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Michał Bulc1*, Jarosław Całka1, Łukasz Zielonka2, Michał Dąbrowski2, Katarzyna Palus1

1Department of Clinical Physiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego Str. 13, 10-719 Olsztyn, Poland
2Department of Veterinary Prevention and Feed Hygiene, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, 10-718 Olsztyn, Poland

Corresponding author: michal.bulc@uwm.edu.pl

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Effect of chemically-induced diabetes mellitus on phenotypic variability of the enteric neurons in the descending colon in the pig

Michał Bule¹*, Jarosław Całka¹, Łukasz Zielonka², Michał Dąbrowski², Katarzyna Palus¹

¹Department of Clinical Physiology, Faculty of Veterinary Medicine, University of Warmia and Mazury, Oczapowskiego Str. 13, 10-719 Olsztyn, Poland

²Department of Veterinary Prevention and Feed Hygiene, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, 10-718 Olsztyn, Poland

*Corresponding author: michal.bulc@uwm.edu.pl

Abstract

Gastrointestinal neuropathy in diabetes is one of numerous diseases resulting in abnormal functioning of the gastrointestinal tract (GIT), and it may affect any section of the GIT, including the descending colon. In the gastrointestinal system, the neurons are arranged in an interconnecting network defined as the enteric nervous system (ENS) which includes the myenteric plexus and the submucosal plexuses: inner and outer. Regular functioning of the ENS is determined by normal synthesis of the neurotransmitters and neuromodulators. This paper demonstrates the effect of hyperglycaemia on the number of enteric neurons which are immunoreactive to: neural isoform of nitric oxide synthase (nNOS), vasoactive intestinal

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peptide (VIP), galanin (GAL), calcitonin gene-related peptide (CGRP) and cocaine amphetamine-regulated transcript (CART) in the porcine descending colon. It was demonstrated that there was a statistically significant increase in the number of neurons within the myenteric plexus immunoreactive to all investigated substances. In the outer submucosal plexus, the CART-positive neurons were the only ones not to change, whereas no changes were recorded for nNOS or CART in the inner submucosal plexus. This study is the first study to discuss quantitative changes in the neurons immunoreactive to nNOS, VIP, GAL, CGRP and CART in the descending colon in diabetic pigs.

Key words: chemical encoding, neurotransmitters, diabetes, descending colon, pig

The mammalian gastrointestinal tract (GIT) is a functionally diversified system which is adapted for digestion and absorption (Furness, 2006; Greenwood-Van Meerveld et al., 2017). Its sections differ in their structure, depending on the type of food. To sustain its vital functions, the GIT is strictly controlled by the nervous system (Furness et al., 2014). The gastrointestinal tract is innervated by both the central nervous system (CNS) and by a unique network of intramural neurons which are arranged as the enteric nervous system (ENS), also called the enteric or second brain (Timmermans et al., 1992; Furness, 2006). The general structure of the ENS is very similar, encompassing the intramural plexuses interlinked with nerve fibres. The number and type of arrangement of the individual plexuses is determined by a given section of the GIT and the animal species Timmermans et al., (1992a), Furness (2012). For the latter, there are differences between rodents and large species, such as pigs (Gonkowski and Rytel, 2019). In the porcine descending colon, the ENS is organized into three separate and clearly visible
plexuses Makowska (2018). The myenteric plexus is situated between circular and longitudinal muscle layer, and it controls regular motility in this GIT section. There are two types of the submucosal plexuses: inner submucosal plexus (ISP) and outer submucosal plexus (OSP). The former is found under the internal side of the circular muscle layer, while the latter is situated near the lamina propria of the mucosal layer (Clarc and Furness 2004). These plexuses mainly control the section functions, resorption and blood flow in the GIT. While in rodents, the submucosal plexus is not divided into two parts (Furness 2006). Importantly, in humans, the ENS is arranged in the same way as in the pig, which makes the pig a very good animal model for the biomedical studies on animal models, especially in the studies investigating the pathologies in the gastrointestinal tract (Brehmer et al., 2010; Swindle et al., 1998).

