Expression of serotonin, somatostatin, and glucagon-like peptide 1 (GLP1) in the intestinal neuroendocrine cells of pigs fed with population rye type and hybrid rye type grains¹⁾

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Summary

Neuroendocrine cells (NEC) are a cell population in the gastrointestinal tract that plays a role in the regulation of the digestion process, satiety and nutrient homeostasis. NE cells express a variety of bioactive hormones that can undergo changes in response to different luminal stimuli, including multiple components, which are present in the diet. In recent years, a modern (hybrid) type of rye grain has been introduced to feed industry. The goal of the present study was to determine immunohistochemically whether the feeding of the pigs with population and hybrid rye grains may evoke adverse changes in the small and large intestines in terms of the expression of serotonin, glucagon-like peptide 1 (GLP1) and somatostatin. Feeding animals with population and hybrid rye grains resulted in a slight increase in serotonin-positive NE cells in the small intestine (but not in the large intestine). After feeding animals with population rye (but not with hybrid rye) grains, there was a decrease in the small intestine GLP1-immunoreactive NE cells was found. No changes in the expression of GLP1 were found in the large intestine of experimental animals. The numbers of somatostatin-IR NEC in the small and large intestines were not affected by feeding with either population or hybrid rye grains. In conclusion, we found that feeding pigs with hybrid and population rye grains started adaptive changes in NEC. However, those changes were not profound, which allows us to speculate that adverse effects of these rye grains have a minor (if any) impact on the gut hormone balance (and indirectly on the health status) of animals.

Keywords: rye, grain, feeding, farm animals, neuroendocrine cells, gastrointestinal tract

Rye is a one of the best cereal crops; it is easy to grow and not very expensive. However, the use of rye

grain in the feeding of livestock (including pigs) is still rather limited due to its lower energy value (when compared with that of other grains) and the presence of so-called “anti-nutritive” substances. In a series of experiments, it was found that population rye grains

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are relatively rich in such anti-nutritional and toxic compounds as ergot alkaloids (33), pentosans (35), alkilorezorcynols (6), phytates, and acid phosphatase (34). In recent years, a new variety of rye (with lower susceptibility to ergot) called hybrid rye grown and developed. Despite the presence of potentially harmful ingredients, both population and hybrid rye grains have been successfully used as a diet component in the feeding of meat calves and lactating dairy cows (28, 30). However, the precise mechanism by which population rye and hybrid rye affect the function and structure of the gastrointestinal tract (GIT) is largely unknown. One hypothesis is that the digested components of various types of rye grains may influence the function of neuroendocrine cells (NEC).

NEC have been recognized as sensory cells with neural determination scattered in the mucosa throughout a distinct part of GIT. These cells are highly organized, capable of responding to food components by producing and secreting gut hormones and neurotransmitters, and form a kind of disseminated system under the central influence. Several types of NEC with different chemical codes have been found in the mammalian intestines. Enterochromaffin cells are the most common in the GIT type of NEC, and their major bioactive product is serotonin (5-hydroxytryptamine; 5-HT) (3). Other subsets of NEC present in the mammalian small and large intestines are D cells (producing mainly somatostatin) and L cells (producing mostly GLP1, but also GLP2 and peptide YY) (15, 19). In a series of functional experiments, it has been established that different types of NEC may modulate certain activities of the gut, including peristalsis, secretion, pain transduction, absorption of nutrients, or even immune response (13, 17, 22, 37). Interestingly, recent studies indicate that gut microbiota can mediate the activity of the gut-brain axis through changes in the production of bioactive substances by subsets of NEC (8), which explains why some authors postulate that gut microbiota should be regarded as a virtual endocrine organ. Besides microbiota, other factors (including changes in diet) are also known to be capable of changing the distribution, proportions, and products of NEC (11). It is not clear whether such activation and upregulation of NEC is beneficial for the host, especially as one of consequences may be hormone overexpression and development of tumors arising from NEC (10).

To at least partially answer the question of how dietary components present in rye grains interact with the intestinal epithelial barrier of fattened animals, we decided to immunohistochemically assess whether feeding pigs with rye grains may change the numbers and distribution of serotonin-, somatostatin- and GLP1-expressing NEC in the small and large intestines. Additionally, were analyzed any differences in the composition of NEC in animals fed with population and hybrid rye grains.

