

# Immunohistochemical distribution of cocaine- and amphetamine-regulated transcript peptide - like immunoreactive (CART-LI) nerve fibers and various degree of co-localization with other neuronal factors in the circular muscle layer of human descending colon

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**Summary.** Cocaine- and amphetamine-regulated transcript peptide (CART) is a neuromediator and/or neuromodulator in nerve structures within the gastrointestinal tract, but knowledge about its distribution, functions and co-localisation with other neuronal factors, especially in humans, is very scarce.

During the present investigation the distribution and immunohistochemical reaction (IR) of CART - like immunoreactive (CART-LI) nerve fibers in the circular muscle layer of human descending colon were studied. Fragments of human colon were processed for double labelling immunofluorescence using a mixture of anti-CART antibodies with antibodies against vesicular acetylcholine transporter (VAcHT), vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase - activating peptide (PACAP), substance P (SP), galanin (GAL) and nitric oxide synthase (NOS).

Thick CART-LI nerve fibers formed a very dense meshwork within the colonic circular muscle layer in all patients studied. The highest number of CART - positive nerves also contained VAcHT and/or VIP. A slightly lower level of co-localisation was observed in the case of CART and PACAP or CART and NOS. Only single nerve fibers were concurrently immunoreactive to CART and SP or CART and GAL. The present study reports for the first time a detailed description of the IR of CART-LI nerve fibers in the circular muscle layer within adult human descending colon.

**Key words:** Cocaine- and amphetamine-regulated transcript (CART), Descending colon, Immunohistochemistry, Human

## Introduction

It is well established that nerve structures in human descending colon can contain a broad spectrum of neuromediators and/or neuromodulators (Krammer et al., 1994; Wattchow et al., 1997; Krantis et al., 1998; Wedel et al., 1999; Porter et al., 2002; Murphy et al., 2007; De Fontgalland et al., 2008; Harrington et al., 2010; King et al., 2010; Southwell et al., 2010). One of them is cocaine-and amphetamine-regulated transcript peptide (CART), which was described for the first time in 1981 in ovine hypothalamus (Spiess et al., 1981). Up till now this peptide has been observed in various parts of nervous system, including neurons of the ENS (Kuhar and Dall Vechia, 1999; Murphy et al., 2000; Ekblad, 2006; Wierup et al., 2007; Gunnarsdottir et al., 2007; Gonkowski et al. 2009a,b).

In spite of the fact that a broad spectrum of various functions of CART has been suggested (in stress responses, ontogenesis of nervous system, sensory information processing and feeding behavior (Douglass et al., 1995; Kuhar and Dall Vechia, 1999; Risold et al., 2006), the exact functions of this peptide in the GI tract have not yet been completely explained. It is known that CART is involved in the inhibition of feeding (Kristensen et al., 1998), exacerbation of colonic

motility (Tebbe et al., 2004) and reduction of gastric acid secretion (Okumura et al., 2000). These functions are probably realized via regulatory circuits located within the central nervous system, because investigations on the motor activity of isolated wall fragments of stomach, as well as small and large intestine, were not able to reveal any contractile or relaxatory activity of CART (Ekblad et al., 2003).

Moreover, some previous studies, which described changes in the number of CART - positive nervous structures in the ENS under pathological processes of GI tract (Gunnarsdottir et al., 2007; Gonkowski et al., 2009b, 2012), as well as the co-localization of CART and VIP, known as an important neuroprotective factor (Wierup et al., 2007), may suggest neuroprotective functions of CART in the digestive system.

It should be pointed out that the knowledge concerning the distribution, immunohistochemical reaction (IR) and possible functions of CART - like immunoreactive (CART-LI) nerve structures in the human GI tract is highly fragmentary. Till now only two studies have reported on the expression of CART in the intestine of children and changes in enteric CART - like immunoreactivity during Hirschsprung's disease (Gunnarsdottir et al., 2007) and drug resistant ulcerative colitis (Gonkowski et al., 2009b).