The disturbances and diseases of the gastrointestinal tract are common both in animals and in humans and are called gastroenteropathies (Gatopoulou et al., 2012). They may be primary or complicate another disease (Demedets e al., 2012). Diabetes mellitus is an example of the latter, and in chronic diabetes with multiple hyperglycaemic crises, the peripheral nervous system becomes pathologically affected, including the enteric neurons and gastrointestinal complications (Chandrasekharan and Srinivasan 2007; Vinik et al., 2003). These complications may affect any parts of the GIT (Chandrasekharan and Srinivasan 2007). In the large intestine, specifically the descending colon, abnormal motility and pathological control of the sphincters may develop, which results in diarrhoea, constipation or faecal incontinence episodes; all of these symptoms significantly reduce the wellbeing and comfort of life in the patients (Gatopoulou et al., 2012). However, before severe gastrointestinal disturbances with clinical symptoms develop, the ENS neurons adapt to an unfavourable microenvironment, i.e. to an increased concentration of glucose in the blood and tissue fluids in diabetes. In neurophysiology, this phenomenon is called neural plasticity (Skobowiat and Całka 2011; Vasina et al., 2006) and, among others, it presents as an increase and/or a reduction of the
number of neurons that are immunoreactive to specific biologically active compounds (Bulc et al., 2017, 2018).

The number of substances called neurotransmitters or neuromodulators is very high, and new ones with such attributed functions are being discovered (Costa and Furness 1982; Keast et al., 1985). These substances are divided based on the inhibiting, stimulating, sensory or gaseous functions (Furness 2000). Vasoactive intestinal peptide (VIP) and galanin (GAL) are among the best-known neurotransmitters (Whittaker, 2005; Foxt-Threlkeld et al., 1991). Both peptides belong to the non-adrenergic non-cholinergic system. The VIP is an important chemical that relaxes the smooth muscles of the gastrointestinal tract, which results in delayed gastric emptying, vasodilatation and secretory inhibition in the GI (Nasser et al., 1995; Burleigh et al., 2005). GAL has both an inhibitory effect, which is a predominant one and it may increase contractility in some animal species, as in the canine ileum (Gonkowski et al., 2010). For an understanding of gastrointestinal pathophysiology, the strong neuroprotective effects of both VIP and GAL are significant which, in turn, makes these two substances vital for the neuronal survival under unfavourable conditions (Arciszewski and Ekblad 2005). Nitric oxide is the main player in gaseous neurotransmission. Due to its very short half-life, it is necessary to use an enzyme, i.e. nitric oxide synthase (NOS), to identify the neurons which use nitric oxide (Sanders et al., 1994; Shah et al., 2004). The neural isoform of NOS (nNOS) is a predominant type of this enzyme in the central and peripheral nervous system. Nitrergic neurotransmission in the gastrointestinal tract has an inhibitory effect on the smooth muscle and controls the blood circulation by changing the lumen of blood vessels but, importantly, nitric oxide may present a neuroprotective function (Schleiffer and Raul 1997). Pain signals due to excessive dilatation of the intestines or the stomach, irritation of the mucosa or disturbances in the blood circulation, are transferred to the central nervous system via the ascending pathways, while a key role is played by the calcitonin gene-related peptide (CGRP) (Nuki et al., 1993; Wolf et al., 2007).
This substance is used as a marker of the primary sensory neurons and is mainly involved in transmitting the nociceptive and sensory stimuli. However, further studies have demonstrated that CGRP may regulate some other processes in the GI, such as food absorption, blood flow regulation in the mesentery (specifically) and gastric acid secretion (Lambrecht et al., 1993; Wolf et al., 2007; Ohna et al., 2008; Bulc et al., 2018). The cocaine amphetamine-regulated transcript (CART) is a relatively late discovery and the substance which is also expressed in the gastrointestinal tract (Ekblad 2006). The function of this peptide in the GI is poorly explained. It is known that it may indirectly control the GI motility, thereby impacting nitrergic neurotransmission (Ekblad et al., 2003). Furthermore, its quantity changes in many gastrointestinal diseases, especially those with inflammatory and neurodegenerative background (Gonkowski et al., 2009; Bulc et al., 2015; Makowska et al., 2017).

