Original article

Inflammation- and axotomy-induced changes in cocaine- and amphetamine-regulated transcript peptide-like immunoreactive (CART-LI) nervous structures in the porcine descending colon

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Abstract

This study reports on changes in CART-like immunoreactive (CART-LI) nerve structures in the porcine descending colon during chemically driven inflammation and after axotomy. The distribution pattern of CART-LI nerve structures was studied using doublelabeling immunofluorescence technique in the circular muscle layer, myenteric (MP), outer submucous (OSP) and inner submucous plexuses (ISP) and also in the mucosal layer of the porcine descending colon in physiological conditions as well as under pathological factors. In the control animals, CART-LI perikarya have been shown to constitute 5.11% ± 0.64, 4.03% ± 1.17 and 0.05% ± 0.04 in MP, OSP and ISP, respectively.

Changes in CART-immunoreactivity depended on the pathological factor and the part of the enteric nervous system (ENS) studied. Numbers of CART-LI perikarya amounted to 2.77% ± 0.64, 2.60% ± 0.36 and 0.26% ± 0.19 during chemically-induced colitis and 3.04% ± 0.88, 2.46% ± 0.8 and 0.43% ± 0.09 after axotomy in MP, OSP and ISP, respectively.

Both studied pathological processes also caused an increase in the number of CART-LI nerve fibers in the circular muscle as well as in the mucosal layer.

Key words: CART, enteric nervous system (ENS), descending colon, pig, immunohistochemistry

Introduction

The first cocaine- and amphetamine-regulated transcript peptide (CART) was isolated for the first time in 1981 from the ovine hypothalamus (Spiess et al. 1981). Now it is known that there are several isoforms of this peptide. CART has been found within the enteric nervous system (ENS) in different parts of the gastrointestinal tract (GI) (for review, see Ekblad 2006) of numerous species including the pig (Gonkowski et al. 2009a) and humans (Gunnarsdóttir et al. 2007, Wierup et al. 2007, Gonkowski et al. 2009b). A broad spectrum of different functions has been proposed for CART, it plays a role in stress responses (Koylu et al. 2006), feeding behavior (Kristensen et al. 1998, Hunter et al. 2004), reduction of

gastric acid secretion (Okumura et al. 2000) and exacerbation of colonic motility (Tebbe et al. 2004). Until now, however the detailed functions of CART within the GI tract, especially under pathological conditions, have not yet been completely explained (Gunnarsdóttir et al. 2007, Gonkowski et al. 2009b). On the other hand, it is well known that neurons of the ENS can change their functional and structural as well as chemical phenotype as a result of adaptive responses to different factors, including a wide array of intestinal and extra-intestinal diseases or nerve injury (Ekblad et al 1999, Vasina et al. 2006, Pidsudko et al. 2008, Gonkowski et al 2010). Many aspects of the etiology and pathogenesis of diseases such as inflammatory bowel disease (IBD), irritable bowel syndrome (IBS) and others, still remains unknown, although increasingly there are reports confirming the major role that this diseases plays in the nervous system (Delvaux 2004, Mawdsley and Rampton 2005, Hou et al. 2009, Pellissier et al. 2010). The effects exerted on ENS by inflammation or axotomy are still poorly understood and changes in the number of CART-LI structures are unknown.

The aim of this study was to indicate possible inflammation- and axotomy-induced changes in CART-immunoreactivity within nervous structures in the porcine descending colon. It is necessary to contribute to a better understanding of the possible functions of the CART peptides in pathological processes within the large intestine of the pig – an important animal model for biomedical research on animal and human physiology and pathology investigations (Crowe and Burnstock 1989, Swindle 1992, Verma et al. 2011). These results should provide a basis for further investigations, necessary for the elucidation of CART function within the GI.

