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Increased expression of CART, nNOS, VIP, PACAP, SP and GAL in enteric neurons of the porcine stomach prepyloric region following hydrochloric acid infusion

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Abstract

Introduction. Stomach hyperacidity leads to damage of the mucus/bicarbonate barrier, ulcerations and the development of stomach cancer. Key regulators of the mucosal barrier/luminal acid balance are neurotransmitters secreted by intramural neurons. The aim of the current study was to determine the expression of gastric neuropeptides and nNOS in the porcine stomach following hydrochloric acid instillation. We report on increased expression of enteric neurotransmitters involved in adaptive reaction to an experimentally-induced hyperacidity state.

Material and methods. The investigation was conducted on eight 12–18 kg pigs. The influence of intragastric infusion of hydrochloric acid on the expression of cocaine- and amphetamine-regulated transcript peptide (CART), neuronal nitric oxide synthase (nNOS), vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase-activating peptide (PACAP), substance P (SP) and galanin (GAL) in the submucous and myenteric gastric neurons of the pig has been studied with double immunofluorescence.

Results. A mimicked hyperacidity state significantly increased the proportion of enteric neurons immunoreactive to CART, nNOS, VIP, PACAP, SP and GAL in the submucous gastric neurons. In the myenteric plexus, a significant increase of the number of VIP-, CART- and GAL-immunoreactive (IR) neurons was found. Similarly, the percentage of myenteric nNOS-IR and PACAP-IR neurons tended to increase, while the fraction of SP-IR cells did not change.

Conclusions. Stomach hyperacidity modifies the expression of the studied neurotransmitters in a specific way depending on the location of the neurons in particular plexuses of the stomach. Increased numbers of neurons expressing CART, nNOS, VIP, PACAP, SP and GAL clearly indicate their regulatory engagement in the restoration of the physiological gastric balance following hyperacidity. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 4, 179–187)*

Key words: pig; stomach; hyperacidity; enteric nervous system; neuropeptides; IHC

Introduction

Stomach digestive function is based on the secretion of gastric juice, whose main ingredients are hydrochloric acid (HCl), pepsinogen, mucus and HCO_3^- ions. HCl and the proteolytic enzyme pepsin participate in the digestion of proteins, while mucus and HCO_3^- protect the mucosal lining against acid-pepsin digestion [1].

Gastric acid secretion is stimulated by various neuronal (vagal, enteric), paracrine (histamine) and hormonal (gastrin) factors, while somatostatin, glucagon-like peptide-1, cholecystokinin and atrial natriuretic peptide reduce secretory stomach activity [2]. Gastric hyperacidity states increase the risk of numerous gastrointestinal disorders and increase morbidity and mortality related to those pathologies [3–5]. Excessive secretion of gastric juice may cause destruction of the mucous protection barrier, the development of erosions, formation of ulcers ac-

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companied by inflammatory state [6, 7] and, finally, the development of cancer [8]. Based on previous observations, this harmful environment might trigger adaptive reaction of the stomach intramural neurons, whose nature, due to a lack of adequate data, remains unclear [9].

Intramural gastric neurons constitute a local emergency system that is called into operation when the gastric mucosa is endangered by acid or other harmful stimuli [10]. Among the active factors are neurotransmitters synthetized and released by enteric neurons [11]. Identification of the hyperacidity-induced neuroactive substances constitutes the first indicator of their possible engagement in the gastric mucosal repair or degradation process. Although available reports implicate certain neurotransmitters like cocaine- and amphetamine-regulated transcript peptide (CART) [12, 13], nitric oxide (NO) [14, 15], VIP — vasoactive intestinal polypeptide [5], pituitary adenylate cyclase-activating peptide (PACAP) [16], substance P (SP) [10, 17, 18], galanin (GAL) [2, 19] in the maintenance of the luminal mucus-hydrochloric acid equilibrium, the specific neuronal populations that synthesize and release those substances still remain obscure.