It is also important to emphasize that one of the possible ways to suggest the functions of CART within the GI tract seems to be investigations on co-localization of this peptide with other, better known neuromediators and/or neuromodulators in the same nerve fibers. So, the aim of the present study was to determine for the first time the distribution of CART-LI nerves in the circular muscle layer of human descending colon, as well as co-localization of CART with other neuronal factors that are well known to be widespread in the GI tract. The obtained results may contribute to a better understanding of distribution and possible function of CART in regulatory processes within the human intestine.

## Materials and methods

Fragments of the descending colon were collected from patients (2 women and 3 men, mean age 61±5 years) hospitalized in the Clinical Hospital of Medical University in Gdansk (Poland) just after colectomy performed due to an incurable intestinal obstruction. The samples were taken from peripheral parts of the excised colon, which have not been shown to express any macroscopic and microscopic pathological changes. Tissue collection for immunohistochemical study did not affect the decision of the surgeon concerning resection dimension. Fragments of descending colon taken by the surgeon were about 3 cm in length, and for immunohistochemical investigations sections about 1 cm length were used. All procedures during the present study have been in accordance with ethical standards known as the Helsinki Declaration 1975 (revised 1983)

and were performed in compliance with the instructions of Bioethical Committee.

Fragments of the descending colon were immediately fixed by immersion in a solution of freshly prepared 4% buffered paraformaldehyde (pH 7.4) for thirty minutes, rinsed for 72 h in phosphate buffer (0.1 M, pH 7.4, at 4°C) and transferred into 18% phosphate-buffered sucrose, where they were kept at 4°C for at least five days until sectioning. Finally, colon fragments were frozen and fixed on glass slides, so that the cutting line was perpendicular to the lumen of gut and cut on a cryostat (-22°C) into 10-μm-thick sections.

Cryostat sections were processed for routine double-labelling immunofluorescence. Briefly, after air-drying at room temperature (rt) for 45 min, sections were incubated with a blocking solution containing 10% normal goat serum, 0.1% bovine serum albumin, 0.01% NaN<sub>3</sub>, Triton x-100 and thimerosal in PBS for 1h (rt). Then, they were incubated (overnight; rt, in a humid chamber) with a mixture of antibodies (Table 1) directed towards cocaine- and amphetamine-regulated transcript protein (CART) and one of the other neuronal factors studied e. i. vesicular acetylcholine transporter (VAcHT) - a marker of cholinergic nerves, vasoactive intestinal polypeptide (VIP), pituitary adenylate cyclase - activating peptide (PACAP), substance P (SP), galanin (GAL) or nitric oxide synthase (NOS) - a marker of nitrergic processes, raised in different species. The commercial antibody against CART (Table 1) used in this study is a monoclonal mouse IgG<sub>2B</sub> clone # 113612, which detects human CART aa 28-116 and recombinant human CART aa 69-116 in ELISA test and western blots, and the immunising antigen to production of this antibody is *E Coli* - derived recombinant human CART Ala37-Leu116. This antibody was also used in previous immunohistochemical studies (Równiak et al., 2010; Bogus-Nowakowska et al., 2011).

Complexes of primary antibodies bound to appropriate antigens were visualized by incubation (1h, rt) with species-specific secondary antisera conjugated to FITC or biotin and the latter antibodies were then visualized by a streptavidin-CY3 complex (1h; rt). Each step of immunolabelling was followed by rinsing the sections with PBS (3x10 min, pH 7.4).

Standard controls, i. e. pre-absorption of the neuropeptide antibodies with appropriate antigens (Table 1) for 18 hours at 37°C, omission and replacement of primary antibodies by non-immune sera were performed to test antibody and specificity of the method.

Nerve profiles were estimated in 4 sections *per* patient (in 5 fields *per* section). For the semiquantitative evaluation of the density of nerves immunoreactive to each substance studied, an arbitrary scale was used, where (-) means the absence of fibers (+) - single fibers, (++) - rare nervous processes, (+++) - depicts a dense and (+++++) - very dense meshwork of fibers. The same method was used for measuring the degree of co-localisation of CART with each of the other neuronal

## CART-LI nerve fibers in the human descending colon

factors studied.