Materials and Methods

The present study was performed on sixteen immature female pigs of the Large White Polish breed (20 kg ± 1 body weight, approximately 10 weeks old), kept in standard laboratory condition with access to species-specific food and water *ad libitum.* All surgical procedures were performed in compliance with the regulations and rules approved by the Local Ethic Committee (No. 85/2008, dated 17.12.2008).

The animals were divided into four experimental groups of four animals each (n=4 each, total N=16): two control groups: one with the animals intact before sacrifice, and one with a "sham operated", treated like inflammation animal group without in-

flammation-caused mixture. One experimental group had induced aseptic colitis, and the second experimental group was axotomised, both using the methods previously described by Gonkowski et al. (2010). The pigs from the "sham operation" and inflammation groups were subjected to median laparotomy and injected with 50 μl of 0.9% NaCl ("sham operation") or 10% formalin solution (inflammation) (in microinjections of 10 μl each) into the wall of the descending colon. Animals from the axotomy group were subjected to bilateral transection of the caudal colonic nerve. After 5 days the animals were euthanized with an overdose of sodium thiopental, and perfused transcardially with 4% buffered paraformaldehyde.

Tissue samples from the descending colon (ca. 3 cm long) were collected from all the animals, post-fixed by immersion in the same fixative for several hours and finally stored in 18% buffered sucrose. Tissue fragments from the colitis group were also examined using a standard histopathological method to confirm the occurrence of inflammation. Ten μm thick cryostat sections were subjected to a routine double-labelling immunofluorescence as described previously by Pidsudko et al. (2001), using a combination of antisera raised in different species and directed towards protein gene-product 9.5 (PGP 9.5; mouse monoclonal, AbD Serotec, SN: 7863-2004, dilution 1:2000, used as a pan-neuronal marker) and CART (rabbit monoclonal, Phoenix Pharmaceuticals, USA, SN: H-003-61; dilution 1:16000). Primary antisera were visualized by species specific secondary antisera: anti mouse AlexaFluor 488 (Invitrogen, serial nr A21202, dilution 1:800), and anti-rabbit AlexaFluor 546 (Invitrogen, serial nr A10040, dilution 1:800).

Double-labelled somata were evaluated using an Olympus BX51 microscope equipped with epi-fluorescence and appropriate filter sets, counted in each ganglionated plexus (i.e. the myenteric – MP, outer submucous – OSP and inner submucous – ISP plexuses), found in the sections studied (10 sections per animal; only neurons with a clearly visible nucleus were included), pooled and presented as mean ± SEM. To prevent double counting of the same perikarya, the sections were located at least only each tenth sections was used. Statistical analysis was performed with the Duncan test using STATISTICA 9.0 (StatSoft Inc., Tulsa, Oklahoma, USA). Differences within percentage quantities of CART-LI neurons in each ganglionated plexus were considered as being statistically significant (when p≤0,05, indicated by small letters, Table 1) or highly significant (when p≤0,01, indicated by capital letters, Table 1).

Table 1. Percentage average number of CART-LI perikarya for total number examined neurons in appropriate type of plexuses.

		MP	OSP	ISP
Control group	N=	8719	3613	8212
	%	5.11[A]	4.03[A]	0.05
	SEM	0.64	1.17	0.04
Sham operated group	N=	8303	3616	8221
	%	5.19[B]	3.97[B]	0.035
	SEM	0.63	0.77	0.04
Axotomy	N=	8187	3516	8582
	%	3.04[AB]	2.46[AB]	0.43
	SEM	0.88	0.8	0.09
Experimental colitis	N=	8348	3621	8433
	%	2.77[AB]	2.60[AB]	0.26
	SEM	0.64	0.36	0.19

Negative controls employed in the immunofluorescence procedure included preabsorption, omission and replacement control.

In order to evaluate the semi-quantity of the density of intraganglionic CART-LI nerve fibers an arbitrary scale was used, where (++++) means a very dense meshwork and (-) depicts the absence of the nerve fibers studied.