In the pig, the anatomy of the enteric nervous system depends on the segment of digestive tract [19]. In the stomach, it is composed of two intramural plexuses: the myenteric plexus and the submucous plexus. It is widely accepted that myenteric neurons are responsible for the control of stomach and gut motor function, whereas submucous neurons mainly regulate mucosal secretion [20–22]. It has also been reported that local gastric neurons respond with structural, functional and neurochemical changes to inflammatory processes, bacterial infections, toxins and intestinal diseases [23]. Consistently, the enteric neurons are known to be highly plastic in their response to inflammation [24, 25]. This adaptive reaction comprises both up and down adjustment of transmitter expression and the induction of new genes that induce *de novo* expression of neurotransmitters formerly absent in the enteric neurons. The adaptive response occurs to promote enteric neurons to survive pathological conditions, but also to facilitate the healing process of the inflamed part of the gastrointestinal tract [26].

Thus, the influence of intragastric HCl instillation on the expression of CART-, neuronal NOS-, VIP-, PACAP-, SP- and GAL- immunoreactivity in the porcine stomach intrinsic neurons was studied. The pig, like humans [27, 28], is a species particularly susceptible to ulcer formation. We have chosen the stomach prepyloric region since this area is pathognomonic for the location of peptic ulcers which, when

localized here, may alter gastric emptying [29]. Moreover, selection of the pig as an experimental animal species is based on close anatomical and physiological resemblance and human-like sensitivity of this species to stressful conditions [30].

Materials and methods

The present investigation was conducted on eight immature female pigs of the Large White Polish breed (12–18 kg *b.w.*, approximately 8 weeks old), which were kept in standard laboratory conditions during the experiment. Animals were purchased from Production and Testing Plant "Balcyny" in Balcyny. All experimental actions were conducted in compliance with the instructions of the Local Ethical Committee in Olsztyn, decision number 05/2010.

Pigs were randomly divided into two groups: a control (group C; n = 4) and an experimental group (HCL group, n = 4). Animals of the experimental group were pre-treated with atropine (Polfa, Warszawa, Poland, 0.4 mg/kg, *s.c.*) and propionyl promasine (Stresnil, Janssen, Beerse, Belgium, 0.8 mg/kg, *i.m.*) 15 min. before the application of the main anesthetic — sodium thiopental (Thiopental; Sandoz, Kundl, Austria; 20 mg/kg *i.v.*). Following this, a 0.25 M aqueous solution of hydrochloric acid with a dose of 5 ml/kg of body weight was administered intragastrically using a stomach tube. On the first day and on the seventh day of experiment, endoscopic examinations (using a video-endoscope Olympus GIF 145 with working length 1030 mm and diameter 9.8 mm) were performed to confirm inflammatory changes caused by HCl treatment within gastric mucosal layer. These examinations were conducted under general anesthesia (as mentioned above). Immediately after gastroscopy, animals of both groups were euthanized by an overdose of sodium thiopental. On the same day, the control animals were also anaesthetized, and then euthanized by an overdose of sodium thiopental. Afterwards, all animals (C and HCL groups) were perfused transcardially with 4% buffered paraformaldehyde (pH 7.4) prepared *ex tempore*.

Stomachs were collected from all animals and were then fixed in 4% paraformaldehyde for 20 min, rinsed in a 0.1 M phosphate buffer solution, pH 7.4, for 72 h at 4°C and then kept at 4°C in a buffered 18% sucrose solution. Next, tissue samples of the prepyloric region of the stomach were collected. The sample was taken from the area located 5 cm from the pylorus. Tissue specimens were cut with a cryostat at -22°C (Microm HM-525, Microm International Gmbh, Walldorf, Germany) into 14-μm-thick sections. To preclude double counting, the analysis covered only neurons with well visible nucleus, which were located at least 100 μm apart from each other.