### Results

The presence of nerve fibers immunoreactive to all neuronal factors studied were observed within the circular muscle layer of human descending colon. Moreover, clear differences in density of nerve processes positive for particular neuronal factors were seen (Table 2, Figs. 1, 2). The majority of nerve fibers were immunoreactive to CART, PACAP-27, VACHT, VIP and/or NOS. A slightly lower density of GAL-LI nerve

processes was observed and only rare nerve fibers showed immunoreactivity to SP. The appearance of nerve fibers depended on the type of neuronal factor for which they were immunopositive. Nerve processes immunoreactive to CART (Figs. 1, 2), VIP (Fig. 1A), PACAP-27 (Fig. 1B), GAL (Fig. 1C), VACHT (Fig. 2A), and/or NOS (Fig. 2C) were thick, very visible and built the large nerve bundles. In contrast, nerves positive for SP (Fig. 2B) were thin and delicate.

Moreover, the co-localisation of CART with all neuronal factors studied was observed and the degree of co-localization varied and depended on the type of

**Table 1.** List of antibodies and reagents used in immunohistochemical investigations.

Antigen	Code	Species	Dilution/Concentration	Supplier
<b>Primary antibodies</b>				
1	2	3	4	5
CART	MAB163	Mouse	1:1000	R&D System, Minneapolis, MN, USA
GAL	RIN7153	Rabbit	1:4000	Peninsula, San Carlos, CA, USA
NOS	AB5380	Rabbit	1:1000	Chemicon Int, Temecula, OH, USA
PACAP-27	IHC 8922	Rabbit	1:20000	Phoenix Pharmaceuticals, INC, Belmont, CA, USA
SP	8450-0505	Szczur/Rat	1:500	Biogenesis Ltd., Poole, England
VACHT	H-V005	Rabbit	1:1400	Phoenix Pharmaceuticals
VIP	VA1285	Rabbit	1:6000	Biomol, Hamburg, Germany
<b>Secondary antibodies and complexes of fluorochromes</b>				
1			4	5
Goat biotinylated anti-rabbit IgG			1:800	CN Biomedicals, Aurora, Ohio, USA
Goat biotinylated anti-rat IgG			1:800	BioTrend, Köln, Germany
Goat FITC - conjugated anti-mouse IgG			1:800	ICN Biomedicals
CY3 - conjugated Streptavidin			1:8000	Jackson
<b>Antigens used in pre-absorption tests</b>				
1		3	4	5
CART		C5977	0.1 $\mu$ M	Sigma, St Louis, MO, USA
GAL		G0278	0.5 $\mu$ M	Sigma
NOS		N3033	1.0 $\mu$ M	Sigma
PACAP-27		052-02	0.3 $\mu$ M	Phoenix Pharmaceuticals
SP		S6883	0.7 $\mu$ M	Sigma
VACHT		V007	0.6 $\mu$ M	Phoenix Pharmaceuticals
VIP		V6130	1.0 $\mu$ M	Sigma

**Table 2.** Density of nerve fibers in the circular muscle layer of human descending colon positive for particular substances, presented in arbitrary units.

Substance	Density of nerve fibers
CART	++++
VIP	++++
PACAP-27	++++
GAL	+++
VACHT	++++
SP	++
NOS	++++

++: rare nerve fibers; +++: dense meshwork of fibers; ++++: very dense meshwork of fibers.

**Table 3.** Degree of co-localisation of CART with other active substances within nerve fibers in the circular muscle layer of human descending colon, presented in arbitrary units.

Substances	Degree of co-localisation
CART/VIP	++++
CART/PACAP-27	+++
CART/GAL	+
CART/VACHT	++++
CART/SP	+
CART/NOS	+++

+: single nerve fibers; +++: dense meshwork of fibers; ++++: very dense meshwork of fibers.