A semi-quantitative evaluation of the density of the CART-LI nerve fibers within the mucosal or muscular layers was based on a count of all the profiles immunoreactive to a given antigen *per* observation field (approximately 0.55 mm^2). Nerve profiles were counted in 4 sections *per animal* (5 fields of view *per* section). The data obtained were pooled and presented as a mean.

All pictures were taken using acquisition and processing software (Cell-F) with a digital ColorView camera connected to a PC.
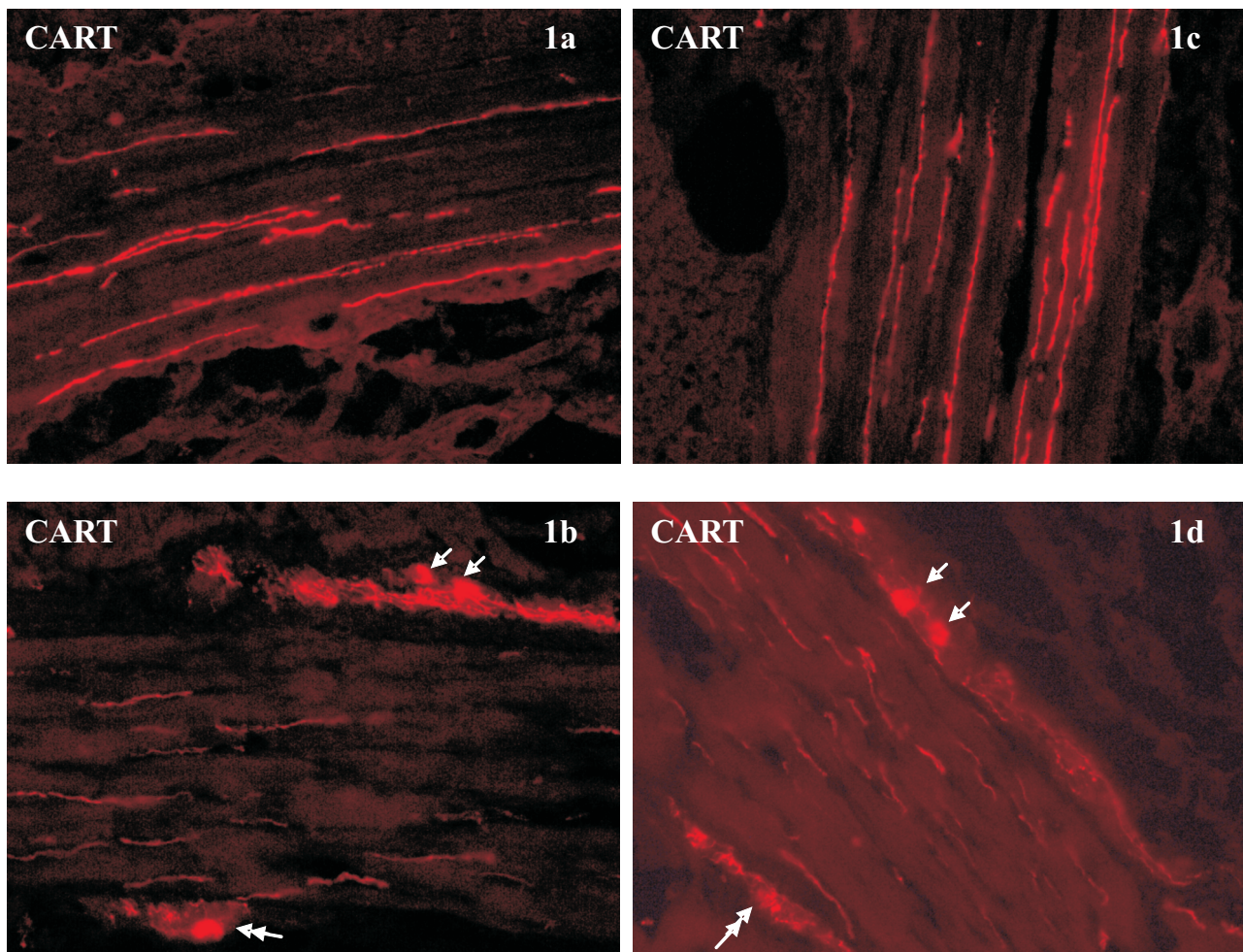

