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Original article

Enteric nervous system in the European beaver (*Castor fiber*) pylorus – an immunohistochemical study

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Abstract

European beaver (*Castor fiber*), the largest rodent species inhabiting a wide area of Eurasia, feeds mainly on dry parts of plants, bark or wood. Such kind of nourishment needs to be properly digested in each part of the gastrointestinal tract. The time of stomach digestion, which directly influences all the following steps of the digestion process, is precisely controlled by the pylorus and its innervation. However, virtually no data is available on the organization of the enteric nervous system in most of the wild animal species, including beavers. On the other hand, a peculiar diet consumed by beavers, suggests that the arrangement of their stomach intramural nerve elements can be atypical. Therefore, the present study investigated the distribution and chemical coding of neurons and nerve fibers in the pylorus of the European beaver.

The experiment was performed on stomachs obtained from a group of 6 beavers caught in Northeastern region of Poland (due to beaver overpopulation). Pyloric wall tissue cryosections were double immunostained with a mixture of antibodies against pan-neuronal marker PGP 9.5 (to visualize enteric neurons) and ChAT (cholinergic marker), nNOS (nitrergic marker), SP, CGRP, Gal (peptidergic markers).

Confocal microscopy analysis revealed that the majority of enteric nerve cells were clustered forming submucosal and myenteric ganglia and all the studied substances were expressed (in various amounts) in these neurons.

We conclude, that the anatomical arrangement and chemical coding of intramural nerve elements in the beaver pylorus resemble those found in other mammalian species.

Key words: European beaver, stomach, pylorus, enteric nervous system, immunocytochemistry

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Introduction

The European beaver is one of the largest rodents in the world. The diet of these animals consists mostly of dry parts of plants, bark or wood. Such kind of nourishment needs to be properly insalivated, digested and transported through the digestive system. The time of stomach digestion, in which the chyme is mixed with the stomach juice containing hydrochloric acid, is extremely important for all the following steps of the digestion process. The pylorus, including its circular muscle band (known as the pyloric sphincter) is an anatomical structure precisely controlling the gastric outflow (Ramkumar and Schulze 2005). The function of the pylorus is regulated by intrinsic (Lindestrom and Ekblad 2002, Ramkumar and Schulze 2005, Furness 2006, Zalecki 2015) and extrinsic (Kressel et al. 1994, Zalecki 2012, Zalecki et al. 2012, Zalecki 2014) nerves. Neurons act via neurotransmitters. The main neurotransmitters exploited by neurons supplying the gastrointestinal tract can be classified as factors activating (acetylcholine, substance P) or inhibiting (nitric oxide – synthetized by its synthase - NOS) the motility, substances involved in transduction of intramural sensory impulses (calcitonin gene-related peptide - CGRP) and other modulatory neuropeptides (as galanin) which can modulate the function of neurotransmitters and/or participate in neuronal reaction to pathology (Lang and Kofler 2011). Although studies on the nerve control of the pyloric sphincter function are conducted for many years, the precise mechanism of this regulation still remains unclear. Several studies on the pylorus innervation have been performed in laboratory rodents (Kressel et al. 1994, Lindestrom and Ekblad 2002) and other animals (Mazzuoli et al. 2008, Zalecki 2015, Zalecki et al. 2016, Zalecki et al. 2018) to elucidate this problem.

Due to the limited access to biological material from wild mammals, the data on the innervation pattern of the pylorus in these species is very incomplete [concern such animals as wild boar (Zacharko-Siembida and Arciszewski 2014)], and no information is available in the beaver. On the other hand this question seems to be intriguing due to the specificity of the diet consumed by these animals, and the importance of the pyloric sphincter for proper digestion. The knowledge on anatomy and histology of the beaver organs is very scarce, and concerns the mammary gland (Franke-Radowiecka et al. 2016) and stomach (Bisaillon and Bherer 1979, Morgan 1868, Ziolkowska et al. 2014). Recent contributions (Ziolkowska et al. 2014) have described histological and ultrastructural properties of the European beaver stomach, indicating its unique morphological features including the cardiogastric gland, large number of chief cells and thick layer of highly specific mucus. Moreover, the authors have pointed to the thick muscular layer in the pyloric region, which indicates its significance in the stomach functioning.