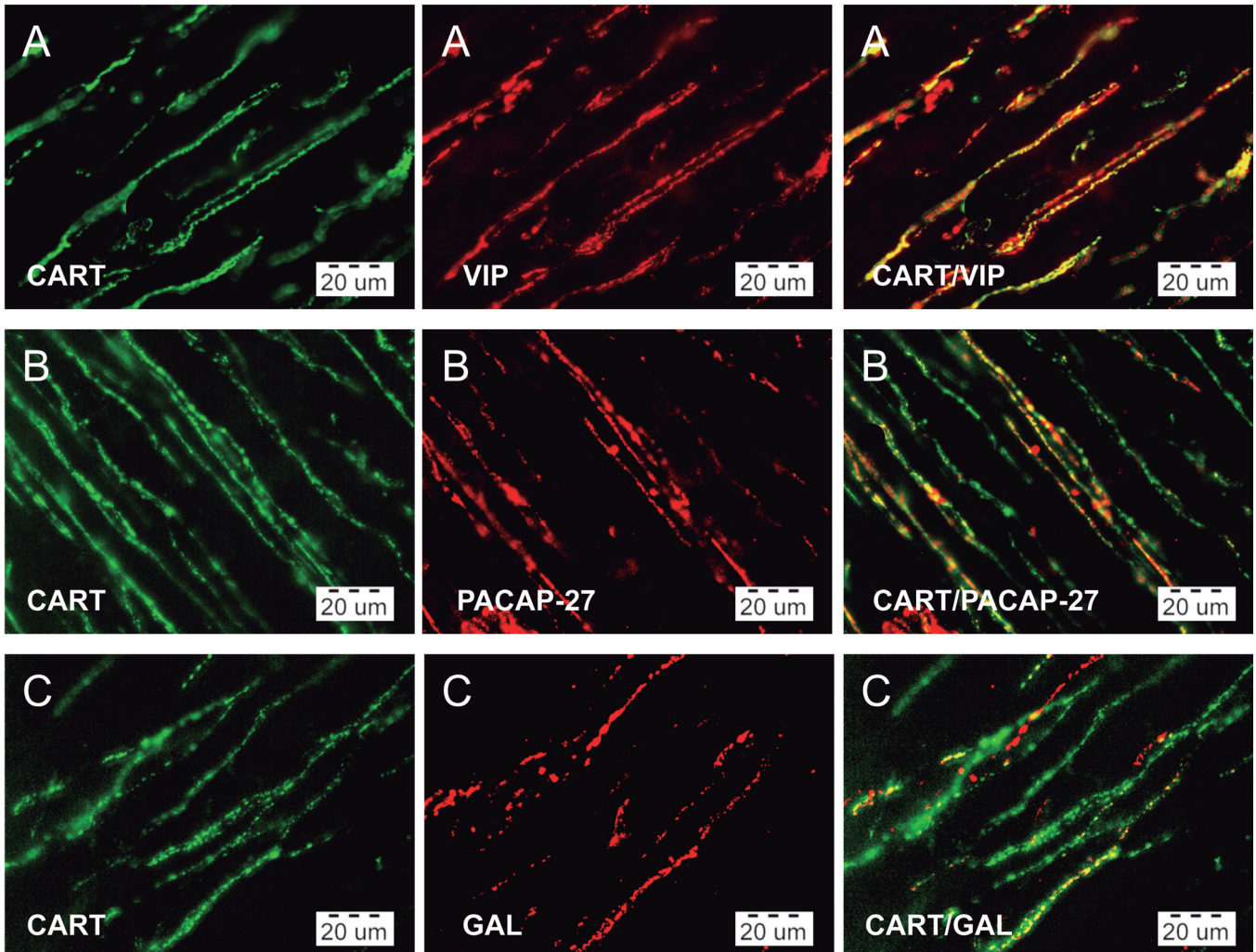
substance (Table 3, Figs. 1, 2). The highest number of CART - positive nerves also contained VAcHT (Fig. 2A) and/or VIP (Fig. 1A). A slightly lower level of co-localisation was observed in the case of CART and PACAP-27 (Fig. 1B) or CART and NOS (Fig. 2C). Only single nerve fibers were concurrently immunoreactive to CART and GAL (Fig. 1C) or CART and SP (Fig. 2B).

In the present study visible differences between particular patients both in the density of fibers immunoreactive to neuronal factors studied and the degree of co-localisation of CART with other substances were not observed.

Moreover, all standard controls of antibodies i. e. pre-absorption, omission and replacement completely eliminated specific stainings (Fig. 3).