Fig. 1. a-d. CART-LI fibers in the descending colon muscular layer in pigs of the control group (1a), after "sham operation" (1b), axotomy (1c) and inflammation (1d); Pictures "b" and "d" show CART-LI neurons in the myenteric plexus (single arrow) surrounded by a dense network of intraganglionic CART-positive fibers and CART-LI neurons in the outer submucous plexus (double arrow); mag. 100x.

Results

The presence of CART-LI neurons was observed within the MP, OSP and ISP. CART-LI nerve fibers in the circular muscular (Fig. 1.a-d) and mucosal layer (Fig. 2.a-d) were found under physiological as well as experimental states. There was no regularity in their distribution.

tically highly significant (p≤0.01) differences between both control and experimental groups. A large number of CART-positive fibers was detected in myenteric plexuses in all the groups (Table 2).

In OSP in the control groups, approximately 4% of CART-LI neurons were observed, whereas in the experimental groups, a decrease in the population of CART-positive neurons by about 1.5% was found
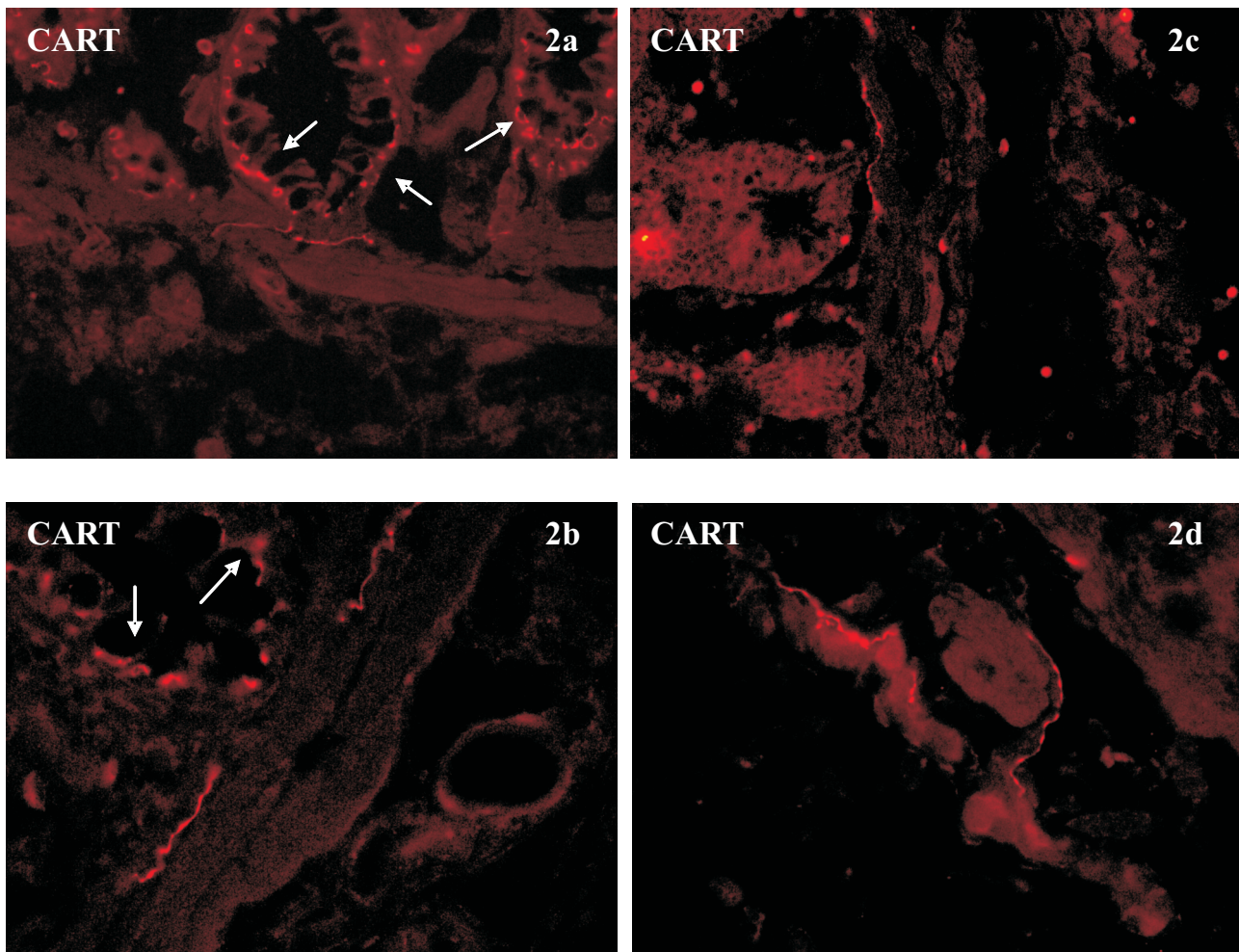