Therefore, the present study aimed to identify the arrangement of intrinsic innervation of the pylorus in the European beaver, as well as to specify the chemical coding of these nerve structures. Double-labeling immunofluorescence was used to investigate the presence of cholinergic (anti ChAT antibodies), nitrergic (anti nNOS antibodies) and peptidergic (substance P, CGRP, Gal) neurons in the pylorus of the European beaver. Such information should allow to understand whether the beaver pylorus is regulated in a way similar to that found in other mammalian species.

Materials and Methods

The stomach samples were harvested from European beavers (*Castor fiber*) (n=6) caught in the Bialowieza National Park, located in Northeastern region of Poland.

Animal captures were allowed due to beaver overpopulation in this area and were conducted by a specialized team from the Polish Hunting Association.

All the experimental procedures, including animal euthanasia, were based on the permission from the National Ethics Commission for Animal Experimentation, Polish Ministry of Science and Higher Education, and from the Regional Directorate for Environmental Protection in Olsztyn (Poland), a government institution responsible for wildlife management.

The beavers were deeply anaesthetized by intramuscular administration of xylazine (0.1 ml/kg body weight, Sedazin, Biowet Puławy, Poland) and ketamine (0.1 ml/kg body weight, VetaKetam, VetArgo, Poland) at doses appropriate for body weight. The stomachs were removed immediately after the heart stopped, cut along the greater curvature and washed in PBS to remove food debris from the mucosa. Next, the tissues were fixed by immersion in the 4% buffered paraformaldehyde (pH 7.4) for 24 h and finally transferred into 18% buffered sucrose solution (pH 7.4) until they sunk to the bottom of the container.

Pyloric wall tissues were cut transversally into 14 μm thick cryostat sections, which were mounted on chrome-gelatin-coated glass slides and processed for double immuno-labeling following the procedure described in detail previously (Zalecki 2012). Antibody details are presented in Table 1. Microscopic tissue slides were analyzed and photo-documentation was prepared under a confocal laser microscope (LSM 700, Zeiss, Germany). The specificity of immunohistochemical labelling was tested by omission of primary antisera

Table 1. Primary and secondary antibodies applied in the experiment (p-polyclonal; m-monoclonal).

Antigen	Species	Code	Dilution	Manufacturer/Supplier
Primary antibody				
ChAT	Rabbit-(p)	AB143	1:1000	Chemicon Int.
CGRP	Rabbit-(p)	C8198	1:2000	Sigma-Aldrich USA
Gal	Rabbit-(p)	T-4330	1:2500	Peninsula, USA
nNOS	Mouse-(m)	N 2280	1:2500	Sigma-Aldrich, USA
PGP 9.5	Mouse-(m)	7863–2004	1:500	AbD Serotec, USA
SP	Rat-(m)	8450-0505	1:150	AbD Serotec, UK
Secondary antibody				
Goat AlexaFluor 555 anti-rat		A21434		
Goat AlexaFluor 488 anti-mouse - cross abs.		A11029	1:500	Invitrogen, UK
Goat AlexaFluor 555 anti-rabbit		A21428		

and their replacement with normal, non-immune sera (rabbit, mouse or rat). No fluorescence was observed in all the control stainings, what confirmed the specificity of the method.

To determine percentages of neurons expressing particular neurotransmitters (ChAT, nNOS, SP, CGRP, Gal), the numbers of neurons simultaneously co-expressing pan-neuronal marker (PGP 9.5) and one of the neurotransmitters were counted in the myenteric and submucosal ganglia. For each substance investigated at least 100 perikarya were counted. The size of the perikarya was determined by confocal laser microscopy software measurements (Zen 2009, ver. 5.5.0.282 and LSM Image Browser, ver. 4.02, Zeiss) in a group of 30 PGP-immunoreactive nerve cell bodies (independently for submucosal and myenteric ganglia). All the results are presented as average percentages ± SEM.