## Discussion

During the present investigation nerve fibres immunoreactive to all neuronal factors studied were found in the circular muscle layer of human descending colon. These results are generally in agreement with previous studies, where a wide range of active factors were investigated within nerve structures of the circular muscle layer in the large intestine of humans and other species (Krammer et al., 1994; Furness et al., 1995, 2004; Wattachow et al., 1997; Krantis et al. 1998; Tebbe et al., 1998; Wedel et al., 1999; Furness, 2000; Porter et al., 2002; Murphy et al., 2007; De Fontgalland et al., 2008; Gonkowski et al., 2010; Gonkowski and Cařka, 2010, 2012; Harrington et al., 2010; King et al., 2010;



**Fig. 1.** Distribution pattern of nerve fibers immunostained for CART (green) and **A)** VIP, **B)** PACAP-27 or **C)** GAL (red) within the circular muscle layer of the human descending colon. Right column of pictures shows overlap of both stainings. Colocalisation of both antigens is presented as yellow or orange. Scale bar: 20 µm.

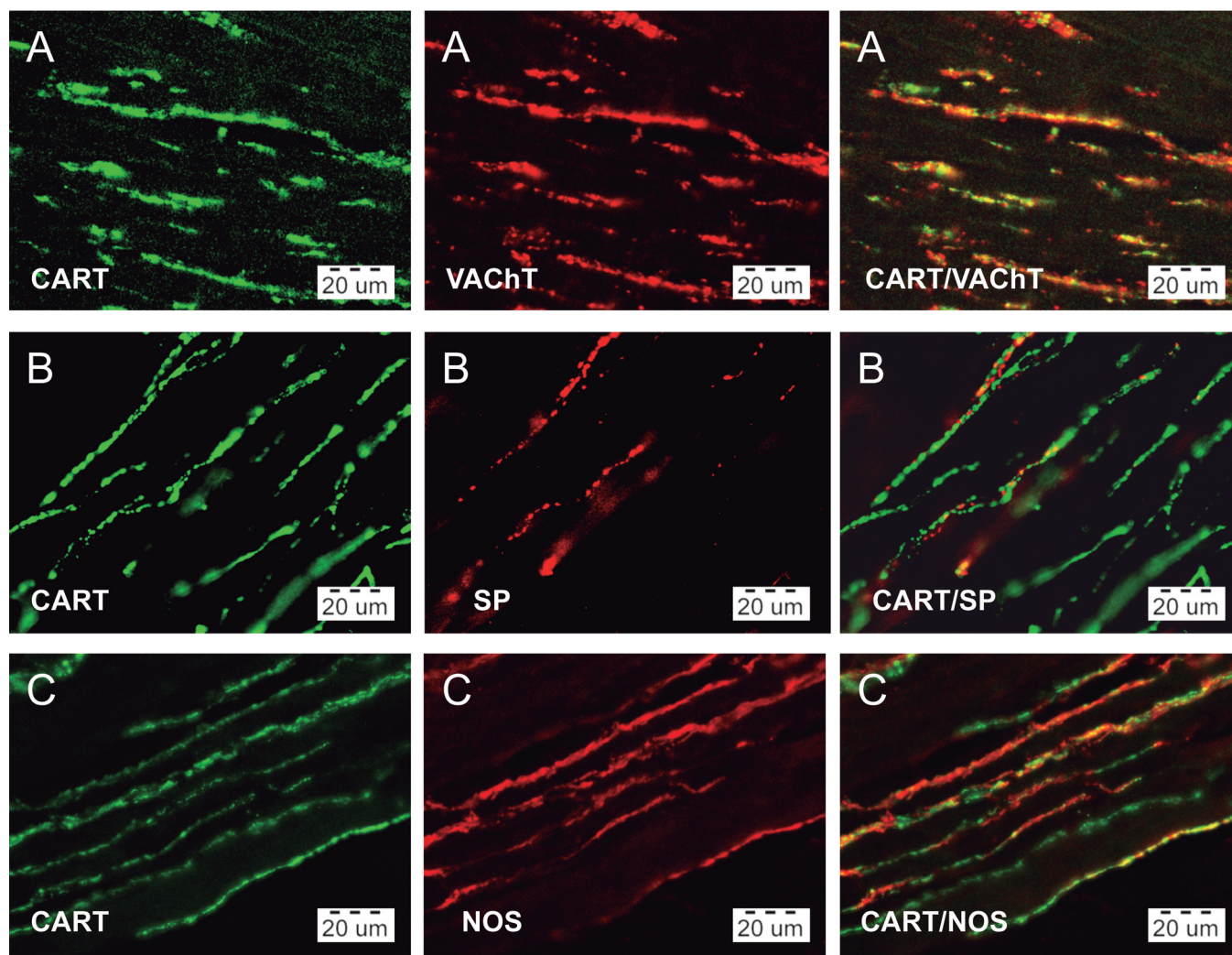
*CART-LI nerve fibers in the human descending colon*