Material and methods

Animals, experimental design, tissue sampling. The animal procedures for the study were in accordance with Polish law. The study was performed at the experimental station of The National Research Institute of Animal Production in Chorzów (Poland). In total, twenty Polish Landrace pigs of both sexes weighing from 30 ± 1.0 to 100 ± 9.5 kg were used in this study. All animals were earmarked by ear tags. The pigs were randomly divided into four groups (two control and two experimental groups, $n = 5$ in each group) and housed in individual balance cages for controlled fattening to determine digestibility of various feeds with different amounts of cereals. Pigs from each experimental group were kept with the corresponding control pigs, since experiments were carried out during two different season of the year. The animals were weighed before the testing. The diet of pigs in the control groups (C1 and C2) contained barley and wheat (50%/50%). The diet of the first experimental group (Dankowskie Rubin population rye group; PR) consisted of wheat (20%), barley (20%), and population rye (60%). The diet of the second experimental group (hybrid rye group; HR) comprised wheat (20%), barley (20%), and hybrid rye (60%). All cereal components of the control and experimental diets were purchased from KWS Lochów Polska Sp. z o. o. (www.kws-zboza.pl) and included a multi enzyme preparation for pig's rations (containing betaglucanase and xylanase Enzym G2G; BAS-POL, Poland; 20 000.0 mg/kg). All animals, weighing initially 30 kg, were fed with their respective diets from until they reached 100 kg of body weight. At the end of the experiment, the animals were killed in a local slaughterhouse, and samples of the small intestine (mid jejunum) and large intestine (colon) were dissected out. The dissected material was further cut into 1cm long segments. Each segment was opened along the mesenteric border, stretched and pinned on a piece of balsa wood. The material was immediately fixed for 24 hours in 4% buffered formaldehyde. Next, the material was transferred for cryoprotection into Tyrode's solution containing 16% sucrose and an addition of sodium azide. After several days of washing (1 change per day), the tissue samples were cut with a cryostat into 10 μ m sections. Every tenth section was mounted on chrome alum gelatin-coated glass slides and stored at -70°C until further analysis.

Immunofluorescence. The protocol used has already been described in details (2). Before double immunofluorescent staining, slides were air-dried for 20 minutes. A hydrophobic pen was used to create a barrier around the stained tissue. In order to block non-specific proteins, the sections were incubated (3×15 min) at room temperature (RT) in 0.01 M phosphate-buffered saline (PBS) enriched with 10% normal goat serum, 0.25% Triton X-100, and 0.25% bovine serum albumin (Sigma-Aldrich). A mixture of two primary antibodies from different animal species was dropped on the sections placed in a humid chamber and left for an overnight incubation (RT). Rat anti-somatostatin monoclonal antibodies (1 : 300, Bio Rad, Oxford, UK; code 8330-0009) were combined either with mouse anti-serotonin monoclonal antibodies (1 : 200, Abcam, Cambridge, UK; code ab16007) or mouse anti-GLP1 monoclonal antisera (1 : 4000, Novus Biologicals, Abington, UK; code NBP1-

05189). For the visualization of the above antigens bound by primary antibodies, species-specific secondary antibodies (IgG) conjugated to either FITC (1 : 400, MP Biomedicals, Cleveland, OH, USA) or Texas Red (1 : 400, MP Biomedicals) were used. Washing with PBS was performed several times after each staining. Finally, the slides were mounted in phosphate buffered glycerol (pH = 8.2). In order to verify the specificity of the antibodies used, negative control stainings were done. In a series of control incubations, the primary antibodies were replaced with an equivalent volume and concentration of normal mouse or rat IgG or primary sera were omitted. Additionally, control staining was done with primary antibodies that had been preabsorbed with an excess of specific antigens. No positive immunoreaction was observed in NEC from control sections.