Fig. 2. a-d. CART-LI fibers in the descending colon mucosal layer in the control (2.a and 2.b) and experimental (2.c and 2.d) groups. Arrows indicate non-neural CART-LI structures in intestinal crypts (pictures "a" and "c" mag. x100, pictures "b" and "d" mag. x200).

Plexuses containing from one to several CART-LI neurons were observed, although a great majority of them had none.

MP in the control and "sham operation" groups had about 5% CART-LI neurons. In pathological conditions, a decrease in their number to about 3% in the axotomy group and 2.77% in the colitis group was observed (Table 1, Fig. 3-4.a-c). Duncan's test found no statistical differences between the control and "sham operation" groups, nor between the axotomy and inflammation groups. There were, however statis-

Table 2. Density of intraganglionic nerve fibers positive for CART in ENS plexuses, presented in arbitrary units.

	MP	OSP	ISP
Control group	++++	++	+
Sham operated group	++++	++	+
Axotomy	++++	++	+
Experimental colitis	++++	++	++

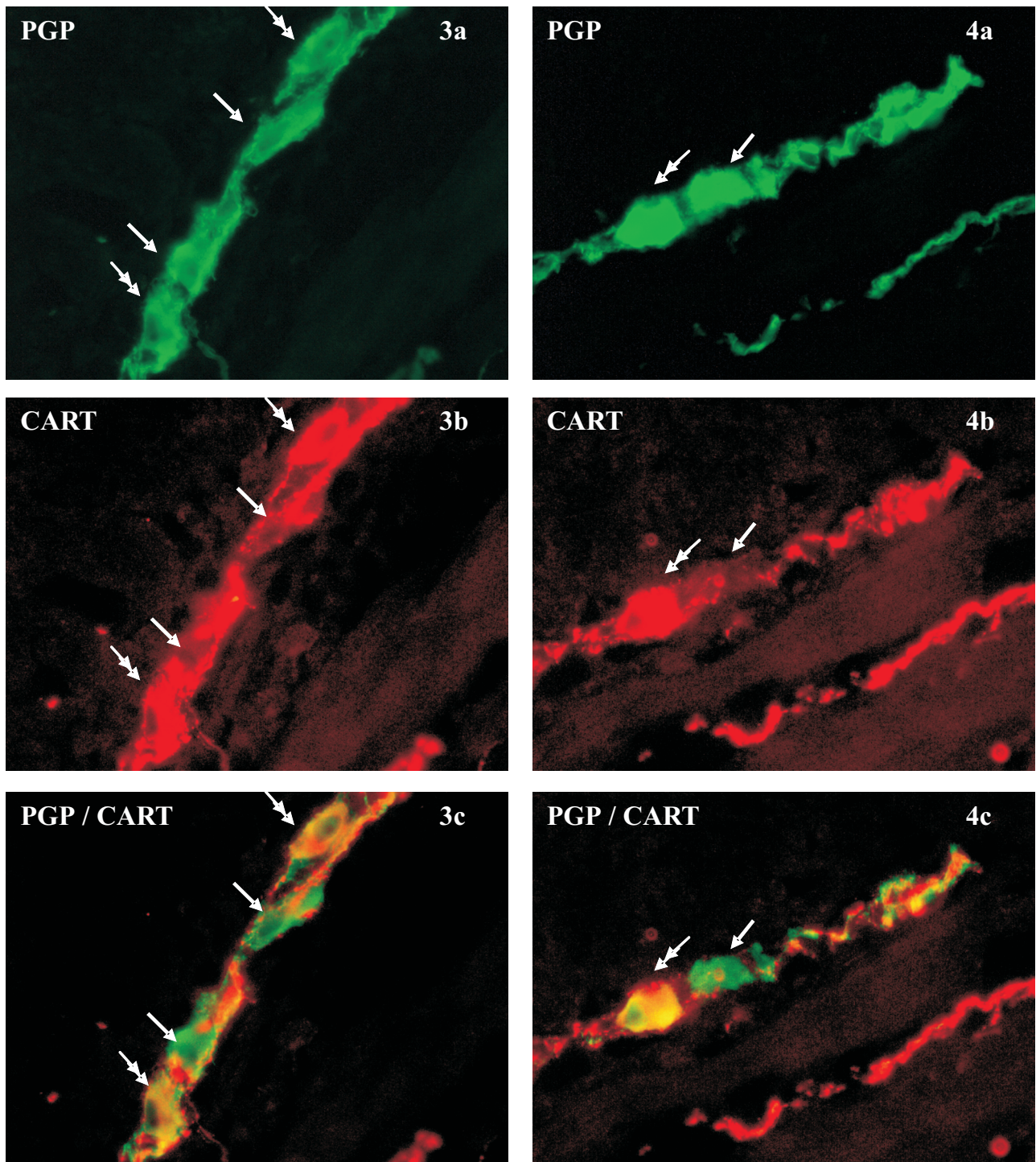(Table 1, Fig. 5-6.a-c). As with MP, Duncan's test revealed no statistical differences between the control

Fig 3-4. Myenteric plexus in the porcine descending colon immunostained for PGP 9.5 (a) and CART (b). Single arrows indicate neurons devoid of CART-LI, colocalisation of both antigens in studied perikarya exemplified with double arrows; mag. x400.

and "sham operated" groups, nor between the axotomy and inflammation group. There were, however, statistically significant differences (p≤0.05) between both control groups and both experimental groups. Additionally, single intraganglionic fibers were observed (Table 2).