Southwell et al., 2010).

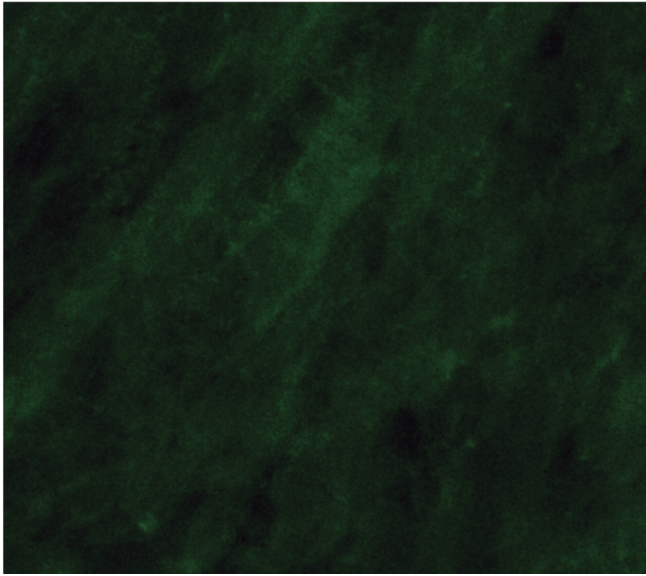
Also, a dense meshwork of CART-LI nerve fibres observed in the present study was similar to that previously observed in children (Gunnarsdóttir et al., 2007), rat (Ekblad et al., 2003), guinea pig (Ellis and Mawe, 2003) and pig (Wierup et al., 2007; Gonkowski et al., 2009a). These results strongly suggest that CART takes part in the regulation of motility of the large intestine, but the exact functions of this peptide in the GI tract are still obscure.

Till now, CART has been described in rats as an inhibitor of gastric acid secretion (Okumura et al., 2000), a reducer of colonic motility via cholinergic pathways (Tebbe et al., 2004) and an inhibitor of relaxation mediated by nitric oxide in colonic muscles in vitro,

(Ekblad et al., 2003), but on the other hand studies of the motor activity of dissected stomach wall fragments and pieces of small and large intestine were not able to reveal any contractile or relaxatory activity of CART (Ekblad et al., 2003). Moreover, some studies, where changes in expression of CART under various pathological factors were observed in the ENS (Gunnarsdóttir et al., 2007; Gonkowski et al., 2009b, 2012) suggest a neuroprotective function of this peptide. Other reports showed neurotrophic roles of CART in the central nervous system during ontogenesis (Risold et al., 2006), but knowledge about the same functions of this peptide in peripheral organs is rather scarce and limited to islet cells of the pancreas (Wierup et al. 2004). Previous investigations also showed differences in



**Fig. 2.** Distribution pattern of nerve fibers immunostained for CART (green) and **A)** VAcHT, **B)** SP or **C)** NOS (red) within the circular muscle layer of the human descending colon. Right column of pictures shows overlap of both stainings. Colocalisation of both antigens is presented as yellow or orange. Scale bar: 20  $\mu$ m.



**Fig. 3.** Labeling using the antibody against CART after pre-absorption with appropriate antigen. Scale bar: 20  $\mu$ m.