Image analysis, quantification and statistics. The stained sections were viewed under a spinning-disc confocal microscope (BX-DSU Olympus, Nagano, Japan) with a fluorescence microscopy MWIY2 filter (545-580 nm) and an MNIBA2 filter (470-490 nm) appropriate to detect fluorochromes used. All images were obtained with a digital color camera (DP-70, Olympus) using the Cell[^]M image system software (Olympus). In all segments of the small and large intestines of the control and experimental

animals, the numbers of NEC IR for serotonin, GLP1, or somatostatin were assessed by cell counting according to a protocol described elsewhere (39). Briefly, cell counts were performed in 10 mm long section of non-interrupted and not damaged intestinal mucosa (at least 5 slides from intestines of each animal from every group). All NEC IR for any of the substances studied were counted and presented as mean $\pm$ S.D. Additionally, semi-quantitative assessments of the numbers of NEC were made in order to evaluate the density of NEC in crypts and villi. The following scale was used: none, single, moderate, numerous. The possible co-localization of the substances studied in the same NEC was assessed visually by filter switching. A one-way analysis of variance test (ANOVA), followed by a Bonferroni post-hoc test and an independent *t* test, was used to analyze statistical differences. A *p* value < 0.05 was considered statistically significant.

Results and discussion

In the small intestine of the control animals from groups C1 and C2, 87.8 ± 9.7 and 90.1 ± 6.7 serotonin-IR cells, respectively, were found, evenly distributed in the epithelial line of the basis of the crypts and villi (figure 1A). In the small intestine of animals from the

Fig. 1A depicts the expression of serotonin in the small intestine of animals from the control (C1) and PR groups. **Fig. 1B** presents serotonin-IR NEC found in the large intestine of animals from the control (C2) and HR groups

PR and HR groups, the numbers of serotonin-cells was slightly higher: 101.2 ± 12.6 and 98.4 ± 10.2 , respectively (figure 1A), but the differences were not statistically significant when compared with the corresponding controls ($p < 0.05$). No statistically significant differences were found between the PR and HR groups, either. In the mucosa of the small intestine of animals from both these groups, a slightly higher proportion of serotonin-expressing cells (compared with controls) were observed at the basis of the epithelial crypts. In the large intestine of the animals from the PR and HR groups, the numbers of serotonin-IR cells (34.2 ± 10.8 and 33.4 ± 10.2 , respectively) were statistically comparable to that in the corresponding controls (36.4 ± 7.1 and 34.8 ± 8.4 , respectively; $p < 0.05$; Fig. 1B). Moreover, no distinct differences were observed in distribution patterns of serotonin-IR NEC in the large intestine. These results indicate that chemical compounds present in both population and hybrid rye grains had only a slight effect on the proportions of enterochromaffin cells in the small intestine and did not affect these proportions in the large intestine. Because these differences were not statistically significant, we speculate that such a slight up-regulation of serotonin-IR NEC should have no noxious effect on the physiology of the porcine GIT. Some dietary components, such as probiotics, crude fibre of cereal origin (31), or L-tryptophan (38), can regulate the intestinal serotonin metabolism. Additionally, it has also been demonstrated that increased numbers of serotonin NEC in the small intestine mucosae accompany several gastrointestinal disorders, such as celiac disease (25), refractory celiac disease (9), and inflammatory bowel disease (32). In the course of inflammatory bowel disease, a proper diet can change the number of NEC to normal (24). It is known that the main action of intestinal serotonin is to promote local inflammation. It has been shown, that enhanced serotonin release is responsible for the stimulation of mast as well as T cells to produce pro-inflammatory cytokines (36), which in turn decrease the function of mucosal serotonin transporter (14). Finally, altered epithelial transport leads to several undesirable conditions, including nausea, vomiting, diarrhea and/or dyspepsia (23). The experimental animals in the present study were clinically healthy (showed none of the abovementioned symptoms), and microscopic analysis of their intestinal tissue revealed no visible signs of inflammation. We therefore speculate that the components present in both kinds of rye grains tested have no or only incidental inflammatory potential. Interestingly, a change in the numbers of serotonin-IR cells was also observed in the large intestine of patients with Crohn's disease (5). Since in the present study we observed no changes in the large intestine, it is reasonable to conclude that the ingested contents of both types of rye grains that had reached the colon had no influence on inflammatory processes in the distal gut.