Sections were processed for the routine double labelling immunofluorescence method, using primary antibodies against the particular active substances studied. Briefly, after

Table 1. Primary and secondary antibodies used in the study

Antigen	Species of origin	Code	Dilution	Supplier
PRIMARY ANTIBODIES				
PGP-9,5	Mouse	7863-2004	1:500	AbDserotec
SP	Rat	8450-0505	1:150	AbDserotec
CART	Rabbit	MAB 163	1:8000	R&D
nNOS	Rabbit	AB5380	1:3000	Chemicon
VIP	Rabbit	VA 1285	1:2000	Biogene
GAL	Rabbit	AB2233	1:2000	Milipore
PACAP	Guinea pig	T-5039	1:2000	PENINSUL
SECONDARY ANTIBODIES				
Alexa Fluor 488	Donkey Anti-Mouse	A21202	1:1000	Life Technologies
Alexa Fluor 546	Donkey Anti-Rabbit	A11010	1:1000	Life Technologies
Alexa Fluor 546	Donkey Anti-Rat	A11081	1:1000	Invitrogen
Alexa Fluor 546	Donkey Anti- Guinea pig	A11074	1:1000	Invitrogen

CART — cocaine- and amphetamine-regulated transcript; GAL — galanin; nNOS — neuronal nitric oxide synthase; PACAP — pituitary adenylate cyclase-activating polypeptide; PGP 9.5 — protein gene product (pan-neuronal marker); SP — substance P; VIP — vasoactive intestinal peptide.

air-drying at room temperature (RT) for 45 min., sections were incubated with a blocking solution containing 10% of normal goat serum, 0.1% bovine serum albumin, 0.01% NaN3, Triton x-100 and thimerosal in phosphate-buffered saline (PBS) (all these reagents purchased from Sigma, Aldrich, Poznan, Poland) for 1 h at RT. They were then incubated (overnight; RT, in a humid chamber) with a combination of antibodies against protein gene-product (PGP-9.5) with substance P or cocaine- and amphetamine- regulated transcript peptide, pituitary adenylate cyclase-activating polypeptide, galanin, vasoactive intestinal polypeptide or neuronal isoform of nitric oxide synthase. Primary antibodies bound to appropriate antigens were visualized by incubation (1 h, RT) with species-specific secondary antisera conjugated to Alexa Fluor 546 and Alexa Fluor 488 (1 h, RT). Each step of immunolabeling was followed by rinsing of the sections with PBS (3 × 10 min, pH 7.4). The specifications of antibodies are detailed in Table 1. Standard controls, *i.e.* pre-absorption of the neuropeptide antibodies with appropriate antigens for 18 h at 37°C, omission and replacement of primary antibodies by non-immune sera were performed to test the antibodies and specificity of the method.

The labeled sections were analyzed using an Olympus BX51 (Olympus, Tokyo, Japan) fluorescence microscope equipped with epi-illumination and appropriate filter sets. Expression of each studied bioactive substance was analyzed in at least 500 PGP-positive neural cells. Finally, the obtained data were pooled, expressed as means ± SEM and statistically analyzed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). The differences were considered statistically significant at $P \leq 0.05$.

Results

Microscopic examination of enteric innervation of the stomach prepyloric region in physiological conditions revealed the expression of CART, nNOS, VIP, PACAP, SP and GAL in neurons of the submucous as well as the myenteric plexus (Figs. 1 and 2, Table 2). In the submucous plexus, intragastric HCl instillation resulted in a significant increase in the proportion of neurons expressing CART, nNOS, VIP, PACAP, SP and GAL. In the neurons of the myenteric plexus, a significant increase in the numbers of VIP-, CART- and GAL-immunopositive cells was found. The percentage of nNOS- and PACAP-positive neurons increased insignificantly, while the number of SP-immunoreactive cells did not changed.

Detailed exploration of the CART expression revealed that in the submucous plexus CART was found in 7% of control neurons and 24% of neurons following HCl treatment. In the myenteric plexus, CART-immunoreactive (-IR) cells constituted 33% of control neurons and 51% of the immunoreactive cells after HCl application.

In the submucous plexus of the control group, nNOS was expressed in 4% of neurons, while in hyperacidic animals it was expressed in 20% of the PGP-9.5-IR cells. Myenteric plexus showed nNOS in 20% of the gastric control neurons and 27% cells of the experimental animals.