Results

Microscopic analysis revealed that the majority of PGP 9.5-positive enteric perikarya were grouped in myenteric (Fig. 1a,b) and submucosal (Fig. 1b,c) ganglia. Most of the myenteric neurons were found between the longitudinal and circular muscle layers, however, solely occurring nerve structures were additionally observed between bundles of circular muscles (Fig. 1b). Interestingly, the myenteric neurons were scarce and most of them were clustered in small groups (up to 5 perikarya), while the larger clusters (up to 10-15 perikarya) were also occasionally encountered. Most of the myenteric perikarya were oval or multipolar in shape with average dimensions of 27.4±1.8 x 20.1±1.2 μm. The submucosal neurons occurred individually or in groups up to 10-14 cells. They were mostly localized in the submucosal layer (Figs. 1b, 3a",b",c"), however, they were also occasionally observed in the muscularis mucosae (Fig. 3d",e") and in the lamina propria of the mucosa. Most of the submucosal perikarya were oval in shape (Figs. 1c, 3c") and measured 27.1±1.9 x 17.4±1.8 μm in diameter.

Analysis of the double-immunolabeled sections revealed that 39.46±1.71% (Fig. 2a,a',a") of the myenteric neurons were immunoreactive for cholinergic marker (ChAT) while 32.45 ± 1.85% for nitrergic marker (nNOS; Fig. 2b,b',b"). The percentages of the myenteric neurons immunoreactive for particular peptides were as follows: 41.53±2.43% immunoreactive for SP (Fig. 2c,c',c"), 27.97 ± 1.10% for CGRP (Fig. 2d,d',d") and 32.52±1.75% for Gal (Fig. 2e,e',e").

In the submucosal ganglia, 42.58±1.77% of perikarya were immunoreactive for ChAT (Fig. 3a,a',a") and 20.40±3.60% for nNOS (Fig. 3b,b',b"). In addition, submucosal neurons expressed immunoreactivity for all the peptides investigated: 40.72±1.15% expressed SP- (Fig. 3c,c',c"), 46.15±4.54% expressed CGRP- (Fig. 3d,d',d") and 40.22±0.94% expressed Gal-immunoreactivity (Fig. 3e,e',e").

All substances investigated were also observed in nerve fibers associated with the submucosal and myenteric perikarya (Figs. 2b,b',b",c,c',c",d,d',d",e,e',e", 3b,b',b",c,c",c",d,d',d",e,e',e"). The most numerous were those immunoreactive to substance P (Figs. 2c',3c') and galanin (Figs. 2e',3e'), while fibers expressing other substances were relatively scarce.

Discussion

The present study has revealed for the first time the innervation pattern of the pylorus in the European bea-

