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Coexpresion of CART with SP, GRP, NPY and galanin in the stomach of the wild boar

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PURPOSE

In 1995, Douglas et al. identified in the rat brain mRNA for a novel signaling molecule called cocaine and amphetamine-regulated transcript (CART). The rat CART gene was found to encode a polypeptide of either 129 or116 residues in length. The biologically active CART (55-102) and CART (62-102) are produced as a result of post-translational processing of both forms of pro-CART. Further studies involving immunohistochemistry revealed that CART in numerous mammals besides central nervous system is also expressed in peripheral neurons of parasympathetic, intracardiac as well as enteric ganglia. Significance of CART at the peripheral system has been barely explored mainly due to lack of specific CART receptor(s) antagonists. The presence of CART in the gut wall may suggest that the peptide also modulates peripherally the function of the gastrointestinal tract (GIT). However, till now, it is not clear what exact role CART exerts in the enteric nervous system (ENS). Enteric neurons harbours a variety of neurotransmitters/neuropeptides which is referred to a chemical neuronal code. The coexistence of CART with several biologically active peptides has been studied in GIT of a few mammals . In last year, some attempts aiming for the neurochemical characterization of porcine CART-containing enteric neurons have been undertaken mainly in relation to small and large intestine, whereas the stomach has attracted relatively less attention. In his previous work, preliminary reported the presence of CART-immunoreactive (IR) neuronal elements in the fundus of the porcine stomach. Therefore, considering the scarcity of data regarding the presence of CART in ENS of the mammalian stomach, this study aimed to immunohistochemically evaluate the presence and chemical code of CART-IR neurons in discrete regions of the wild boar stomach (atrium, corpus and pylorus).

RESULTS

Distribution pattern of CART-immunoreactivity in the wild boar stomach is summarized in table 1.

No statistical differences were found between CART-positive/galanin-positive myenteric neurons present in the antrum (34.8 ±1.6%), corpus (33.2 ±1.7%) and pyloric part (38.7 ±1.9%) of the wild boar stomach. No visible differences in distribution patterns of CART-IR/galanin-IR nerve fibres were found between all regions wild boar stomach. In general, CART-IR/galanin-IR nerve fibres were frequently seen in the circular smooth muscle layer (but not in the longitudinal muscle layer). Lamina muscluaris mucosae contained single to moderate numbers of CART-IR/galanin-IR nerve fibres. Between myenteric neurons and submucous neurons, CART-IR/galanin-IR nerve fibres were incidentally found. Galanin-IR submucous neurons were frequently encircled by CART-containing nerve terminals.

Relatively small population of CART-IR/ SP-IR myenteric neurons (less then <1%) was also found in the corpus of the wild boar stomach. Statistically higher ($P < 0.05$) proportions of CART-IR/SP-IR myenteric neurons were found in the antrum (5.2 ± 0.3%) and pylorus (4.9 ± 0.4%) of the wild boar stomach Single CART-IR/SP-IR nerve fibres were also detected in lamina muscularis muscosae of the antrum, corpus and pylorus of the wild boar. In the smooth muscle layer of the antrum and pylorus (but not in the corpus) of the wild boar stomach, the densities of CARTIR/SP-IR nerve fibres were assessed as moderate.

In the antrum of the wild boar stomach, 4.6 ±0.5% of CART-IR myenteric neurons showed immunoreactivity to NPY. Statistically similar proportions of CART-IR/NPY-IR myenteric neurons were found in the corpus (3.8 ±0.4%) and pyloric part (4.2 ±0.7%) of the wild boar stomach. In all regions of the wild boar stomach, single CART-IR/NPY-IR nerve fibres were noted only in the circular (but not longitudinal) smooth muscle layer. No presence of NPY was visualized in CART-IR nerve fibres present in lamina muscularis mucosae and those running between myenteric and submucous neurons.

In neither CART-IR enteric neurons nor CART-positive nerve fibres the presence of GRP was found.

CONCLUSIONS

In Iconclusion, in this study, the existence and distribution patterns of CART in discrete regions of the wild boar stomach were described in details. Colocalization studies revealed that in this animal species, a functional cooperation of CART with several neuropeptides is likely.

