy of the Enteric Nervous System: Potential Feedback and Impact on Cancer Progression. Int J Mol Sci. 2020;21(9):339, doi: 10.3390/ijms21093391.
18. Gonkowski S, Gajęcka M, Makowska K. Mycotoxins and the Enteric Nervous System. Toxins (Basel). 2020;12(7):461, doi: 10.3390/toxins12070461.
19. Gonkowski S, Kamińska B, Landowski P, Całka J. Immunohistochemical distribution of cocaine- and amphetamine regulated transcript peptide - like immunoreactive (CART-LI) nerve fibers and various degree of co-localization with other neuronal factors in the circular muscle layer of human descending colon. Histol Histopathol. 2013; 7:851–858, doi: 10.14670/HH-28.851.
20. Gonzalez LM, Moeser AJ, Blikslager AT. Porcine models of digestive disease: The future of large animal translational research. Transl. Res. 2015;166:12–27, doi: 10.1016/j.trsl.2015.01.004.
21. Hay DL, Garelja ML, Poyner DR, et al. Update on the pharmacology of calcitonin/CGRP family of peptides: IUPHAR Review 25. Br J Pharmacol. 2018;175(1):3-17, doi: 10.1111/bph.14075.
22. Holzer P, Holzer-Petsche U. Constipation Caused by Anti-calcitonin Gene-Related Peptide Migraine Therapeutics Explained by Antagonism of Calcitonin Gene-Related Peptide's Motor-Stimulating and Prosecretory Function in the Intestine. Front Physiol. 2022;12:820006, doi: 10.3389/fphys.2021.820006.
23. Huang R, Wu JH, Wang SL. Effect of moxibustion on colonic mucosal injury and expression of CGRP positive nerve fibers of distal colonic mucosa in ulcerative colitis mice. Zhongguo Zhen Jiu. 2021;41(10):1127-34, doi: 10.13703/j.0255-2930.20201226-k0001.
24. Inoue R, Nishimune H. Neuronal Plasticity and Age-Related Functional Decline in the Motor Cortex. Cells. 2023;25:12(17):2142, doi: 10.3390/cells12172142.

25. Jakob MO, Kofoed-Branzk M, Deshpande D, et al. An Integrated View on Neuronal Subsets in the Peripheral Nervous System and Their Role in Immunoregulation. Front Immunol. 2021;12:679055, doi: 10.3389/fimmu.2021.679055.
26. Kaleczyc, J.; Klimczuk, M.; Franke-Radowiecka, A.; Sienkiewicz, W.; Majewski, M.; Łakomy, M. The distribution and chemical coding of intramural neurons supplying the porcine stomach—The study on normal pigs and on animals suering from swine dysentery. Anat. Histol. Embryol. 2007;36:186–193, doi: 10.1111/j.1439-0264.2006.00744.x.
27. Kwiatkowski P, Godlewski J, Kieżun, et al. Colorectal cancer patients exhibit increased levels of galanin in serum and colon tissues. Oncol Lett. 2016;12(5):3323-3329, doi: 10.3892/ol.2016.5037.
28. Le Berre C, Naveilhan P, Rolli-Derkinderen M. Enteric glia at center stage of inflammatory bowel disease. Neurosci Lett. 2023;13:809:137315, doi: 10.1016/j.neulet.2023.137315.
29. Li F, Zou YY, Cui Y, et al. Calcitonin gene-related peptide is a promising marker in ulcerative colitis. Dig Dis Sci. 2013;58(3):686-93, doi: 10.1007/s10620-012-2406-y.
30. Li ZS, Furness JB. Immunohistochemical localization of cholinergic markers in putative intrinsic primary afferent neurons of the guinea-pig small intestine. Cell Tissue Res. 1998;294(1):35-43, doi: 10.1007/s004410051154.
31. Makowska K, Fagundes KRC, Gonkowski S. Influence of bisphenol A and its analog bisphenol S on cocaine- and amphetamine-regulated transcript peptide-positive enteric neurons in the mouse gastrointestinal tract. Front Mol Neurosci. 2023;16:1234841, doi: 10.3389/fnmol.2023.1234841.
