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Original article

Distribution and immunohistochemical characteristics of cocaine- and amphetamine-regulated transcript-positive nerve elements in the pelvic ganglia of the female pig

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Abstract

Cocaine- and amphetamine-regulated transcript (CART) peptides are widely expressed not only in the brain but also in numerous endocrine/neuroendocrine cells as well as in neurons of the peripheral nervous system. The present study investigated the distribution patterns of CART-like immunoreactivity in the pelvic plexus (PP) of the female pig. The co-expression of CART with principal neurotransmitter markers: choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), serotonin (5-HT) or biologically active neuropeptides: pituitary adenylate cyclase-activating polypeptide (PACAP), substance P (SP), calbindin was analyzed using double immunohistochemical stainings. Amongst neurons immunopositive to Hu C/D panneuronal marker as many as $4.1 \pm 1.2\%$ in right and $4.4 \pm 1.6\%$ in left pelvic ganglia were found to express CART. The vast majority of CART-IR ganglionic neurons were predominantly small in size and were evenly scattered throughout particular ganglia. Immunoreactivity to CART was also detected in numerous nerve terminals (which frequently formed pericellular formations around CART-negative perikarya) as well as in numerous nerve fibres within nerve branches interconnecting the unilateral pelvic ganglia. Immunohistochemistry revealed that virtually all CART-IR neurons were cholinergic in nature and CART-IR basket-like formations frequently encircled TH-positive/CART-negative perikarya. None of CART-IR ganglionic neurons showed immunoreactivity to SP, PACAP, 5-HT or calbindin. CART-IR nerve fibres ran in a close vicinity to serotonin-containing cells or faintly labelled SP-expressing neurons. On the other hand, PACAP-IR, SP-IR (but not 5-HT-positive) nerve terminals were found to run in close proximity to CART-IR neurons. Our results indicate that: 1) CART present in PP may influence the activity of pelvic ganglionic neurons/SIF cells, 2) PP should be considered as a potential source of CART-like supply to pelvic viscera and 3) functional interactions between CART and SP or PACAP are possible at the periphery.

Key words: CART, neuropeptides, chemical coding, pelvic plexus, pelvic ganglia, pig

Introduction

In the mammalian autonomic nervous system, the pelvic plexus (PP) is, from anatomic point of view, a very curious cross-road for sympathetic and parasympathetic nerve fibres. Preganglionic sympathetic impulses to pelvic ganglia are conveyed via the hypogastric nerve, whereas the pelvic nerves provide parasympathetic input to pelvic organs. Therefore, numerous ganglia distributed throughout PP contain both noradrenergic and cholinergic neurons. In a series of anatomical investigations, some differences in the structure of male and female PP as well as between large and small mammals (mainly rodents) have been revealed. In females of large animals as well as in women, PP takes the shape of a triangle located along both sides of the rectum reaching the uterus and containing 10 to 20 evenly distributed middle-sized ganglia (Woźniak and Skowrońska 1967, Li and Masuko 2001) including the paracervical ganglia (Łakomy and Szatkowska 1992). In male mammals, PP (similarly as indicated above in females) extends caudally on both sides of the rectum and reaches the border of the vas deferens and seminal vesicles (Tsaknakis 1971). However, in males of large mammals PP comprises of one large ganglion called the anterior pelvic ganglion (APG) and several smaller in size ganglia scattered throughout the plexus (Kaleczyc et al. 2003a, Ali et al. 2004). In males and females of rodents, the structure of PP is even more simplified since in animal species such as the rat, guinea-pig or mouse one large major pelvic ganglion (MPG) and only a few small accessory pelvic ganglia are present (Dhami and Mitchell 1991, Mitchell 1993, Keast 1995, Wanigasekara et al. 2003). Noteworthy, in rodents the sexual dimorphism in neuronal numbers of MPG has been found (Greenwood et al. 1985). Retrograde tracing experiments have clearly indicated that ganglionic neurons in PP provide neuronal supply to pelvic organs including the vas deferens (Keast 1992, Kaleczyc 1998), prostate (Zacharko et al. 2004), urinary bladder (Pidsudko 2014), uterus (Houdeau et al. 1998), rectum and penis (Domoto and Tsumori 1994).