Hyperacidic conditions in the submucous plexus caused an increase in the ratio of VIP-IR neurons from 2% in controls to 11% in experimental pigs,
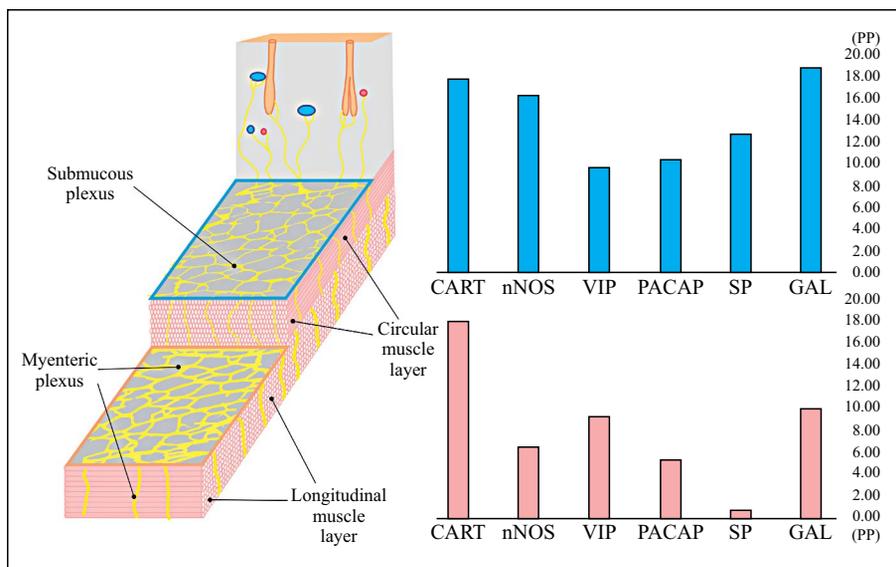
Figure 1. Diagram showing percent point (pp) increase of the number of CART-, nNOS-, VIP-, PACAP-, SP- and GAL-immunoreactive neurons in submucous plexus (blue) and myenteric plexus (orange) in the porcine prepyloric region following hydrochloric acid intragastric instillation to pigs. For abbreviations: see the description of Table 1.
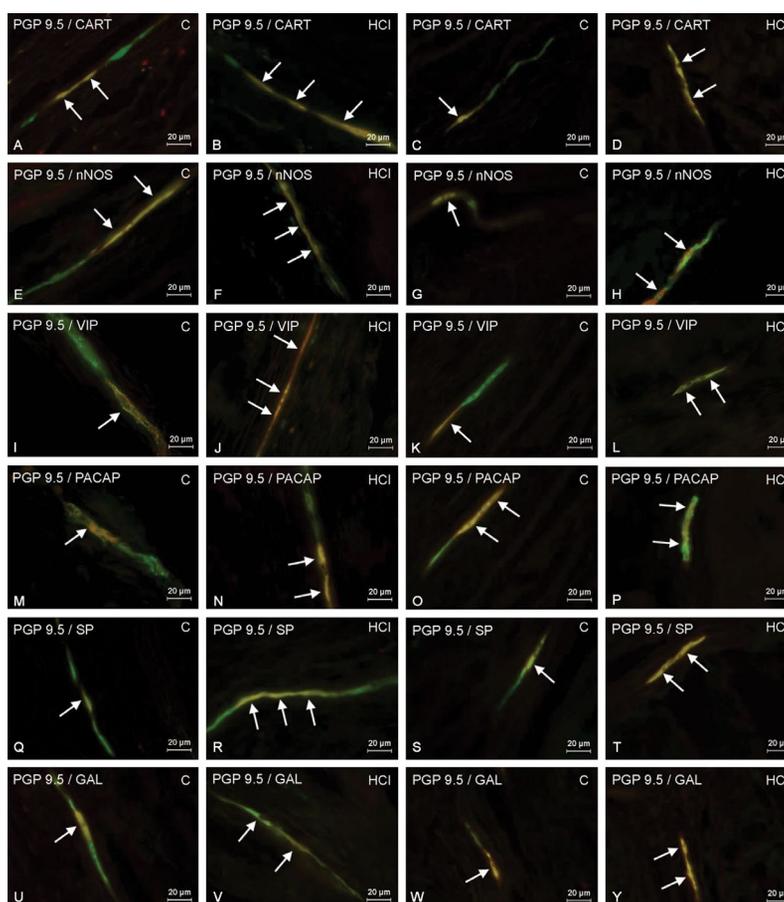