32. Makowska K, Gonkowski S, Zielonka L, et al. T2 Toxin-Induced Changes in Cocaine- and Amphetamine-Regulated Transcript (CART)-Like Immunoreactivity in the Enteric Nervous System Within Selected Fragments of the Porcine Digestive Tract. Neurotox Res. 2017;31(1):136-147, doi: 10.1007/s12640-016-9675-8.
33. Makowska K, Gonkowski S. Changes Caused by Bisphenols in the Chemical Coding of Neurons of the Enteric Nervous System of Mouse Stomach. Int J Environ Res Public Health. 2023 Mar 14;20(6):5125, doi: 10.3390/ijerph20065125
34. Makowska K, Gonkowski S. The Influence of Inflammation and Nerve Damage on the Neurochemical Characterization of Calcitonin Gene-Related Peptide-Like Immunoreactive (CGRP-LI) Neurons in the Enteric Nervous System of the Porcine Descending Colon. Int J Mol Sci. 2018;19(2):548, doi: 10.3390/ijms19020548.

35. 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;30;15(1):200, doi: 10.3390/nu15010200.
36. Makowska K, Rytel L, Lech P, et al. Cocaine- and amphetamine-regulated transcript (CART) peptide in the enteric nervous system of the porcine esophagus. C R Biol. 2018;341(6):325-333, doi: 10.1016/j.crvi.2018.06.006.
37. Mishra A, Lal G. Neurokinin receptors and their implications in various autoimmune diseases. Curr Res Immunol. 2021;2:66-78, doi: 10.1016/j.crimmu.2021.06.001.
38. Myers JP, Antoniou MN, Blumberg B. et al. Concerns over use of glyphosate-based herbicides and risks associated with exposures: a consensus statement. Environ Health. 2016;15:19, doi: 10.1186/s12940-016-0117-0.
39. Palus K, Bulc M, Całka J. Changes in VIP-, SP- and CGRP- like immunoreactivity in intramural neurons within the pig stomach following supplementation with low and high doses of acrylamide. Neurotoxicology. 2018;69:47-59, doi: 10.1016/j.neuro.2018.09.002.
40. Palus K, Bulc M, Całka J. Effect of Acrylamide Supplementation on the CART-, VAChT-, and nNOS-Immunoreactive Nervous Structures in the Porcine Stomach. Animals (Basel). 2020; 10(4):555, doi: 10.3390/ani10040555.
41. Palus K, Bulc M, Całka J. Glyphosate affects the neurochemical phenotype of the intramural neurons in the duodenum in the pig. Neurogastroenterol Motil. 2023;35(3):e14507, doi: 10.1111/nmo.14507.
42. Rytel L, Palus K, Calka J. Co-expression of PACAP with VIP, SP and CGRP in the porcine nodose ganglion sensory neurons. Anat Histol Embryol. 2015;44:86–91, doi: 10.1111/ahe.12111.
43. Rytel L, Wojtkiewicz J, Snarska A, et al. Changes in the Neurochemical Characterization of Enteric Neurons in the Porcine Duodenum After Administration of Low-Dose Salmonella Enteritidis Lipopolysaccharides. J Mol Neurosci. 2021;71(8):1556-1566, doi: 10.1007/s12031-019-01473-y.
44. Spencer NJ, Hu H. Enteric nervous system: sensory transduction, neural circuits and gastrointestinal motility. Nat Rev Gastroenterol Hepatol. 2020;17(6):338-351, doi: 10.1038/s41575-020-0271-2.

45. Sundler, F.; Ekblad, E.; Håkanson, R. Occurrence and distribution of substance P- and CGRP-containing nerve fibers in gastric mucosa: Species differences. Adv. Exp. Med. Biol. 1991;298:29–37, doi: 10.1007/978-1-4899-0744-8_3.
46. Szymanska K, Gonkowski S. Bisphenol A-induced changes in the enteric nervous system of the porcine duodenum. Neurotoxicology. 2018;66:78-86, doi: 10.1016/j.neuro.2018.03.008.
47. Szymanska K, Gonkowski S. Neurochemical characterization of the enteric neurons within the porcine jejunum in physiological conditions and under the influence of bisphenol A (BPA). Neurogastroenterol Motil. 2019;31(6):e13580, doi: 10.1111/nmo.13580.