	Wild Boar		
	Antrum	*Corpus*	*Pylorus*
Myenteric ganglia			
Neurons	41.7 ± 3.2%	36.0 ± 2.2%	35.8 ± 3.5%
Nerve fibres	+++	+++	+++
Submucous ganglia			
Neurons	-	-	-
Nerve fibres	+	+	+
Smooth muscle layer			
Longitudinal muscle	+++	+++	+++
Circular muscle	++++	++++	++++
Submucous layer	-	-	-
Mucous layer			
Lamina muscularis mucosae	++	++	++
Mucosal glands	-	-	-

Table 1. Expression patterns of CART in enteric nervous system of the stomach of the wild boar. Semi-quantitative scale: absent (-), single (+), moderate (++), numerous (+++) and very numerous (++++)

MATERIAL AND METHODS

Five wild boars (weighing ca. 10-15 kg) of both sexes were used in the study.

The stomach samples were collected from the bodies of hunted animals killed during the hunting season 2011-2012. In each animal, samples of the antrum, corpus and pyloric part were cut-off and immediately washed in cold (4 C) 0.01 M phosphate-buffered saline (PBS; pH=7.3). Next, the tissues were fixed by immersion for 48 h in cold (4 C) Stefanini's solution. After the fixation, the stomach samples were placed for several days in cold 10% sucrose-containing cryoprotective Tyrode's solution. Finally, the materials were mounted on a wooden block, embedded in O.T.C. compound and left to freeze in dry ice. Using a cryostat, serial longitudinal and transverse sections of 10 mm thickness were made. Every fifth section was placed on glass slide and stored at 20 C for further immunohistochemical studies.

The cryostat sections were rehydrated at room temperature (RT) with three changes in 0.01 M phosphate-buffered saline (PBS; pH 7.3) containing 0.25 % bovine serum albumin and 0.25 % Triton X-100. To visualize CART-positive gastric neurons, a combination of mouse antibodies raised against general neuronal marker Hu C/D proteins (1:800; Molecular Probes, OR, USA) and rabbit anti-CART antibodies (1:10000; Phoenix Pharmaceuticals, Burlingame, CA, USA) were used. To examine the colocalization of CART with neuropeptides, rabbit CART antibodies were mixed with one of the following: guinea pig antibodies raised against galanin (1:300; Peninsula, San Carlos, CA, USA); rat antibodies raised against substance P (SP; 1:200, AbDSerotec, Oxford, UK), rat anti-NPY antibodies (1:700; Biomol International, Exeter, UK). To visualize bound primary antibodies, the following secondary antisera were used: Texas Red-conjugated anti-rabbit goat IgG (1:400; MP Biomedicals, Cleveland, OH, USA), FITC-conjugated anti-mouse goat IgG (1:400; MP Biomedicals); FITC-conjugated anti-rat goat IgG (1:400; MP Biomedicals), FITC-conjugated anti-guinea-pig goat IgG (1:400; MP Biomedicals).

As a control, the specificity of positive staining was tested using a pre-absorption study. In the control staining, the specific antiserum was replaced by the same antiserum, which had been preincubated with the corresponding antigen (10100 µg of blocking substance per 1 ml of diluted antiserum). In these staining with the preabsorbed antisera, no specific immunoreactions were observed. Incubation of cryostat sections in solution lacking primary antibodies (substituted with a normal serum) also served as a negative control.