It is well known that functions of autonomic neurons (including those located in pelvic ganglia) are mediated through two major neurotransmitters (noradrenaline and acetylcholine) which are supported by a variety of biologically active substances called in general neuropeptides (for a review see Dail 1996, Kaleczyc et al. 2003b, Podlasz and Wąsowicz 2008). Over the past years, cocaine- and amphetamine-regulated transcript (CART) has been added to the list of active substances known to be present in both central and peripheral neurons. The discovery and name of

CART originates directly from the experiment of Douglass et al. (1995) who were able to identify in the rat brain genes specifically expressed after the acute intraperitoneal administration of cocaine and amphetamine. CART peptides have been extracted from several mammalian neuroendocrine tissues, sequenced and analyzed, which have led in turn to the finding that the CART gene encodes two, long and short, isoforms which may be processed into two biologically active proteins of either 116 or 129 amino acids, respectively (Thim et al. 1998). CART mRNA and peptides are predominantly found in several hypothalamic regions of the central nervous system known to be associated with food intake (Koylu et al. 2000, Sergeev et al. 2001). Besides the apparent role in appetite regulation, the participation of CART in mechanisms underlying some neurological disorders like ischemic stroke, dementia or seizures have been described (for review see Zhang et al. 2012). Interestingly, highly selective expression of CART has been also described in peripheral ganglia (including enteric) and in certain organs of many animal species including the rat (Ekblad et al. 2003, Fenwick et al. 2006, Richardson et al. 2006, Ivanusic et al. 2012), mouse (Gautron et al. 2012), sheep (Arciszewski et al. 2008), cow (Janiuk et al. 2013) and pig (Wierup et al. 2006, 2007). In previous reports we and other researchers also described the presence of CART and the co-localization patterns with neuroactive peptides in porcine peripheral ganglia like enteric ganglia of the stomach (Zacharko-Siembida and Arciszewski 2014a), small and large intestines (Gonkowski et al. 2009, Burlínski et al. 2014), dorsal root ganglia (Zacharko-Siembida et al. 2014) and ganglia of the urinary bladder (Zacharko-Siembida and Arciszewski 2014b). Considering the wide distribution of CART peptides in the porcine peripheral nervous system we speculate it is likely that in this animal species CART may be expressed in other (so far not investigated) ganglionic neurons. Therefore, in the present experiment, we immunohistochemically studied the expression and co-localization patterns of CART with several neuropeptides in PP of the female pig.

Materials and Methods

Animals, anesthesia and tissue preparation

The experimental procedures were carried out in accordance with specific Polish and international laws and regulations, and stayed in accordance with principles approved by the Local Ethical Committee at the University of Life Sciences in Lublin, Poland. Special efforts were undertaken to minimize the poss-

Table 1. Characteristics of primary antibodies used in the study.

| Antigen | Host | Dilution | Source and code | Reference |
|--|------------|----------|---|-------------------------|
| CART (61-102) | Rabbit | 1:10000 | Phoenix Pharmaceuticals, USA, code H-003-61 | Bulc et al. 2015 |
| Hu C/D | Mouse | 1:800 | Molecular Probes, USA, code A-21271 | Lin et al. 2003 |
| Tyrosine hydroxylase (TH) | Mouse | 1:100 | Sigma-Aldrich, Germany, code T.2928 | Zalecki 2012 |
| Choline acetyltransferase (ChAT) | Sheep | 1:100 | Abcam, UK, code ab 18736 | Hoogduijn et al. 2009 |
| Calbindin D-28k | Mouse | 1:1500 | SWant, Switzerland, code 300 | Celio 1990 |
| Pituitary adenylate cyclase-activating polypeptide (PACAP) | Guinea-pig | 1:300 | Bachem, Switzerland, code T-5039 | Wojtkiewicz et al. 2012 |
| Substance P (SP) | Rat | 1:400 | AbD Serotec, UK, code 8450-0505 | Zalecki 2012 |
| Serotonin (5-HT) | Mouse | 1:100 | Abcam, UK, code ab16007 | Wells et al. 2011 |

ible stress in the animals. Five (n=5) 6 weeks old female piglets (weighing approx. 25 kg each) were used in the study. After sedation with azaperone (Stresnil, Pharmacia Upjon, Poland 0.5 mg/kg b.w.) the animals were killed by an overdose of sodium pentobarbital (Morbital, Biowet Puławy, Poland 50 mg/kg b.w.). A midline incision was made and the sympathetic caudal mesenteric ganglion was identified for orientation purposes. Then, according to the description provided by Tsaknakis (1971) the afferent branches (hypogastric and pelvic nerves) as well as the efferent braches (nerves reaching the rectum, ureters and urinary bladder) of the right and left PP were visualised. Finally, the whole bilateral PPs (containing small pelvic ganglia and paracervical ganglion) were gently dissected out and immediately washed in 0.9% NaCl. The right and left dissected PP were immersed in 15% saturated picric acid and 2% paraformaldehyde fixative solution for 24 h at room temperature (RT). For cryoprotection the material was placed in a container filled with 18% sucrose solution and kept for 5-6 days until it sunk to the floor of the flask. Next, the material was formed in approx. 1 cm³ blocks, frozen at -20°C and cut using a cryostat into 10 µm-thick serial sections. Every fifth section was mounted onto a glass slide (SuperFrost[®] Plus, Menzel-Gläser, Thermo Scientific, Braunschweig, Germany) and stored at -20°C until use.