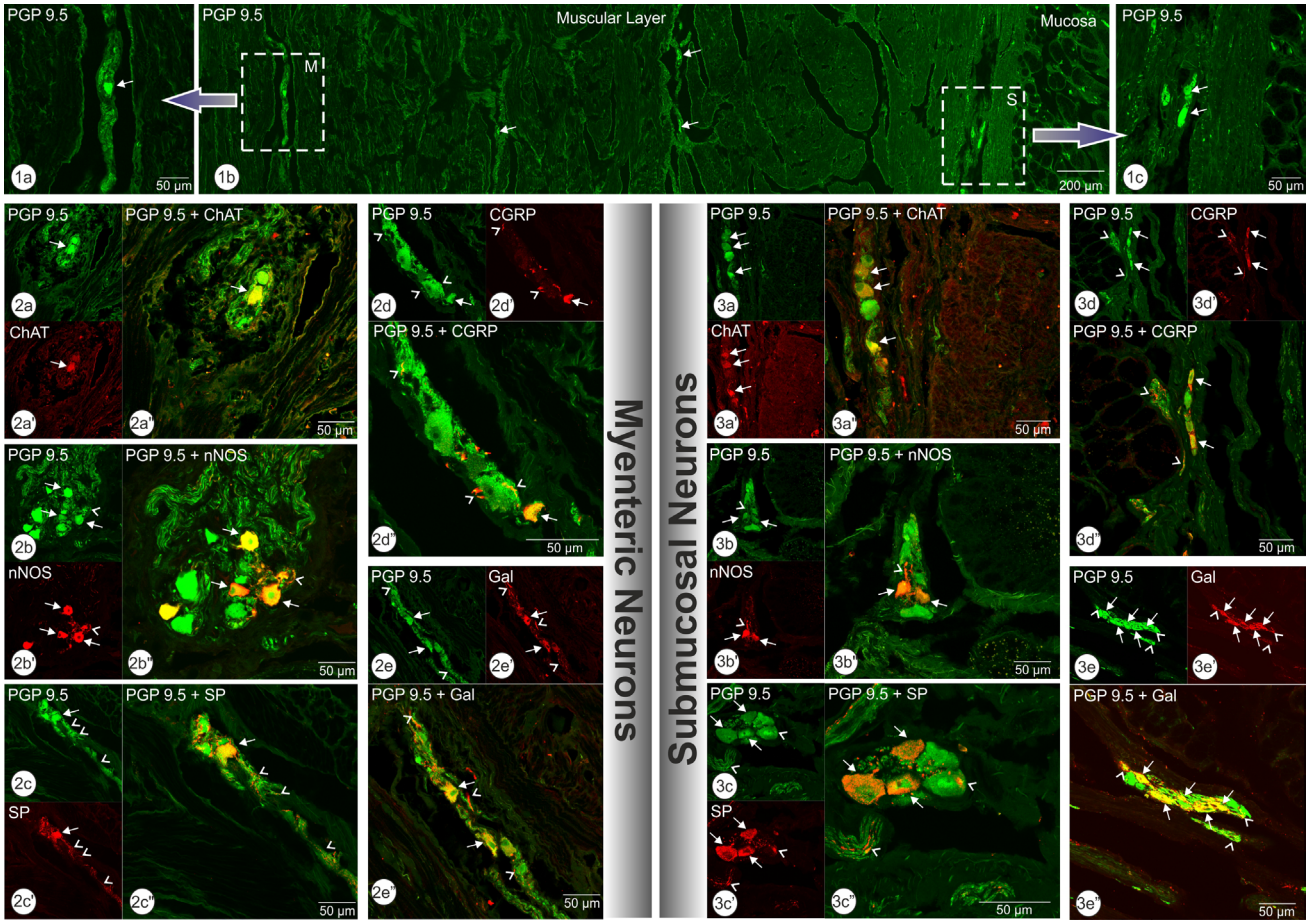

Fig. 1. (a, b, c)
Set of microphotographs showing the transverse cross-section of the pyloric wall in the European beaver immunolabelled with anti-PGP 9.5 antibodies. Scale bars are included in the pictures.
(a, c) pictures showing high magnification views of the myenteric (a) and submucosal (c) ganglia. Arrows point to the PGP 9.5 immunoreactive neuronal perikarya.
(b) an image showing the entire cross section of the pyloric wall (picture composed of many microphotographs processed with the use of confocal laser microscope software). Muscular (M) and submucosal (S) ganglia are marked with dotted line rectangles [high magnification pictures of these selected areas are presented in the picture (a) and (c)]. Occasional nerve structures located between muscle bundles (at different depths) are marked by arrows. Very thick muscular layer can be observed.

Fig. 2. (a, b, c, d, e, a', b', c', d', e', a", b", c", d", e")
Set of microphotographs showing exemplary sections of the myenteric neurons double-immunostained for PGP 9.5 (a, b, c, d, e) and ChAT (a'), nNOS (b'), SP (c'), CGRP (d'), Gal (e'). Arrows point to neurons which simultaneously co-expressed PGP 9.5 and one of the neurotransmitters. Arrowheads point to double-immunostained nerve fibers. Both fluorescent channels were digitally overlapped in figures (a", b", c", d", e"). Scale bars are included in the pictures.