Figure 2. The immunoreactivity of the studied neuropeptides in the submucous plexus (A, B, E, F, I, J, M, N, Q, R, U, V) and myenteric plexus (C, D, G, H, K, L, O, P, S, T, W, Y) co-localized with protein gene product 9.5 (PGP 9.5) in the stomach prepyloric region in control (**C**) and hydrochloric acid-treated (HCL) pigs. The photographs have been created by digital superimposition of two color channels; PGP 9.5-positive (green) with the other studied neuronal factors (red). Neurons showing co-localization of PGP 9.5 and CART, nNOS, VIP, PACAP, SP, GAL are indicated with arrows. For abbreviations, see the description of Table 1.

Table 2. The percentage of PGP-9.5-immunoreactive prepyloric stomach enteric neurons simultaneously expressing CART, nNOS, VIP, PACAP, SP and GAL in individual control and hydrochloric acid-treated pigs

		Stomach prepyloric region				
		Submucous plexus			**Myenteric plexus**	
		Control	**HCl**		**Control**	**HCl**
CART	1	4.8	23.8	1	28.6	48.2
	2	5.6	23.4	2	28.0	49.2
	3	9.0	25.6	3	42.75	54.0
	4	8.0	25.2	4	32.4	52.4
	Average	6.85 ± 0.98	24.5 ± 0.53*	Average	32.94 ± 3.41	50.95 ± 1.35*
NOS	1	3.4	19.2	1	24.2	27.6
	2	3.6	23.4	2	21.0	30.8
	3	5.2	17.0	3	15.8	26.2
	4	2.2	19.8	4	20.6	23.0
	Average	3.6 ± 0.61	19.85 ± 1.32*	Average	20.4 ± 1.73	26.9 ± 1.61
VIP	1	1.2	11.6	1	10.2	17.4
	2	3.0	9.4	2	9.4	19.0
	3	0.6	9.8	3	7.6	18.2
	4	1.6	13.8	4	6.6	16.6
	Average	1.6 ± 0.50	11.15± 1.00*	Average	8.45 ± 0.82	17.8 ± 0.51*
PACAP	1	0.2	13.4	1	4.2	8.4
	2	1.4	9.4	2	5.6	6.6
	3	1.8	12.2	3	1.4	8.2
	4	0.4	9.8	4	2.2	11.2
	Average	0.95 ± 0.38	11.2 ± 0.95*	Average	3.35 ± 0.95	8.6 ± 0.95
SP	1	36.2	50.4	1	8.2	12.2
	2	40.2	49.4	2	9.6	13.6
	3	41.0	55.2	3	7.2	10.8
	4	35.6	48.2	4	10.4	11.6
	Average	38.25 ± 1.37	50.8 ± 1.53*	Average	8.85 ± 0.71	9.55 ± 0.59
GAL	1	23.6	44.2	1	13.8	23.2
	2	25.8	43.4	2	10.2	19.2
	3	19.4	38.6	3	15.6	22.8
	4	22.6	40.2	4	12.4	27.4
	Average	22.85 ± 1.33	41.6 ± 1.32*	Average	13.0 ± 1.14	23.15 ± 1.67*

*For abbreviations. see the description of Table 1. Means and standard errors of the mean for each group of animals and studied antigens are provided. The significance of differences was evaluated using Student's t test for independent samples. The differences were considered statistically significant at *P ≤ 0.05.

whereas in the myenteric plexus an increase from 8% in control animals to 18% in experimental animals was noted.

PACAP was found in 1% of submucous neurons of untested animals and 11% of neurons of investigated pigs. In the myenteric plexus, PACAP-IR cells constituted 3% of the referred PGP-9.5-IR control population and 9% in HCl-treated animals.

In the submucous plexus, SP expression was found in 38% of the enteric neurons, while in experimental group it constituted 51% of the total population. The myenteric plexus contained 9% of the SP-positive cells in physiological control and 10% in the experimental group.