Immunohistochemistry

In general, for immunohistochemical studies the staining protocol described elsewhere was used (Arciszewski and Zacharko-Siembida 2007). Briefly, frozen sections were first dried out for 15 min at RT. In order to raise the penetration of antibodies and to

rehydrate the sections, three time rinsing (15 min each) with 0.01M phosphate buffered saline (PBS, pH 7.4) supplemented with 0.25% Triton X-100, 10% normal goat serum and 0.25% bovine serum albumin (Sigma Aldrich, Germany) was applied. Next, the sections were placed in a dark humid chamber and incubated overnight (RT) with a mixture of primary antibodies. Visualization of CART in pelvic ganglia neurons was achieved by using the primary antibodies raised against CART and Hu C/D proteins (which served as general neuronal marker). In order to study the co-localization patterns of CART with selected biologically active substances, anti-CART sera were mixed with one of the primary antibodies listed in Table 1. Next day, the sections were rinsed in PBS (3x15 min) and incubated (RT, humid chamber) for 1 h with Texas Red-conjugated anti-rabbit goat IgG (1:400; MP Biomedicals, Solon, OH, USA) mixed with one of the corresponding secondary antisera: FITC-conjugated anti-mouse goat IgG (1:400; MP Biomedicals, Solon, OH, USA), FITC-conjugated anti-sheep rabbit IgG (1:400; MP Biomedicals, Solon, OH, USA) or goat FITC-conjugated anti-guinea pig IgG (1:400; MP Biomedicals, Solon, OH, USA). Following three washes in PBS (15 min each) the sections were finally mounted in phosphate-buffered glycerol (pH 8.2). The specificity of the immunoreaction was assessed in all cases by omitting or replacing the primary antibodies with normal non-immunoreactive serum from the labeling protocol. For additional controls, a series of stainings with primary antisera that had been preabsorbed with an excess amount of synthetic blocking antigen were performed. The immunolabelled sections were viewed under a spinning disk confocal microscope (BX-DSU Olympus, Nagano, Japan) equipped with interference filters optimized for detection of red and green fluorochromes (MINIBA2 and MWIY2; respectively).

Counting procedure and statistics

All numerical data were presented as mean \pm SEM. Relative numbers of CART-immunoreactive (IR) right and left pelvic ganglia neurons were quantified. In each of the PP ganglia studied at least 100 of Hu C/D-IR neurons with well-visible nucleus were studied for the simultaneous presence of CART. The proportions of CART-IR neurons were presented as a percentage of the total number of perikarya. The long axis of CART-positive neurons (with well visible nucleus) was measured. Based on the diameter, CART-IR neurons were arbitrarily classified as small (up to 35 μ m), middle (between 35 and 50 μ m) and large (above 50 μ m) in size. For co-localization studies, all the cell bodies that were found to be immunopositive to CART (but no less than 50) were examined for the presence of the second substance. The proportions of CART-IR neurons co-expressing neuropeptides were presented as percentages of the total number of CART labeled neurons. The following semi-quantitative scale was used to visually assess the density of the nerve fibres IR to CART: absent, single, moderate, numerous and very numerous.

In order to determine differences between homologous and non-homologous neuronal phenotypes of the right and left PP a two-way analysis of variance (ANOVA) test was applied. Statistical significance was determined at $p < 0.05$ confidence level using the Bonferoni's post-hoc test.

Results

CART expression pattern

In the experimental animals, numbers of pelvic ganglia (including paracervical ganglion) found and studied in the left and right PP varied from 7 to 12. In both right and left pelvic ganglia, immunoreactivity to CART was detectable both in neuronal cell bodies (Fig. 1A), neuronal terminals located around ganglionic neurons (Fig. 1B) as well as in the network of nerve branches forming PP. No substantial differences in CART distribution patterns were found between the paracervical ganglion and remaining pelvic ganglia