48. Szymanska K, Makowska K, Gonkowski S. The Impact of T-2 Toxin on Vasoactive Intestinal Polypeptide-Like Immunoreactive (VIP-LI) Nerve Structures in the Wall of the Porcine Stomach and Duodenum. Toxins (Basel). 2018;10(4):138, doi: 10.3390/toxins10040138.
49. Uesaka T, Young HM, Pachnis V, et al. Development of the intrinsic and extrinsic innervation of the gut. Dev Biol. 2016;417(2):158-67, doi: 10.1016/j.ydbio.2016.04.016.
50. Wierup N, Gunnarsdóttir A, Ekblad E, Sundler F (2007) Characterisation of CART-containing neurons and cells in the porcine pancreas, gastro-intestinal tract, adrenal and thyroid glands. BMC Neurosci 8:51, doi : 10.1186/1471-2202-8-51.
51. Ziegler A, Gonzalez L, Blikslager A. Large Animal Models: The Key to Translational Discovery in Digestive Disease Research. Cell Mol Gastroenterol Hepatol. 2016;2:716-724, doi: 10.1016/j.jcmgh.2016.09.003.

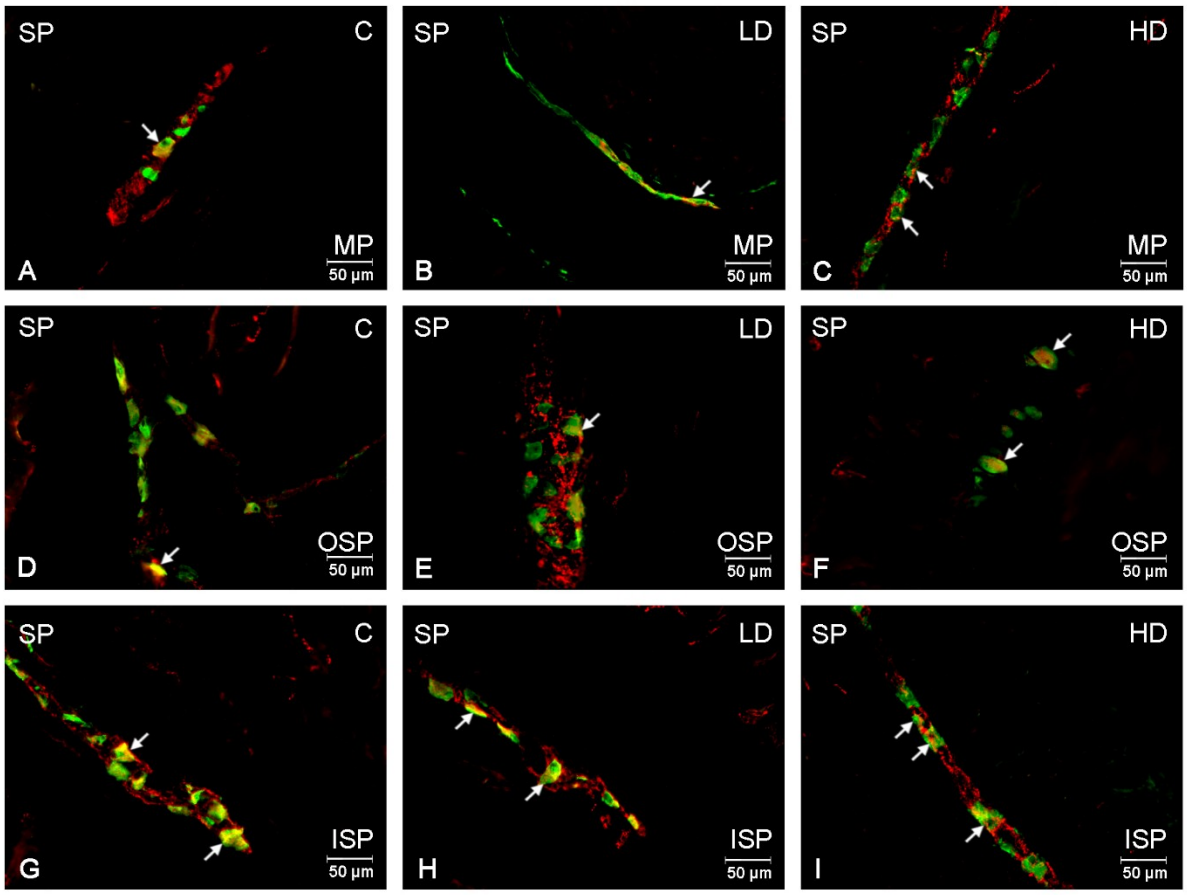

Figure 1. Microphotographs showing enteric neurons immunopositive to SP in the porcine descending colon; **A.** Neurons immunoreactive to HuC/D (panneuronal marker) and SP in the MP of control pigs; **B.** Neurons immunoreactive to HuC/D and SP in the MP of low dose group; **C.