Although, ISP were found to contain CART-LI neurons, their population was very small. In the control groups, there were not more than 0.05% CART-positive neurons. In the experimental groups, there was an increase in the number of CART-LI neurons to 0.26% in the colitis and 0.43% in the
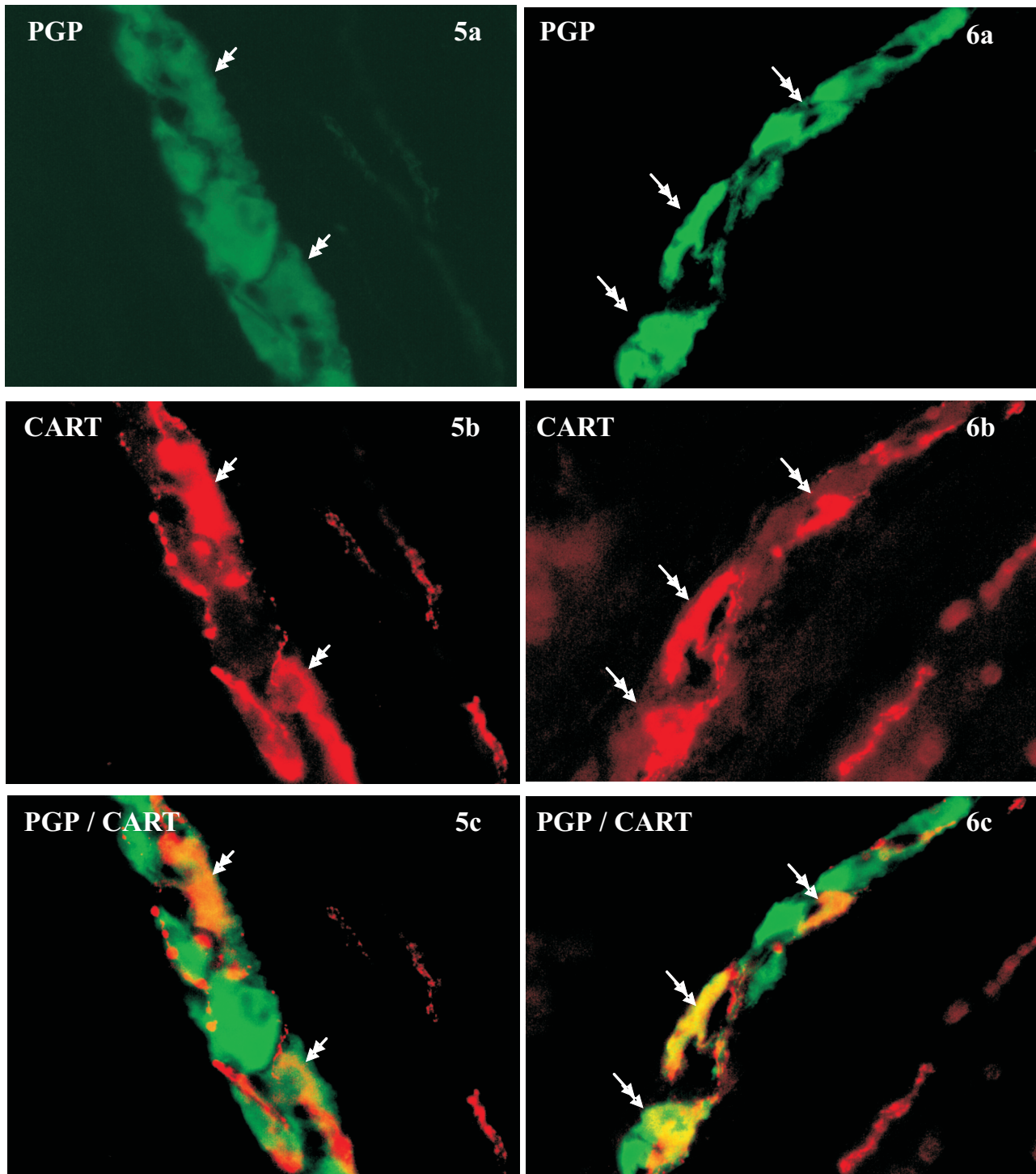
Fig. 5-6. Outer submucous plexus in the porcine descending colon immunostained for PGP 9.5 (a) and CART (b). Colocalisation of both antigens in studied perikarya exemplified with double arrows; mag. 400x.

axotomy group (Table 1). The number of CART-positive neurons was insufficient to conduct a more complete statistical analysis. In ISP intraganglionic CART-positive fibers were observed sporadically in all surveyed conditions (Table 2).

CART-positive nerve fibers were also observed in the muscle layer and mucosa of the descending colon. The influence of pathological effects on the density of CART-LI fibers in the field of vision was also observed. In the control groups on average in the field of vision around 21 fibers were observed. Axotomy induced an increase in CART-positive fiber density to about 46 in the field of vision, while experimental colitis provoked an increase to less than 26 fibers per field (Table 3).

Table 3. Mean number of nerve fibers per studied area (Mean number ± SEM).