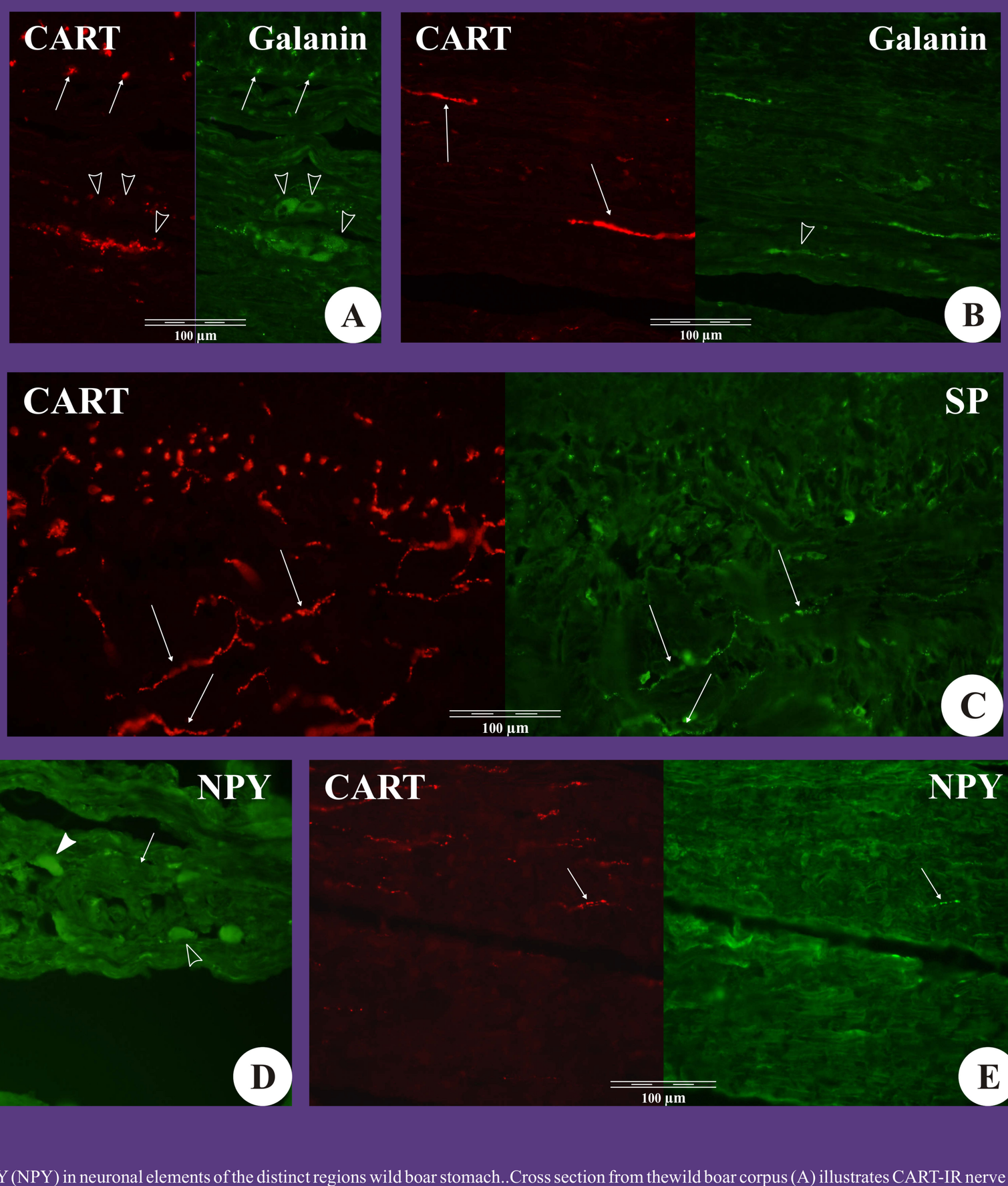

Fig. 2. Coexpression patterns of CART with galanin, substance P (SP) and neuropeptide Y (NPY) in neuronal elements of the distinct regions wild boar stomach..Cross section from thewild boar corpus (A) illustrates CART-IR nerve fibres running between galanin-positive/CART-negative submucous neurons; note that colocalization of CART and galanin is also seen in nerve fibres of the circular smooth muscle layer (arrows). (B) CART-IR/galanin-IR (arrows) nerve fibres were also observed in lamina muscularis mucosae of the pylorus of the wild boar stomach; empty arrowhead indicates galanin-IR nerve fibres lacking CART. Arrows in (C) represent CART-IR/SP-IR nerve fibres found in the smooth muscle layer of the pylorus of the wild boar stomach. (D) In myenteric ganglion from the wild boar stomach antrum, CART-IR/NPY-IR neurons are marked with arrowheads, whereas CART-positive/NPY-negative and CARTnegative/NPY-positive periakrya are indicated with arrow and empty arrowheads (respectively). In the wild boar stomach pylorus (E), the vast majority of the circular muscle-supplying CART-IR nerve fibres are devoid of NPY, but in single nerve fibres (arrow), the colocalization of both neuropeptides can be found.