The objective of the paper was to investigate the quantitative variability of the neurons in the enteric nervous system of the porcine descending colon in response to streptozotocin-induced hyperglycaemia. The double immunohistochemical staining method was applied, with the anti-VIP, -GAL, -nNOS, -CGRP and -CART antibodies. In the available literature, there are few studies with the pig as an diabetic experimental animal model (Juranek et al., 2010). The anatomy, histology and physiological processes (especially in the gastrointestinal tract) and the metabolic rate in the pig are far more like those in humans than in rodents which are commonly used in metabolic research (Swindle and Smith 1998; Swindle, 2012). Furthermore, with no direct life-threatening effect, the pathological mechanism of gastrointestinal complications in diabetes has not been so widely investigated as, for instance, diabetic retinopathy or nephropathy. Therefore, an understanding of the role in this process played by the enteric neurons and their synthesized neurotransmitters may be an important stage in understanding the pathophysiological background of diabetic gastroenteropathy.
Material and methods

Animals and diabetes induction

A total number of 10 juvenile pigs of White Large Polish breed were used in this study. The experiments had been approved by Local Ethical Committee in Olsztyn (Poland) (decision number 13/2015/DTN) and according to the Act for the Protection of Animals for Scientific or Educational Purposes of 15 January 2015 (Official Gazette 2015, No. 266), applicable in the Republic of Poland with special attention paid to minimizing any stress reaction. After week acclimatisation period pigs were randomly divided into two groups (control and experimental with chemically induced diabetes 5 animals in each). After adaptative time diabetes was induced by a single intravenous injection of streptozotocin under premedication induced by atropine (0.05 mg/kg body weight /BW/, given intramuscularly; Atropinum sulf. WZF, Warszawskie Zakłady Farmaceutyczne Polfa S.A., Poland), azaperone (2 mg/kg BW, given intramuscularly Stresnil, Janssen Pharmaceutica, Beerse, Belgium) (STZ, (Sigma–Aldrich, St. Louis, MO, USA, 0130; 150 mg/kg). Directly before injections STZ was dissolve disodium citrate buffer solution (pH = 4.23, 1 g streptozotocin/10 ml solution). The needle was inserted into ear venous and STZ infusion time was about 5 minutes. In order to eliminate nausea and vomiting after streptozotocin infusion, pigs were fasted for 18 h before the experiment and the control animals were injected with equal amounts of vehicle (citrate buffer). After diabetes induction animals were kept in cages adopted to pigs. Animals with both groups receive standard diet (rapeseed meal 6.0%, soybean meal 9.0%, wheat 54.0%, barley 28.5%, others 2.5%) and water ad libitum. Blood glucose level was measure before STZ injection, 48 h after induction of diabetes. Next measurements were made in each week of the experiment. Blood glucose concentrations were measured in plasma using an Aceent-200 (Cormay) biochemical analyser, with the colorimetric measurement at a wavelength of 510 nm/670 nm.

Tissue collection and immunofluorescent procedure
Six weeks after diabetes induction animals in both groups were deeply anesthetized via intravenous administration of pentobarbital 60 mg/kg m.c. (Vetbutal, Biowet, Poland). Afterwards pigs were perfused, and gastrointestinal tract were prepared as previously described (Bulc et al., 2015). Next 2-cm-long fragments of the descending colon from the place where nerves from inferior mesenteric ganglia supply the intestine were collected. The samples were postfixed by immersion by the same fixative for 1 h, rinsed several times with PB and finally transferred to 30 % sucrose solution and stored at 4 °C until sectioning. The tissue blocks were cut in frontal or sagittal planes using a Microm HM 560 cryostat (Carl Zeiss, Germany) at a thickness of 12 μm and mounted on gelatinized glass. Subsequently, sections were double stained by first incubating with primary antisera overnight. Following antibodies were used marker mouse Hu C/D proteins (1:1000; Invitrogen USA; code A-21271), rabbit anti CART antibodies (1:12,000; Phoenix Pharmaceuticals, Burlingame, CA, USA, code H 00), rabbit anti nNOS antibodies (1:4000: Chemicon, Billerica, MA, USA, code. AB 5380), rabbit anti VIP (1: 6000; Biomol, Hamburg, Germany, code VA1285), rabbit anti GAL (1:1000; Millipore, Billerica, MA, USA, code AB 2233) and rat anti CGRP (1: 4000; Millipore, code MAB317). After overnight incubation with BSA, the sections were incubated with secondary antibodies (donkey anti mouse Alexa Fluor 488, 1:1000 Invitrogen USA; code A21202, and donkey anti-rabbit Alexa Fluor 546 1:1000 Invitrogen, USA; code A10040). The slides were viewed and photographed using an Olympus BX51 microscope equipped with epifluorescence and appropriate filter sets, coupled with a digital monochromatic camera (Olympus XM 10) connected to a PC and analysed with Cell Dimension software (Olympus, Tokyo, Japan).