** Neurons immunoreactive to HuC/D and SP in the MP of high dose group; **D.** Neurons immunoreactive to HuC/D and SP in the OSP of control pigs; **E.** Neurons immunoreactive to HuC/D and SP in the OSP of low dose group; **F.** Neurons immunoreactive to HuC/D and SP in the OSP of high dose group; **G.** Neurons immunoreactive to HuC/D and SP in the ISP of control pigs; **H.** Neurons immunoreactive to HuC/D and SP in the ISP of low dose group; **I.** Neurons immunoreactive to HuC/D and SP in the ISP of high dose group; All pictures were created by digital superimposition of two colour channels (green for HuC/D and red for SP). MP – myenteric plexus; OSP outer submucosal plexus; ISP – inner submucosal plexus.

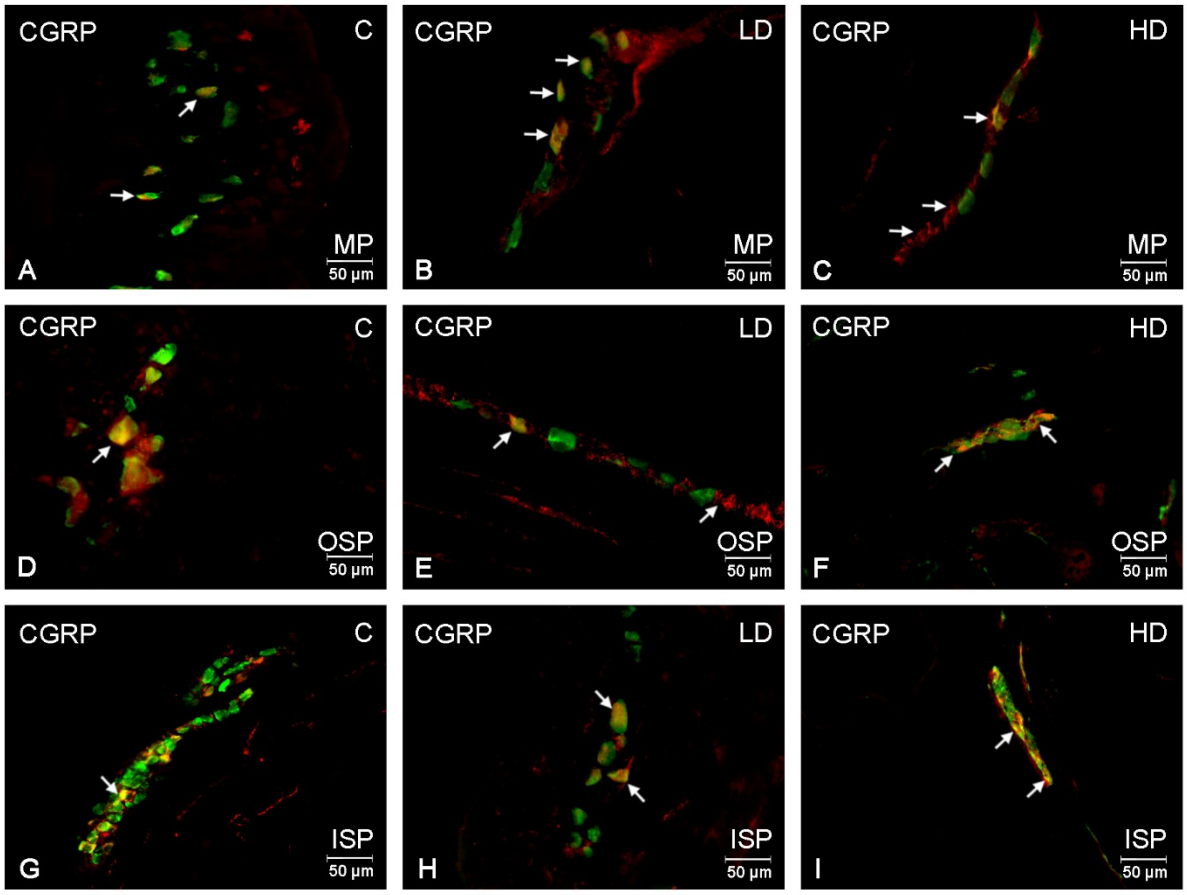

Figure 2. Microphotographs showing enteric neurons immunopositive to CGRP in the porcine descending colon; **A.** Neurons immunoreactive to HuC/D (panneuronal marker) and CGRP in the MP of control pigs; **B.** Neurons immunoreactive to HuC/D and CGRP in the MP of low dose group; **C.** Neurons immunoreactive to HuC/D and CGRP in the MP of high dose group; **D.** Neurons immunoreactive to HuC/D and CGRP in the OSP of control pigs; **E.