Fig. 3. (a, b, c, d, e, a', b', c', d', e', a", b", c", d", e")
Set of microphotographs showing submucosal neurons double-immunostained for PGP 9.5 (a, b, c, d, e) and ChAT (a'), nNOS (b'), SP (c'), CGRP (d'), Gal (e'). Arrows point to neurons which simultaneously co-expressed PGP 9.5 and one of the neurotransmitters. Arrowheads point to double-immunostained nerve fibers. Both fluorescent channels were digitally overlapped in figures (a", b", c", d", e"). Scale bars are included in the pictures.

ver. Although the stomach in this species is characterized by some unique features (e.g. the cardiac gland), the general pattern of the intrinsic innervation of the pylorus is similar to that found in other species. Enteric perikarya are grouped into ganglia localized in the submucosa (some cells observed also in the muscularis mucosae) and between the longitudinal and circular muscle layers. In classical terms, submucosal ganglia are distributed between the deep face of the muscular layer and the lamina muscularis mucosae, and together with the accompanying nerve fibers form the submucosal plexus. According to many authors, the term submucosal plexus is strictly reserved for the intestinal structure, while in the stomach, the term submucosal ganglia

should be applied. Such interpretation mainly results from a limited number of submucosal perikarya and nerve fibers observed in the stomach of many species, especially in small laboratory mammals. However, in some publications dealing with the innervation of the stomach terms submucosal ganglia and submucosal plexus are used interchangeably. It should be noticed that the latter term was used mainly in papers on the stomach innervation in larger mammals (cat, pig, sheep) (Edin et al. 1979, Nozdrachev et al. 1981, Yamamoto et al. 1995, Van et al. 1996, Kaleczyc et al. 2007) however, occasionally, this term was even applied in relation to the rat stomach (Lee et al. 1987) and pylorus (Kressel et al. 1994) structure. What is more, some authors divide the intestinal submucosal plexus into further sublayers (Balemba et all. 1998). It should be emphasized, that precise localization of submucosal perikarya in different species is not homogenous throughout the same gastrointestinal segment – in some localizations the mucosal or "muscularis mucosa attached" perikarya are also present. The most likely explanation for irregular organization and non-obligatory existence of the submucosal cells in different species (Meier-Ruge and Bruder 2005, Furness 2006, Kramer et al. 2011) result from the fact that neural crest progenitor cells destined to became submucosal neurons are slightly displaced during embryonic migration (Stohr 1949). Moreover, submucosal neurons are mainly responsible for the innervation of the mucosa, while myenteric neurons control motility (Furness 2006). Therefore, in general, all neurons localized beneath the muscular layer can be referred to as submucosal neurons, in contrary to neurons localized in the muscular layers, which respectively should be referred to as myenteric neurons.

Although the localization of myenteric neurons in the beaver is similar to that observed in other species, the number of cells clustered together seem to be relatively small. These results confirm the presence of interspecies differences between rodents and omnivore mammals, such as pigs, in which the pyloric myenteric neurons are numerous and frequently grouped into clusters comprising up to 30 perikarya (Kaleczyc et al. 2007, Zalecki 2015, Zalecki et al. 2016). In rodents, the number of myenteric neurons is reduced, and they occur as small scattered ganglia or singular perikarya intermingled between the muscle bundles (Kressel et al. 1994), or they have been even not determined (Lindestrom and Ekblad 2002). Scattered groups of perikarya observed in the European beaver pylorus seem to stay in contrast with the large thickness of its circular muscle layer. In the light of data showing complexity of the pyloric sphincter extrinsic innervation (Ramkumar and Schulze 2005), small number of myenteric neurons observed in the European beaver pylorus suggests significant role of extrinsic nerves in the regulation of pyloric muscles activity in this species.