Standard controls, i.e. pre-absorption for the neuropeptide antisera (20 μg of appropriate antigen per 1 mL of corresponding antibody at working dilution (VIP 064-24, Phoenix Pharmaceutical), GAL 026-06, Phoenix Pharmaceutical, nNOS N3033, Sigma, St Louis, MO, USA, protein CART C5977 Sigma–Aldrich, St and CGRP ab158017, Abcam). Also, omission and
replacement of the respective primary antiserum with the corresponding non-immune serum, completely abolished immunofluorescence and eliminated specific staining.

**Counting of the nerve structures and statistical evaluation**

To evaluate the percentage of exanimated neurons, at least 700 Hu C/D labelled cell bodies in a particular plexus (MP, OSP, and ISP) of each studied animal were examined. Only neurons with well-visible nucleus were counted. To prevent double counting of Hu C/D immunoreactive neurons, the sections were located at least 100 μm apart. The data pooled from all animal groups were statistically analysed using Statistica 13 software (StatSoft Inc., Tulsa, OK, USA) and expressed as a mean ± standard error (SEM) of mean. Significant differences were evaluated using Student’s t-test for independent samples (*P < 0.05, **P < 0.01, and ***P < 0.001).

**Results**

All STZ pigs developed diabetes within 7 days showing blood glucose concentration over 20 mmol/L. The mean glucose concentration in diabetic group during time of experiment was 20.57 mmol/L ± 0.94 while in control group was at physiological level 5.07 mmol/L ± 0.12. Exact level of glucose concentration are presented in table 1. It should be stressed that although chronic hyperglycaemia in diabetic animals was remarkably higher than in controls, all pigs which received STZ survived the duration of the experiment in a good general condition and none of the animals required exogenous insulin supplementation.

During present investigation all exanimated substances occurred in both submucosal and myenteric plexuses in descending colon (Tab 2, 3; Fig 1A-S). Neurons immunoreactive to nNOS (nNOS-IR) constituted the most abundant population among all substances studied. In control animals in MP their number constitute 30.10 ± 0.95% (Fig 1A-C). Higher glucose level in experimental group led to increase of nNOS-IR neurons inside MP (to 39.22 ± 1.04%) (Fig. 1 D-F). In OSP in control animals the number of nNOS-IR neurons was estimated at 33.45 ±
1.44% (Fig 1. G-I), while in diabetes group we have observed an increase level of nNOS-IR cell bodies (41.36 ± 0.65%) (Fig 1 J-L). Meanwhile in ISP we have noted statistically significant changes in experimental group comparing to control ones (42.36 ± 0.78% vs 43.27 ± 0.92%) (Fig 2 M-O, Fig 1 P-S).

Another investigated substance was VIP (Tabl. 3). In the MP in control animals neurons immunoreactive to VIP (VIP-IR) constituted 4.78 ± 0.12% (Fig 2 A-C) and increased in experimental animals to 9.65 ± 0.24% (Fig 2. D-F). In OSP the number of VIP-IR neurons was similar to MP and in control constituted (5.14 ± 0.39% (Fig 2. G-I) and also increased in experimental animals (7.44 ± 0.42%) (Fig 2 J-L). In turn, in ISP the number of VIP-IR neurons was high in control (8.36 ± 0.74%) (Fig 2 M-O) and elevated in diabetes animals (13.11 ± 0.56%) (Fig 2 P-S).

In turn, GAL-IR neurons in MP of control animals were estimated at 3.27 ± 0.11% (Fig 3 A-C) and increased significantly in experimental group (to 14.28 ± 0.74%) (Fig. 3 D-F). In OSP the number of GAL-IR neurons was relatively small (1.56 ± 0.17%) (Fig 3 G-I) but in diabetes pigs was substantially increased (11.89 ± 0.56%) (Fig 3 J-L). In ISP, comparing with MP and OSP, number of GAL-IR neurons was quite high in control (10.90 ± 0.41%) (Fig. 3 M-O) and also in experimental pigs (31.89 ± 1.23%) (Fig. 3 P-S).