	Circular muscle layer	Mucosal layer
Control group	20.93 ± 1.44	0.33 ± 0.62
Sham operated group	21.07 ± 1.87	0.27 ± 0.59
Axotomy	46.47 ± 2.39	1.8 ± 0.54
Experimental colitis	25 ± 0.54	3.55 ± 0.83

Discussion

The results of this study demonstrate that CART-immunoreactive structures are present in all the studied parts of the porcine colonic nervous system. This is consistent with the results obtained in other studies (Ekblad et al. 2003, Ekblad 2006, Gunnarsdóttir et al. 2007, Wierup et al. 2007, Arciszewski et al. 2009, Gonkowski et al. 2009a,b). Although the knowledge concerning the role(s) of CART within the ENS is very limited, the presence of CART-LI structures and their changes in number observed in these experimental conditions clearly indicate their biological effects within the alimentary tract. CART has been described as an inhibitor of gastric emptying and acid secretion (Okumura et al. 2000), an inhibitor of NO-mediated relaxation in the colon (Ekblad et al. 2003), and a reducer of colonic motility *via* cholinergic pathways (Tebbe et al. 2004) as well as in stress responses (Koylu et al. 2006).

High concentrations of CART-LI fibers present in the circular muscle observed during this investigation suggest a significant role of CART peptides in the regulation of gut functioning. This information is confirmed in a previous study of Gonkowski et al. (2009a). It may be concluded that the presence of CART-LI neurons in MP and OSP combined with a high concentration of CART-immunoreactive fibers demonstrates the significant role of CART peptides in the regulation of the gut motility. A very small number of CART-LI neurons in ISP – the nervous structure supplying the intestinal mucosa and containing primarily secretomotor neurons (Timmermans et al. 2001, Furness 2006) – may explain the marginal role of these peptides in terms of regulation of secretion in the descending colon. It is surprising that previous studies conducted on animal mature ENS revealed the presence of a large number of CART-LI fibers in the muscle and intestinal mucosa (Ekblad et al 2003, Ellis and Mawe 2003, Wierup et al. 2007). This may indicate a regulatory role of CART peptides in the gut secretion in adult organisms.

It is suggested that changes in the expression of CART at the stage of ontogenetic development can be related to its neurotrophic function (Risold et al. 2006). However, the precise functions of CART peptides in the ontogeny of individual species require further investigations.

The present investigation revealed that some pathological stimuli can change CART-immunoreactivity within the porcine descending colon, and these changes have different influences on the subdivision of colonic ENS studied. These results suggest the role of CART in the regulation of gut functions in physiological conditions as well as during inflammatory process and neurectomy. Two previous studies in this subject concerning the human GI revealed changes in CART-immunoreactivity in ENS during Hirschprung's disease (Gunnarsdóttir et al. 2007) and ulcerative colitis (Gonkowski et al. 2009b).

Ekblad (2006) suggests the neuroprotective role of CART peptides. Generally, however it is accepted that injury to neurons should result in an increase in the expression of these neurotransmitters, which promote their regeneration (Arciszewski and Ekblad 2005). The decrease in the number of CART-LI neurons observed in the present investigation, combined with an increase in the number of CART-positive nerve fibers may suggest that it is connected with intensive transport of CART peptides to nerve endings, confirming their possible neuroprotective role. In previous investigations conducted on human tissues (Gonkowski et al 2009b), the effects observed appear to be similar. Unfortunately there are no earlier studies that describe possible changes of co-localisation of CART peptides with other, biologically active substances of recognized functions. Further studies in this area may explain the mechanism of action of this group of peptides.

In summary, the results obtained in this study suggest a significant role for CART in the function of the porcine descending colon ENS in physiological, as well as some pathological conditions, such as probably multiagent(s) inflammatory or surgery enforced neurectomy. This knowledge in a new area of modern medicine may increase our range of possibilities in the therapy of intestinal diseases and post-surgery treatment.

Acknowledgements

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