Immunohistochemistry revealed that after feeding with population rye grain there was a slight, but not statistically significant ($p < 0.05$), decrease in the number of GLP1-IR NEC (119.2 ± 15.8 in PR vs. 140.4 ± 14.2 in C1). In both PR and C1 groups, no visible changes in the distribution of GLP-1-IR cells were noted (they were located mostly in the epithelial cells of the mucosa; Fig. 2A). However, the proportions of GLP1-IR NEC found in the small intestine of pigs fed with hybrid rye grain (127.2 ± 8.8) were statistically the same as those in the corresponding control group (130.6 ± 16.8) or the PR group ($p < 0.05$). As many as 22.6 ± 14.1 and 26.2 ± 6.1 GLP1-IR NEC were found in the large intestine of the control animals C1 and C2, respectively, and feeding with neither population nor hybrid rye grains statistically changed these proportions (23.4 ± 7.3 in PR and 25.0 ± 10.1 in HR; Fig. 2B). Previous studies clearly indicate that GLP1 released postprandially from intestinal L cells increases insulin secretion by pancreatic β -cells (16), and this process is enhanced after eating food rich in proteins (21). Therefore, it is believed that the modern treatment of diabetes may be based on the increase of GLP1 levels. Some initial steps have been made, since it has been shown that mice fed with a diet supplemented with Y-27632 (inhibitor of ROCK protein kinases) showed increased numbers of GLP1-IR NEC in the small intestine (27). Considering our results in this light, we may speculate that feeding pigs with population (but not hybrid) rye grain may evoke a barely detectable lower tolerance of glucose. It must be remembered that the ablation of GLP1-producing L cells in transgenic mouse leads to increased loss of body weight and morphological changes in the structure of the small intestine (20). Interestingly, increased numbers of L cells have also been found in mice with Roux-en-Y gastric bypass surgery, but not in those fed with a chow/high-fat diet (26). It is important to mention that in patients with functional dyspepsia an increased plasma GLP1 concentration has been observed (4). Some authors even observed that the numbers of GLP1-IR colonic NEC decreased substantially after weaning, which suggests a functional plasticity of these cells in relation to the type of food (29). In addition to its role as incretin, GLP1 also decreases the contractility of the smooth muscle of the large intestine (1), and, because we found no differences in the numbers of colonic GLP1-producing NEC in present study, we postulate that feeding with rye grain (population or hybrid) may be neutral for colonic motility.

In the control pigs, somatostatin-IR NEC were extremely rare in both the small (15.8 ± 3.3 in C1 and 17.0 ± 2.4 in C2; Fig. 3) and the large intestines (8.8 ± 2.4 in C1 and 9.0 ± 2.7 in C2). These proportions were not changed when pigs were fed with either population rye (13.6 ± 3.8 in small intestine and 8.0 ± 1.2 in large intestine) or hybrid rye (16.4 ± 4.9 in small intestine and 9.4 ± 2.1 in large intestine; Fig. 3). Moreover, no

Fig. 2. In Fig. 2A shows populations of GLP1-expressing NEC found in the small intestine of control (C2) and hybrid rye grain-fed animals (HR). In microphotographs (Fig. 2B) sparse GLP1-immunoreactive NEC are seen in the large intestine of the control (C1) and experimental (PR group) pigs

Fig. 3. Single somatostatin-immunoreactive NEC found in the small intestine of control (C2) and experimental (HR group) animals

significant differences in the numbers of somatostatin-IR cells were found between animals from the PR and HR groups. It is not clear whether NEC plasticity also includes changes in the expression of somatostatin. No

change in the numbers of somatostatin-IR NEC was found during refractory celiac disease (9). However, in patients with inflammatory bowel syndrome the somatostatin-IR NEC of the duodenum were affected

by dietary guidance (24). In rats with experimentally induced colitis, the numbers of somatostatin-IR NEC were significantly lower than they were in the controls (12). It has been found that somatostatin released from the small intestinal L cells plays a role in inhibition of GLP secretion (18). Recently, it has been found that somatostatin secreted within the intestinal mucosa acts as a down-regulator of pro-inflammatory mediators (7). Nevertheless, in the present study, we found that the numbers of somatostatin-IR cells in both the small and the large intestines after feeding with either population or hybrid rye grains, were still dramatically low, which allows us to conclude that these grains had no influence on somatostatin expression.

In conclusion, feeding with population and hybrid rye grains had only a very slight effect on populations of serotonin-IR, GLP1-IR (but not somatostatin-IR) NEC. Therefore, it seems that neither of these rye grains is capable of disturbing the homeostasis of serotonin-IR, GLP1-IR and somatostatin-IR NEC, which suggests their usefulness in animal feeding. The results of the present study may be applicable in a wider strategy aimed at developing a new nutritional plan for pigs in which hybrid rye grain plays the main role.

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