CGRP immunoreactive neurons (CGRP-IR) in control animals inside MP were estimated at 16.20 ± 1.06% (Fig 4 A-C), while in hyperglycaemic animals their number was statistically higher (20.98 ± 1.36%) (Fig 4 D-F). Also, in submucosal plexuses we have noted increased number of CGRP-IR neurons. In OSP they constituted 18.97 ± 0.71% in control animals (Fig 4 G-I) and 28.64 ± 0.98 % in experimental group (Fig 4 J-L). In turn, in ISP we have noted 22.41 ± 0.56% of CGRP-IR neurons in control animals (Fig 4 M-O) and 30.87 ± 0.67% in experimental group (Fig 4 P-S).
The last investigated substances was CART (Tabl 3). In control group CART-immunoreactive (CART-IR) neurons in MP constituted 9.41 ± 0.49% (Fig 5A-C) and increased in experimental pig to 18.47 ± 0.45% (Fig 5D-F). Moreover, in both submucosal plexuses we have noted statistically significant changes in the number of CART-IR neurons (in OSP: 2.23 ± 0.19% in control (Fig 5 G-I) vs 2.17 ± 0.56% in experimental group (Fig 5 J-L) and in ISP: 2.01 ± 0.17% in control (Fig 5 M-O) vs 3.77 ± 0.14% in diabetic animals (Fig 5 P-S).

**Discussion**

These studies have for the first time demonstrated the quantitative changes in the ENS neurons of the descending colon in response to a pathological stimulus, i.e. hyperglycaemia. The experiment we performed lasted 6 weeks. Pigs received a single dose of streptozotocin (150 mg / kg). This dose is complies with previous experience using the pig model (Grusner et al., 1993; Juranek et al., 2010). The applied dose caused the occurrence of persistent hyperglycaemia at the mean level of 20 mmol / L. The glucose level indicates that we can classify the developed diabetes as mild type of diabetes. It should be emphasized that both duration of the experiment and the obtained glycaemic levels were sufficient to induce changes in the neurons of the enteric nervous system. It should be underline that in the cause of enteric neurons the time of influence of pathological factor is shorter than in the case of peripheral nevus system (Gonkowski et al., 2009, 2010, 2019). First of all enteric neurons create small population lying into gastrointestinal wall. this causes that they are constantly exposed to the pathological factor like high glucose level.

Until now, studies (including those on the gastrointestinal tract) have been conducted with rodents with chemically induced diabetes (Belai et al., 1996; Rees and Alcolado 2005). In their studies, the authors used the pig as an experimental model, i.e. a species which is more widely accepted as a good animal model in biomedical studies (Swindle, 2012; Grüssner et al., 2012). The discussed experiment is obviously a preliminary study, and the application of labelling the
neurons with the specific antibodies presents a good tool for the visualization of the individual neurons in the respective ENS plexuses. Therefore, it is possible to determine in which section of the ENS the number of neurons immunoreactive to the specific substances increases or decreases in a quite precise manner.

This paper focuses on an investigation of the expression of the neurotransmitters classified as the non-adrenergic non-cholinergic system. First, the numeric variability of NOS-IR neurons was studied. Gaseous neurotransmission plays an important role in the regular functioning of the GI (Schleiffer and Raul 1997). Until now, the presence of this neurotransmitter has been identified in the gastrointestinal tract of a few species, including humans. It is emphasized that there are differences in the amount and distribution of nitric oxide between the individual GI sections as well as between the individual animal species (Shah et al., 2004; Szymańska et al., 2018). In the rat, for example, the highest number of NOS-positive neurons is found in the ileum whereas in the guinea pigs, it is in the descending colon (Schleiffer and Raul 1997; Young et al., 1997). In the discussed study, the number of NOS-IR neurons was relatively high in the enteric plexuses of the descending colon, which is consistent with previous studies carried out with the porcine gastrointestinal tract and gives evidence of an important role of this gas in the GIT (Szymańska et al., 2018). An inhibitory effect of NOS on the contractility and a relaxing effect both on the gastrointestinal smooth muscles and the blood vessel muscles are a relatively well-elucidated function of NOS in the GIT (Barbies et al., 1994). The above-mentioned effect was recorded in different sections of the gastrointestinal tract. In the presented studies, the authors reported an increase in the number of NOS-IR neurons in the MP plexus and OSP, while no changes were demonstrated in the ISP. To date, there has not been any data on the variability of nitrergic innervation in this section of the GIT in diabetes. There have been a few reports mainly on the quantitative changes in the NOS-IR neurons in the stomach and the small intestines (Belai and Burnstock 1999; Bulc et al., 2019).
Previous research carried out by the authors with the porcine diabetes model have also confirmed the involvement of the NOS neurons in gastric small intestinal disturbances in response to hyperglycaemia (Bulc et al., 2019). The abnormalities in the gastrointestinal functions are one of the more common complications of diabetes. They may affect any GI section, including the descending colon (Demeds et al., 2013; Yarandi and Srinivasan 2014). The above-mentioned studies show that nitric oxide may be partially involved in the pathogenesis of these abnormalities via an increase in the number of neurons which produce this gas, and, particularly in the MP and ISP, the gastrointestinal motility may be reduced, which may clinically result in retention of the intestinal content and constipation episodes.

