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# Cocaine- and amphetamine-regulated transcript (CART) peptide in the enteric nervous system of the porcine esophagus

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*Abbreviations :*
CART, cocaine- and amphetamine/regulated transcript peptide
CGRP, calcitonin gene related peptide
CY3, cyanine 3
ENS, enteric nervous system
FITC, fluorescein isothiocyanate
GAL, galanin
-LI, -like immunoreactive
LENK, leu-enkephalin
nNOS, neuronal isoform of nitric oxide synthase
NPY, neuropeptide Y
VAChT, vesicular acetylcholine transporter
VIP, vasoactive intestinal polypeptide
PGP 9.5, protein gene/product 9.5
SOM, somatostatin
SP, substance P.

ABSTRACT

Cocaine- and amphetamine-regulated transcript peptide (CART) is widely distributed within the central and peripheral nervous system. In the brain, CART is considered as the main anorectic peptide involved in the regulation of food intake. Contrary to the central nervous system, a lot of aspects connected with the distribution and functions of CART within the enteric nervous system (ENS) still remain unknown. The aim of the present study was to investigate, for the first time, the population of CART-like immunoreactive (CART-LI) neurons within the porcine esophagus and the denotation of their neurochemical coding. During this experiment, the distribution of CART-LI neurons and the colocalization of CART with other neuronal active substances were examined using standard double- and triple-immunofluorescence techniques in enteric plexuses of cervical, thoracic, and abdominal esophagus fragments. The obtained results showed that CART is present in a relatively high percentage of esophageal neurons (values fluctuated from $45.2 \pm 0.9\%$ in the submucous plexus of the thoracic esophagus to $58.1 \pm 5.0\%$ in the myenteric plexus of the same fragment of the esophagus). Moreover, CART colocalized with a wide range of other active neuronal substances, mainly with the vesicular acetylcholine transporter (VAChT, a marker of cholinergic neurons), neuronal isoform of nitric oxide synthase (nNOS, a marker of nitrergic neurons), vasoactive intestinal polypeptide (VIP) and galanin (GAL). The number of CART-positive neuronal cells and their neurochemical coding clearly depended on the fragment of esophagus studied and the type of enteric plexus. The obtained results suggest that CART may play important and multidirectional roles in the neuronal regulation of esophageal functions.



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## 1. Introduction

The gastrointestinal (GI) tract is supplied by different types of neurons. The extrinsic innervation of the esophagus, the stomach, and the intestine comprises projections of parasympathetic [1], sympathetic [2], and spinal neurons [3]. Parasympathetic efferent innervation of the major part of the digestive tract (from the esophagus to the transverse colon) is supplied by the vagal nerve and neurons located within dorsal motor nucleus of the vagal nerve (DMV) within the brainstem [4,5]. However, the posterior segments of the GI tract (from the descending colon to the anus) are innervated mainly by projections of neurons positioned in dorsal intermediolateral column of the sacral spinal cord (neuromeres S1–S4) [4,6]. Sympathetic efferent innervation of the digestive tract is realized by postganglionic nerves, which are the projections of neuronal cells located in the sympathetic chain ganglia and prevertebral ganglia of the abdominal and pelvic areas, such as the celiac, superior, and inferior mesenteric or pelvic ganglia [2,7]. The exact distribution of these neurons clearly depends on the innervated by them fragments of the digestive tract. The third component of the extrinsic innervation of the digestive tract is an afferent innervation, which conveys sensory and pain stimuli from the GI tract to the central nervous system. Sensory neurons supplying the digestive tract are located in the nodose ganglia of the vagal nerve [8] or the dorsal root ganglia [3,9] in various neuromeres (according to the innervated fragment of the GI tract).

Apart from the extrinsic innervation, the GI tract is supplied by the enteric nervous system (ENS), located in the wall of the digestive tract and containing millions of neuronal cells grouped in ganglionated plexuses [10–13]. The types of these plexuses depend on the animal species and the fragment of the digestive tract. In large domestic mammals (including the pig), the ENS in the esophagus and stomach is built of two types of plexuses: the myenteric plexus–located between longitudinal and circular muscle layers and submucous plexus–near the lamina propria of the mucosal layer, whereas, in the intestine, the submucous plexus is divided into outer submucous plexus–positioned along the inside of the circular muscle layer and inner submucous plexus–situated like the submucous plexus in rodents [14]. Enteric neurons show wide variations in terms of their morphology, functions, and electrophysiological properties [10,15].

The most important criterion useful in the division of enteric neurons into various subclasses is their neurochemical coding [15]. Apart from acetylcholine–the classic neuromediator of the ENS, several dozens of other neuronal active substances acting as neuromediators and/or neuromodulators have been described in enteric neuronal structures [16,17]. The most important of them are: vasoactive intestinal polypeptide (VIP), nitric oxide (NO), galanin (GAL), substance P (SP), and many other ones [16,18,19]. From a wide range of neuronal factors, that have been described in enteric nervous structures, cocaine- and amphetamine-regulated transcript peptide (CART) is one of the less known substances. Till now CART has been described in various parts of both central and peripheral nervous systems, especially within the ENS [20,21], where this peptide has been noted in the myenteric and submucous enteric plexuses and muscular and in mucosal intestinal layers of various mammal species, including humans, and the number of them clearly depended on the fragment of the digestive tract studied [21–23].

Contrary to the central nervous system, where first of all CART is known as a main anorectic peptide involved in the regulation of food intake [21], exact roles of this peptide in the ENS still remain unclear. Admittedly previous studies showed that CART may reduce gastric acid secretion and stimulate colonic motility [21]. The mechanisms of these actions, which most likely proceed with the participation of the central nervous system [20,24] are unknown. Moreover, especially high expression of CART in myenteric plexuses and intramuscular nerve fibers [25,26] can suggest the important role of this peptide in intestinal motility, and changes in its expression under various pathological processes [14,27] denote neuroprotective and/or neurotrophic activities.

Contrary to the stomach and intestine, the knowledge concerning the distribution and functions of CART in the esophageal ENS is very limited [23]. Therefore, the aim of the present investigation was the exact determination of the distribution of CART, as well as the colocalization of this peptide with other better known neuronal factors in the ENS of the esophagus in the domestic pig. It should be underlined that this species, due to considerable similarities to human with respect to the anatomy and physiology of enteric neurons, is often used as an animal model for studying the processes taking place in the human digestive tract [28].

## 2. Materials and methods

The present experiment was carried out using six immature female pigs of the Large White Polish breed (approximately 8 weeks old and 18 kg of body weight). During the investigation, pigs were kept under standard laboratory conditions. The animals were housed in pens with an area of about 4 $m^2$ (three pigs in one pen) with unlimited access to water and nourished twice a day with complete feed appropriate to species and age. All procedures connected to the experiment were performed according to the instructions of the Local Ethical Committee, Olsztyn (Poland).

After a four-day adaptation period, pigs were pretreated with Stressnil (Janssen, Belgium, 75 μl/kg of body weight, i.m.) and, after 15 min, were subjected to euthanasia by an overdose of sodium thiopental (Thiopental, Sandoz, Kundl, Austria) given intravenously. Then animals were perfused transcardially with 4% buffered paraformaldehyde prepared ex tempore. Two-centimeter-long segments of the cervical, thoracic, and abdominal esophagus were collected. They were post-fixed by immersion in 4% buffered paraformaldehyde for 30 min, rinsed in phosphate buffer for three days, and stored at 4 °C in 18% buffered sucrose solution for at least two weeks. Then samples were frozen at −25 °C and cut into