** Neurons immunoreactive to HuC/D and CGRP in the OSP of low dose group; **F.** Neurons immunoreactive to HuC/D and CGRP in the OSP of high dose group; **G.** Neurons immunoreactive to HuC/D and CGRP in the ISP of control pigs; **H.** Neurons immunoreactive to HuC/D and SP in the ISP of low dose group; **I.** Neurons immunoreactive to HuC/D and CGRP in the ISP of high dose group; All pictures were created by digital superimposition of two colour channels (green for HuC/D and red for CGRP). MP – myenteric plexus; OSP outer submucosal plexus; ISP – inner submucosal plexus.

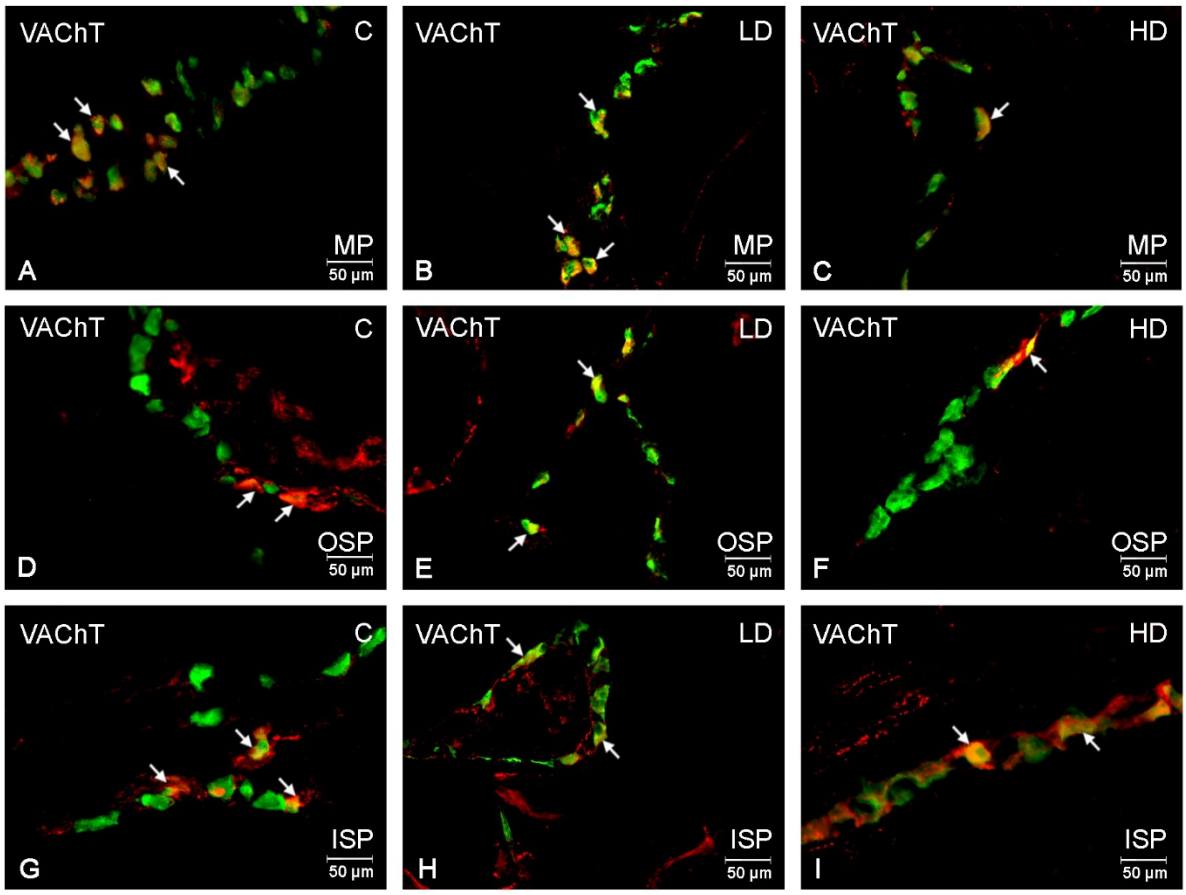

Figure 3. Microphotographs showing enteric neurons immunopositive to VAChT in the porcine descending colon; **A.** Neurons immunoreactive to HuC/D (panneuronal marker) and VAChT in the MP of control pigs; **B.** Neurons immunoreactive to HuC/D and VAChT in the MP of low dose group; **C.** Neurons immunoreactive to HuC/D and VAChT in the MP of high dose group; **D.** Neurons immunoreactive to HuC/D and VAChT in the OSP of control pigs; **E.** Neurons immunoreactive to HuC/D and VAChT in the OSP of low dose group; **F.** Neurons immunoreactive to HuC/D and VAChT in the OSP of high dose group; **G.