The inhibitory effect in the GI tract is linked not only to nitric oxide since VIP and GAL may present similar properties, i.e. the peptides which were investigated in the gastrointestinal tract in the presented study. Both peptides have been already found and described in the GIT in many animal species as well as in humans (Gonkowski et al 2010; Makowska, 2018; Palus et al., 2019; Whittaker, 1989). While the physiological function of these peptides is well explained and it is known that they exert an inhibitory effect similar to the inhibition expressed by NO and galanin also modulates the secretion of VIP and NO (Brehmer et al., 2006), the role of these substances in pathological processes in the GIT is much less understood. Numerous studies have already confirmed that these compounds are involved in such diseases as dysentery, inflammatory bowel conditions and bisphenol-A and acrylamide intoxication (Kaleczyc et al., 2007; Palus et al., 2018a; Szymanska et al., 2018). In diabetes, studies on the altered expression of these substances have been conducted on rats (Belai and Burnstock 1990; Belai et al., 1985). The analysis of these papers reveals that for VIP in the early state of hyperglycaemia, the population of VIP neurons becomes lower and increases later in the course of the disease (Belai et al., 1985). This effect has not been reported for galanin, and its quantity was increased without an initial reduction (Chandrasekharan and Srinivasan 2007). Within the scope of
research conducted with pigs, this paper has, for the first time, demonstrated an increase in both VIP- and GAL-immunoreactivity in the enteric neurons of the descending colon. The increase of number of VIP-IR and GAL-IR cell bodies was recorded in all plexuses on the investigated tissue. For GAL, this increase was significant in the ISP. Undoubtedly, these findings give evidence to the important role of these peptides in diabetic hyperglycaemia. However, their exact role in diabetes is still not fully understood. Both compounds have been proven to have neuroprotective and anti-inflammatory properties (Arciszewski and Ekblad 2005). In the first case, their increase may protect the neurons against apoptosis induced by hyperglycaemia. It has been known for a long time that diabetes, especially with frequent and long-lasting hyperglycaemic episodes, is accompanied by inflammation that results from the activation of nuclear factor-kappa, for instance (Vincent et al., 2004). This leads to potentiated activation of macrophages and the production of numerous proinflammatory cytokines. VIP and GAL can inhibit macrophage infiltration and additionally stimulate the glial cells to produce the anti-inflammatory cytokines (Botella et al., 1992; Brenneman et al., 1995, 2003; Vasina et al., 2006). Therefore, the increased number of the neurons immunoreactive to these substances may indicate an attempt to limit the consequences of hyperglycaemia, including inflammation.