CART expression within nervous structures of the GI tract between juvenile (Gonkowski et al., 2009a) and adult pigs (Wierup et al., 2007), suggesting that the functions of this peptide are correlated to developmental events. Notwithstanding this, the expression of CART in nerve in the circular muscle layer of descending colon is similar in children (Gunnarsdottir et al., 2007) and adult humans (this study), although the number of CART-LI structures undergoes changes during ontogenesis in nervous structures outside the GI tract (Risold et al., 2006).

As already mentioned, one of the possible ways to learn and establish the functions of CART within the GI tract is studies on co-localization of this peptide with other, better known neuronal factors in the same nervous structure.

The current knowledge about the co-localisation of CART with other substances within nerve structures of the GI tract is also fragmentary, but it is known that it depends on the segment of digestive tract, the type of enteric nervous structures, as well as the animal species studied. In rat stomach, small and large intestine CART-positive neurons in the myenteric plexus also contained choline acetyltransferase (ChAT), VIP and NOS (Couceyro et al., 1998; Ekblad et al., 2003), while in the submucous plexus of rat small and large intestine the co-localisation of CART and NOS was not found (Ekblad et al., 2003). On the other hand, in the ileum of guinea pig CART-LI enteric neurons were also immunoreactive to ChAT, tachykinins, calbindin, NOS and VIP (Ellis and Mawe, 2003) and in the large intestine of children the co-localisation of CART and VIP and/or NOS was

observed (Gunnarsdottir et al., 2007).

In the present study the majority of CART-LI nerve fibers were also immunoreactive to VAcHT and/or VIP, which is in agreement with previous investigations (Couceyro et al., 1998; Ekblad et al., 2003; Gunnarsdottir et al., 2007). VAcHT is a marker of acetylcholine, which is generally known as a very prevalent and important excitatory neurotransmitter within the ENS, acting on intestinal muscles and glands (Furness, 2000). VIP is also widely distributed in nerve structures within the GI tract, but in contrast to acetylcholine it is known as an inhibitor of smooth muscle contractions (Geldre and Lefebvre, 2004; El-Mahmoudy et al., 2006; Kasperek et al., 2007).

A slightly lower level of co-localisation was observed in the case of CART and PACAP, as well as CART and NOS, which is a marker of nitrergic neurons. Both PACAP and nitric oxide (NO) are involved in inhibitory processes within the GI tract. They induce muscle relaxation in the stomach, small and large intestine and exert inhibitory effects on gut secretion of various hormones and electrolytes (Murray et al., 1991; Cox, 1992; Sarna et al., 1993; Schleiffer and Raul, 1997; Kuwahara et al., 1998; L uffler et al., 1999).

During this experiment only single CART-LI nerve fibers were also immunoreactive to GAL and/or SP. The effects of these substances on the GI tract are multifaceted and depend on the species and segments of digestive system. It is known that GAL induces the contraction of the muscles in the ileum of the rat, guinea-pig, rabbit and pig (Botella et al., 1992), while in the canine pylorus and ileum it displays relaxatory activity (Fox-Threlkeld et al., 1991). However, SP shows, for instance, strong contractive action on intestinal muscle in rats (L rdal et al., 1993) and dogs (Thor et al., 1982) in which is contrast to humans, where this function of SP was quite reduced (L rdal et al., 1997).

To sum up, the obtained results showed that CART is present in a dense meshwork of nerve fibres within the circular muscle layer of descending colon of adult humans. These fibres can derive from extrinsic neurons supplying the GI tract, as well as from the enteric nervous system, and the majority of them are involved in the regulation of gut motility. Moreover, the co-localisation of CART with a number of other various neuronal factors was observed in fibres studied. This fact can suggest that CART is present in various types of nerves and can play diverse functions both in motor activity and intestinal secretion in the GI tract. Interestingly, CART also co-localised with substances exhibiting opposed action, namely, for example, with acetylcholine and VIP. Most likely, CART may act as a co-transmitter and/or neuromodulator for neuronal factors, which co-localize with this peptide in the same nerves, but its exact functions within the GI tract, as well as problems concerning possible interplay and interactions of CART with other active factors in the nervous system are not fully explained and require

## *CART-LI nerve fibers in the human descending colon*

further investigation.

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