** Neurons immunoreactive to HuC/D and VAChT in the ISP of control pigs; **H.** Neurons immunoreactive to HuC/D and VAChT in the ISP of low doses group; **I.** Neurons immunoreactive to HuC/D and VAChT in the ISP of high dose group. All pictures were created by digital superimposition of two colour channels (green for HuC/D and red for VAChT). MP – myenteric plexus; OSP outer submucosal plexus; ISP – inner submucosal plexus.

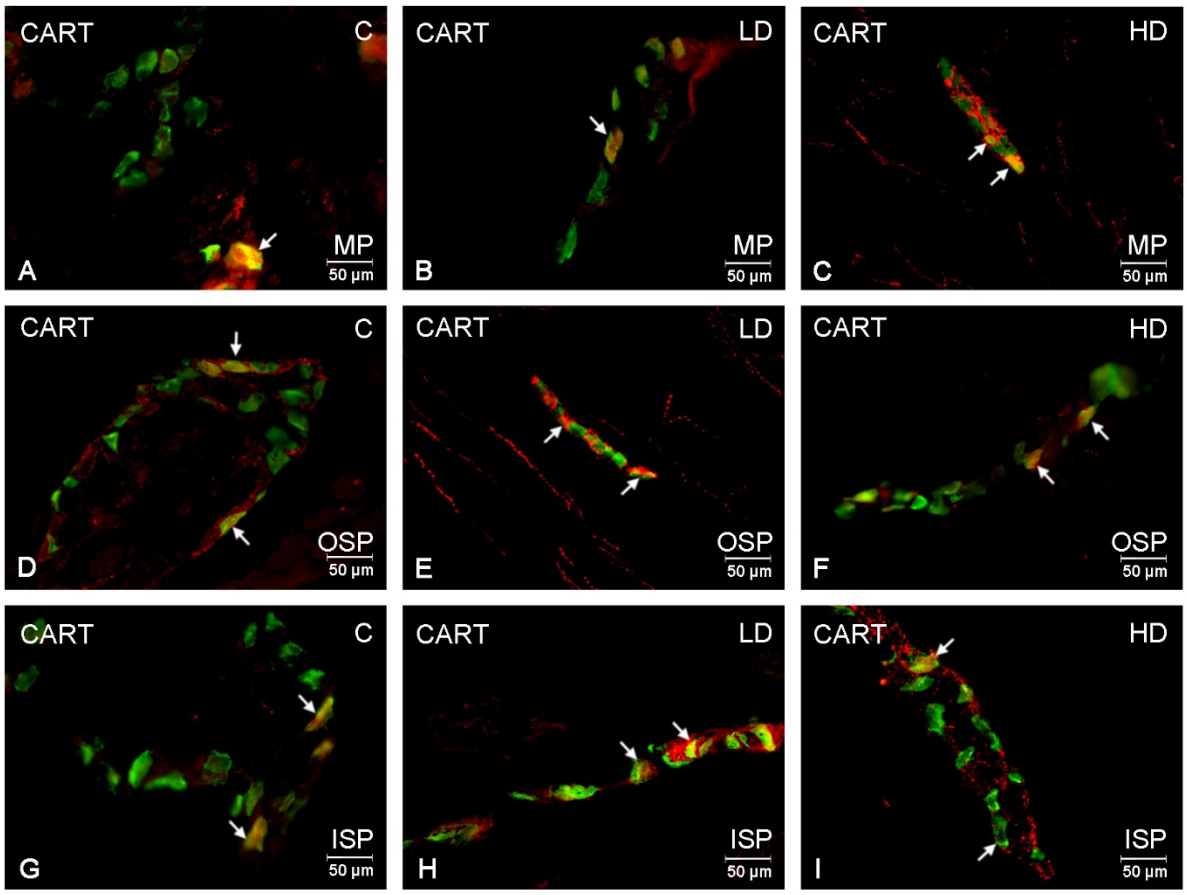

Figure 4. Microphotographs showing enteric neurons immunopositive to CART in the porcine descending colon; **A.** Neurons immunoreactive to HuC/D (panneuronal marker) and CART in the MP of control pigs; **B.** Neurons immunoreactive to HuC/D and CART in the MP of low doses group; **C.** Neurons immunoreactive to HuC/D and CART in the MP of high dose group; **D.** Neurons immunoreactive to HuC/D and CART in the OSP of control pigs; **E.** Neurons neurons immunoreactive to HuC/D and CART in the OSP of low doses group; **F.** Neurons neurons immunoreactive to HuC/D and CART in the OSP of high dose group; **G.** Neurons neurons immunoreactive to HuC/D and CART in the ISP of control pigs; **H.