GAL-IR submucous nerve cells constituted 23% of the reference neurons in control animals, while

HCl treatment caused their increase to 42%. In the myenteric plexus, GAL-expressing neurons in the control group constituted 13% of the total, and in HCl-treated animals they accounted for 23%.

Gastroscopic examination of control and HCl group on the first day and control on the seventh day did not reveal any inflammatory symptoms in the gastric mucosa, while HCl group on the seventh day past gastric HCl instillation revealed inflammatory changes of the mucosa like hyperemia and superficial erosions.

Discussion

This experiment has shown, for the first time, that hyperacidity conditions cause a significant increase in the expression of CART, nNOS, VIP, PACAP, SP and GAL in the submucous gastric neurons of the pig. In the neurons of myenteric plexus, a significant increase of VIP-, CART- and GAL-immunoreactivity was found. nNOS and PACAP showed tendency to increase, although this was not statistically significant, while SP expression did not change. The current data clearly show that in the myenteric plexus SP plays a minor, if any, role in the adaptation of myenteric neurons to the hyperacidic gastric *milieu*. This finding is generally consistent with our previous report that intragastric hydrochloric acid infusion, mimicking hyperacidity state, affected the chemical plasticity of the stomach extrinsic sympathetic neurons [9]. Moreover, we identified specific neurotransmitters involved in the pathology and indicated their precise allocation in the ENS structures and chemically specific neuronal groups which participated in the adaptive response. This allocation bears functional connotation, since neurons forming myenteric plexus are thought to be responsible for control of the stomach and gut motor functions, whereas submucous neurons mainly regulate mucosal secretion [20–22]. An increased expression of the studied substances in both plexuses (except SP in the myenteric plexus) suggests that a hyperacidity-induced adaptive reaction in the stomach wall affects both secretory and motor functions.

It has been well-documented that disproportionate stress [31, 32] or gastrin upregulation [33] underlies the formation of stomach ulcers and excessive secretion of acids is conductive to the development of gastric cancer [34]. Opposing those effects, mucus and bicarbonate secretion form the first line of defense in protecting gastric epithelial cells from acids and digestive enzymes [1, 11]. It has been increasingly realized that enteric neurotransmitters are actively engaged in the regulation of both gastric acid secretion as well as the formation of the mucous barrier [35].

One of the factors suppressing gastric acid secretion is CART [12, 13]. Since its discovery in 1981, CART [36] has been reported to be present in the stomach enteric neurons of numerous species, including those particularly susceptible to ulcer formation, such as humans [27] and pigs [24]. CART has been also implicated as regulator of nitric oxide release [13]. Intriguingly, many authors have reported on the ulcer-protective influence of NO in the stomach [14, 15]. Indeed, NO is an important component of the gastroduodenal defense system due to its inhibitory effects on gastric acid secretion [37]. Moreover in the stomach, enteric neurons co-localize NOS with VIP and in isolated myenteric ganglia VIP induced NO release, and NO facilitated VIP release [38]. VIP attenuates HCl secretion *via* downregulating gastrin secretion [16]. This multi-factorial anti-acidic action is additionally supported by calcitonin-gene-related peptide (CGRP) which has been detected in the stomach neurons [26, 39]. An inhibitory action on gastric acid secretion is also exerted by GAL which was found to be overexpressed in applied here model of hyperacidity [40]. Therefore, CART-, nNOS-, VIP-, CGRP and GAL-immunoreactive enteric neurons of the submucous plexus are very likely to contribute to a complex inhibitory mechanism that prevents gastric hyperacidity and, consequently, gastric mucosal destruction. In contrast, VIP-related PACAP has an opposite, stimulatory action on HCl release [16]. This opposite action of PACAP confirms the existence of a two-way neuronal inhibitory/excitatory control of the acid secretion.