Sensory transmission, such as nociceptive information, plays an important role in the brain receiving alerts of noxious processes in the intestines (Philips and Powley 2006). CGRP is one of the major substances involved in this process. Until now, the presence of CGRP has been confirmed in different animal species and in different sections of the gastrointestinal tract (Timmermans et al., 1992b). Obviously, in both cases there are evident differences between the species, and the expression level also depends on the specific GIT section. Furthermore, the amount of CGRP varies in different pathological conditions (Makowska and Gonkowski 2018, 2019). There are scarce data on the number of CGRP-positive neurons in the descending colon in the diseases affecting this GI section. In diabetes, the quantity of CGRP has only been
investigated in the ileum (Chandrasekharan and Srinivasan 2007). Previous studies conducted by the authors have demonstrated that the expression of CGRP-positive neurons also increases in the stomach during diabetes, as well as in the colon (Feher et al., 2006). What remains crucial, however, are the consequences of the increased number of CGRP neurons and what function they may have, particularly in the descending colon. In the discussed studies, the authors reported a substantial increase in CGRP expression in the submucosal plexuses, whereas it is also known that this peptide controls blood flow in the mucosa and water resorption (Barada et al., 2000; Ohno et al., 2008), which may disrupt water and electrolyte absorption and result in diarrhoea. The control of blood flow is important for inflammation and oedema formation but, contrarily, it may positively impact on the regenerative processes in the mucosa, which has been reported for gastrointestinal ulcers, in which CGRP had a positive effect on mucosal healing (Li et al., 2013). The potentiated expression of CGRP in the myenteric plexus neurons may significantly impact the motility control in this GI section, which may result in diarrhoea, when combined with the effect on resorption. Moreover, it has been reported that CGRP is involved in diarrhoea development in rodents (Evangelista et al., 2003). In addition, diarrhoeas with non-bacterial aetiology have been discussed as one of the numerous gastrointestinal complications in diabetes (Evangelista et al., 2003; Kaiser et al., 2017).

CART is the last of the investigated substances; this peptide has been quite recently discovered. Initially, it was believed to be involved in the hypothalamic regulation of food intake (Jaworski and Jones 2006). Later studies demonstrated its presence in different sections of GIT in different mammalian species (Ekblad, 2006; Gonkowski et al., 2009; Palus et al., 2018b). While the wide distribution of this substance in different GIT sections has been investigated, the role of CART in the physiological and pathological processes is poorly understood. The conducted studies indicate that CART is mainly involved in controlling the motility of the gastrointestinal tract (Ekblad et al., 2003; Ellis and Mawe, 2003). This may be
partially supported by the current study because CART was predominant in the myenteric plexus neurons which regulate GI motility. It was also demonstrated that CART may have an impact on the GI functions by controlling the release of other neurotransmitters, mainly NO (Ekblad et al., 2003).

**Conclusion**

This paper analysed the phenotypic profile of the enteric neurons in the porcine descending colon in response to a long-term increase of glucose in the blood. The results of an immunofluorescent analysis demonstrated that the investigated substances found in the enteric nervous system are involved in adaptive and neurodegenerative processes in diabetes. The variety of functions of these compounds indicates their multipronged role in diabetes. Obviously, these findings (as they represent fundamental research) unequivocally demonstrate their role in the pathophysiology of diabetic gastroenteropathy. Further in-depth research is warranted to understand the exact functions which these substances may have on the molecular level in diabetes.

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Figure 1. serum glucose concentration before induction of diabetes and glucose accumulation after streptozotocin injection (up 1 week to 6 weeks)
Table 2. Functions of the all tested neurotransmitters and their expression changed in particular plexuses in descending colon in pig

| Investigated substances | neural isoform of nitric oxide synthase (nNOS) | vasoactive intestinal peptide (VIP) | galanin (GAL) | calcitonin gene-related peptide (CGRP) | cocaine amphetamine-regulated transcript (CART) |
|-------------------------|-----------------------------------------------|-----------------------------------|--------------|---------------------------------------|-----------------------------------------------|
| Function in gastrointestinal tract | inhibitory effect on the smooth muscle, controls the blood circulation by changing the lumen of blood vessels, neuroprotective function | relaxes the smooth muscles of the gastrointestinal tract, delayed gastric emptying, contraction of blood vessels, neuroprotective effects | inhibitory effect on the smooth muscle, neuroprotective effects | marker of the primary sensory neurons, involved in transmitting the nociceptive and sensory stimuli, regulation of food absorption and acid secretion | control the gastrointestinal motility |
| Changed in expression in myenteric plexus |  |  |  |  |  |
| Changed in expression in inner submucosal plexus |  |  |  |  |  |
Table 3. nNOS, VIP, GAL, CGRP and CART immunoreactive cell bodies in the porcine descending colon under physiological conditions (Control Group) and during experimentally induced diabetes (Experimental Group)