** Neurons neurons immunoreactive to HuC/D and CART in the ISP of low doses group; **I.** Neurons immunoreactive to HuC/D and CART in the ISP of high dose group; All pictures were created by digital superimposition of two colour channels (green for HuC/D and red for CART). MP – myenteric plexus; OSP outer submucosal plexus; ISP – inner submucosal plexus.

Table 1. Percentage of SP-, CGRP-, VAChT-, and CART- immunoreactive enteric neurons of the porcine descending colon in control, and experimental groups (administrated with low and high doses of glyphosate). SEM- Standard error of the mean (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Investigate Substance	**Control group**			**Low dose group**			**High dose group**		
	MP	**OSP**	**ISP**	**MP**	**OSP**	**ISP**	**MP**	**OSP**	**ISP**
SP	6.87	4.98	8.89	6.90	5.08	9.09	15.08	12.34	17.56
±SEM	0.33	0.25	0.77	0.99	0.88	0.80	1.03**	0.98**	1.88***
CGRP	15.08	19.87	21.31	19.97	22.90	24.97	28.53	28.90	32.04
±SEM	0.80	1.90	0.97	1.45**	1.62*	1.83*	2.99***	1.96**	2.74***
VAChT	20.92	22.00	23.06	21.09	21.98	18.53	16.36	17.09	15.77
±SEM	1.44	1.82	1.39	0.87	1.82	0.76**	1.80*	0.45**	0.70***
CART	8.99	3.89	2.98	8.55	3.08	3.87	14.94	3.40	3.44
±SEM	0.90	0,09	0.12	0.76	0.30	0.06	1.04**	0.81	0.63