The results of double-immunofluorescence staining revealed a large population of cholinergic and nitrergic enteric neurons in the European beaver pylorus. According to many studies, these are two main populations of neurons controlling gastrointestinal tract motility – cholinergic neurons activate muscular layer contractions, while nitrergic nerve cells evoke the relaxation response (Tomita et al. 1999, Furness 2006). What is more, acetylcholine is known to be the main neurotransmitter of the intramural ascending nerve pathways, while nitric oxide is utilized by neurons involved in the descending circuits. The occurrence of both these neurotransmitters in the myenteric neurons of the studied species is consistent with results obtained in the stomach of other animals (Ramkumar and Schulze 2005, Furness 2006) and suggests mechanisms for nerve regulation of the beaver's pyloric sphincter muscle similar to those found in other mammals. The occurrence of the cholinergic and nitrergic submucosal neurons, which are mainly responsible for the mucosa innervation, indicates the involvement of both neuropeptides in the regulation of the pyloric mucosa secretory activity in the studied species. The results obtained are in line with data showing cholinergic and non-cholinergic secretomotor innervation of the mucosa (Cooke and Reddix 1994) and suggest the existence of similar nerve circuits in the beaver pylorus.

Substance P, a member of tachykinin neuropeptide family, plays a multiple roles in the regulation of gastrointestinal tract function and is extensively expressed by the enteric neurons (Furness 2006). Interestingly, in the beaver pylorus, SP-immunoreactive neurons constitute large nerve cell populations in both, the myenteric and submucosal ganglia. According to many studies SP is one of the key factors regulating the smooth muscles activity (Schmidt and Holst 2000) and it is indirectly involved in the physiological regulation of the mucosa function. Moreover, SP is one of the key factors involved in the neuro-immune cross-talk (Vilisaar and Arsenescu 2016). The large number of SP-immunoreactive neurons observed in the beaver tissues indicates their significant involvement in the nerve regulation of the beaver pylorus. The present results are highly congruent with findings showing regulatory function of SP in the pylorus of different animals including the cat and rat (Lidberg et al. 1983, Lidberg 1985).

CGRP, a neuropeptide found in the enteric nervous system in many animals, is expressed by secretomotor and intrinsic primary afferent neurons (IPAN's) (Furness 2006). Such cells are known to gather information on conditions occurring in the gastrointestinal

tract and to initiate intrinsic nerve reflexes. The presence of CGRP-immunoreactive neurons in the submucosal and myenteric ganglia of the beaver pylorus is in agreement with earlier determined IPAN's in both of these nerve plexuses (Furness 2006), and suggests the occurrence of CGRP-related neuronal sensory circuits in the beaver's pylorus.

Galanin, a peptide exhibiting wide number of physiological properties, was observed in the gastrointestinal tract of many animals (Lang et al. 2007). Although galanin is highly involved in neuronal plasticity [which occurs as a reaction to pathology (Lang and Kofler 2011, Zalecki et al. 2016, Zalecki et al. 2018)], it is also engaged in the regulation of gastrointestinal function in physiological conditions. Physiological studies have revealed that galanin does not affect the basic pyloric motility, but inhibits the action of previously activated pyloric sphincter (Allescher et al. 1989, Rattan and Tamura 1998). Considering the large thickness of the beaver pyloric sphincter, the relatively large number of galaninergic perikarya suggests their involvement in the regulation of the pyloric relaxation mechanism.

The present study has revealed for the first time the innervation pattern of the European beaver pylorus. Although the diet of this species significantly differs from that consumed by the majority of other rodents or mammals, the general organization of intramural nerve elements found in the pyloric wall resembles that observed in other species. The application of double--immunofluorescence stainings revealed the presence of cholinergic, nitrergic and peptidergic (SP-, CGRP-, Gal-immunoreactive) neurons in submucosal and myenteric ganglia. These neuronal populations were observed in the gastrointestinal tract and pylorus of several animal species (Rattan and Tamura 1998, Ramkumar and Schulze 2005, Furness 2006). Years of studies on the digestive system innervation have provided many data on the physiological roles played by individual neurotransmitter substances, which, in general, are universal for most of the mammals (Furness 2006). It is obvious, that some minor differences were observed as well, however, due the scanty availability of wild animal tissues, we assume that the neurotransmitters investigated in the beaver enteric nervous system play roles similar to those found in the majority of other mammals. The present findings contribute to our knowledge on the morphology of the gastrointestinal tract in the European beaver, one of the largest rodents in the world.

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