| Descending colon | Control Group | Experimental Group |
|------------------|---------------|---------------------|
|                  | nNOS          | nNOS                |
| Myenteric plexus | 30.10 ± 0.95% | 39.22 ± 1.04%       |
| Outer submucosal plexus | 33.45 ± 1.44%  | 41.36 ± 0.65%       |
| Inner submucosal plexus | 42.36 ± 0.78%  | 43.27 ± 0.92%       |
| VIP              | 4.78 ± 0.12%  | 9.65 ± 0.24%        |
|          | GAL     | GAL     |
|----------|---------|---------|
| Outer    | 5.14 ± 0.39% | 7.44 ± 0.42% |
| submucosal | *       |         |
| Inner    | 8.36 ± 0.74% | 31.89 ± 1.23% |
| submucosal | ***     |         |
|          | GAL     | GAL     |
| Myenteric| 3.27 ± 0.11% | 14.28 ± 0.74% |
| plexus   | ***     |         |
| Outer    | 1.56 ± 0.17% | 11.89 ± 0.56% |
| submucosal | ***     |         |
| Inner    | 10.90 ± 0.41% | 31.89 ± 1.23% |
| submucosal | ***     |         |
|          | CGRP    | CGRP    |
| Myenteric| 16.20 ± 1.06% | 20.98 ± 1.36% |
| plexus   | **      |         |
| Outer    | 18.97 ± 0.71% | 28.64 ± 0.98% |
| submucosal | ***     |         |
| Inner    | 22.41 ± 0.56% | 30.87 ± 0.67% |
| submucosal | **      |         |
|          | CART    | CART    |
|          |         |         |
|                | Value 1          | Value 2          |
|----------------|------------------|------------------|
| Myenteric plexus | 9.41 ± 0.49%     | 18.47 ± 0.45%    |
| Outer submucosal plexus | 2.23 ± 0.19%     | 2.17 ± 0.56%    |
| Inner submucosal plexus | 2.01 ± 0.17%     | 3.77 ± 0.14%    |

Statistically significant data (*p>0.05, **p>0.01, ***p>0.001)

Red font in experimental group indicates a statistically significant increase of investigated substances.
Figure 1. Immunofluorescent microphotographs showing nNOS immunoreactive perikarya in descending colon in the myenteric plexus of the control (A-C) and in experimental group (D-F); outer submucosal plexus of the control (G-I) and in experimental group (J-L); inner submucosal plexus of the control (M-O) and in the experimental group (P-S). Photographs in the right column have been created by digital superimposition of two-color channels; Hu C/D (green) and nNOS positive (red) The arrows indicated studied cells bodies
Figure 2. Immunofluorescent microphotographs showing VIP immunoreactive perikarya in descending colon in the myenteric plexus of the control (A-C) and in experimental group (D-F); outer submucosal plexus of the control (G-I) and in experimental group (J-L); inner submucosal plexus of the control (M-O) and in the experimental group (P-S). Photographs in the right column have been created by digital superimposition of two-color channels; Hu C/D (green) and VIP positive (red) The arrows indicated studied cells bodies
Figure 3. Immunofluorescent microphotographs showing GAL immunoreactive perikarya in descending colon in the myenteric plexus of the control (A-C) and in experimental group (D-F); outer submucosal plexus of the control (G-I) and in experimental group (J-L); inner submucosal plexus of the control (M-O) and in the experimental group (P-S). Photographs in the right column have been created by digital superimposition of two-color channels; Hu C/D (green) and GAL positive (red) The arrows indicated studied cells bodies
Figure 4. Immunofluorescent microphotographs showing CGRP immunoreactive perikarya in descending colon in the myenteric plexus of the control (A-C) and in experimental group (D-F); outer submucosal plexus of the control (G-I) and in experimental group (J-L); inner submucosal plexus of the control (M-O) and in the experimental group (P-S). Photographs in the right column have been created by digital superimposition of two-color channels; Hu C/D (green) and CGRP positive (red) The arrows indicated studied cells bodies
Figure 5. Immunofluorescent microphotographs showing CART immunoreactive perikarya in descending colon in the myenteric plexus of the control (A-C) and in experimental group (D-F); outer submucosal plexus of the control (G-I) and in experimental group (J-L); inner submucosal plexus of the control (M-O) and in the experimental group (P-S). Photographs in the right column have been created by digital superimposition of two-color channels; Hu C/D (green) and CART positive (red) The arrows indicated studied cells bodies