Stomach epithelial functions are mainly regulated by the submucous plexus which in cooperation with primary afferent sensory neurons innervate gastric mucosal and submucosal vessels and through regulation of the mucosal blood flow affect mucosal secretion [20]. Mucus secretion protects epithelial cells from acids and digestive enzymes, abrasion by food particles, and pathogens. An increase in mucus thickness is a routine defensive response to luminal insults. Mucus traps secreted bicarbonate on the surface of the epithelium, forming a neutral pH layer that enables epithelial protection and repair [41]. Nitric oxide, a potent vasodilator [42], increases epithelial mucus secretion *via* activation of guanylyl cyclase in stomach epithelium [43]. Our study revealed increased expression of CART and SP in the submucous plexus of experimental pigs. It is clear that CART, through stimulation of NO release [13] parallelly to SP [44] may modulate local blood flow and affect mucus secretion. Indeed, the occurrence of NO-synthesizing neurons demonstrated in this study throughout the submucous plexus, coupled with its high perme-

ability range (up to 300 μm) [45], enables NO-ergic neurons to affect multiple targets in the stomach's epithelium and in the sub-epithelial layer of the mucosa which comprises blood vessels, non-epithelial cells and other enteric nerve cells.

Stomach acidification, parallel to mucus, is known to increase secretion of bicarbonate ions by the epithelium and their retention within the mucus gel layer [11]. The main role of bicarbonate is to neutralize hydrogen ions and pepsin invading from the lumen [35]. Consistently, bicarbonate secretion has been linked with CGRP-dependent NO formation and local hyperemia [46]. It was also reported that GLP-2 which is released by luminal nutrients, stimulates in human and pig tissues bicarbonate secretion *via* VIP-ergic submucous neurons which express GLP-2 receptor [47, 48]. Although this stimulation is mediated by NO-ergic pathway and comprises local hyperemia, it appears to be independent of the above-mentioned capsaicin-sensitive afferent connections [49].

In humans, vagal stimulation results in parallel bicarbonate and gastric acid secretion [50]. Available data show that PACAP stimulates bicarbonate secretion in the duodenum [38, 51] as well as gastric acid secretion in the stomach [16]. Our previous findings revealed in aspirin-evoked pig model of stomach inflammation increased expression of PACAP as well as *de novo* expression of VIP, NOS and GAL in numerous stomach-supplying perikarya located in the dorsal motor vagal nucleus (DMX) [52]. Thus, activation of the parasympathetic stomach supplying DMX-located neurons triggers interneuronal signaling mechanisms which promote the release of the neurotransmitters into the gastric submucosal space. In the light of these findings, vagal mediators PACAP, VIP, NO and GAL released into submucosal space are likely to affect multiple aspects of the gastric secretion and possibly the function of enteric neurons. The fact that PACAP, VIP, NO and GAL are up-regulated simultaneously in vagal DMX, as well as myenteric and submucous neurons indicates their complementary role in the adaptive neuronal processes, including hyperacidity-evoked gastritis. Nevertheless, the exact role of particular mediators still remains unclear and deserves further exploration.

Gastric motility is regulated by neural circuits that affect smooth muscle contractility. Current studies have shown that the gastric inhibitory vagal motor circuit that comprises DMX-located preganglionic cholinergic neurons and postganglionic neurons of the myenteric plexus also mediates inhibition of gastric emptying [53]. Vagal stimulation of the myenteric neurons relaxes the smooth muscles by releasing VIP and NO [54]. This increased relaxation may account

for gastrointestinal motility disorders [38]. Thus, as revealed in the current experiment, the hyperacidity-evoked increased expression of VIP and NOS in myenteric neurons is congruent with the above hypothesis, especially in view of delayed gastric emptying accompanying hyperacidity-induced gastric and duodenal ulcers [29].

In conclusion, this study disclosed that stomach hyperacidity in a specific way modifies the expression of CART, nNOS, VIP, PACAP, SP and GAL in the porcine gastric neurons. Increased expression of the majority of the studied biologically active substances in both the submucous and myenteric plexuses points to the complexity of neural regulation of the gastric response to hyperacidity. The current data strongly support functional studies indicating an active role of the studied neuromodulators in local blood flow regulation, control of gastric acid secretion, mucous/bicarbonate layer formation, control of stomach motility, inflammation and neuroprotection. Nevertheless, further studies are needed to reveal exact mechanisms of regulatory action of the studied neurotransmitters in the stomach prepyloric region.

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