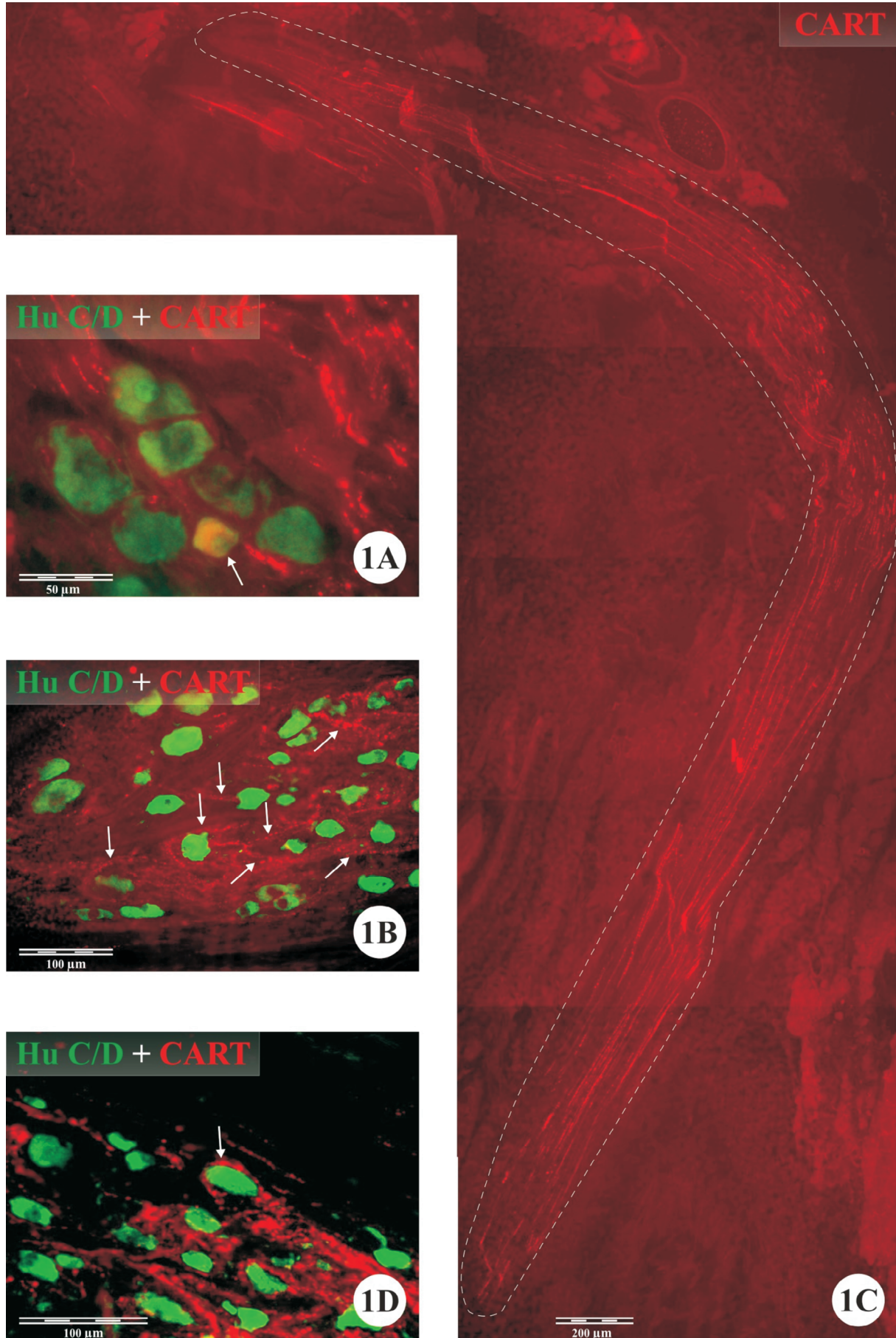
as well as between the left and right PP which entitled us to present the results as general. The pelvic ganglia neurons expressing Hu C/D were bright and very well visible in relation to the background. In the right pelvic ganglia, the average percentage of Hu C/D-IR neurons which additionally expressed CART was $4.1 \pm 1.2\%$ whereas in the left pelvic ganglia, the analogous proportion amounted to $4.4 \pm 1.6\%$ (Fig. 1A). No statistical differences ($p < 0.05$) were found between average numbers of CART-IR neurons observed in the left and right pelvic ganglia. Both in the right and left pelvic ganglia, the vast majority (approx. 95%) of CART-IR neurons were classified as small-sized whereas in only sparse (approx. 5%) CART-expressing perikarya the long axis was larger than 35 μ m (those neurons were classified as middle-sized). CART-expressing neurons were randomly scattered throughout the pelvic ganglia with no specific cellular organization. Very numerous, mainly varicose CART-positive nerve terminals were frequently detected in nerve strands connecting the neighboring ganglia (Fig. 1C). Moreover, depending on the ganglion studied, numerous or very numerous numbers of CART-IR nerve fibres were located between neurons in the right and left pelvic ganglia. Both in the right and left pelvic ganglia, CART-IR nerve fibres frequently encircled CART-lacking ganglionic neurons forming a basket like formations (Fig. 1D).

Co-localization studies

In both the right and left PP, neither CART-expressing ganglionic neurons nor CART-positive nerve fibres were immunoreactive to TH (Fig. 2A). Likewise, the abundant CART-IR nerve fibres observed in ganglionic internodal nerve strands were devoid of TH, however both types of fibres frequently run in a very close proximity. CART-IR ganglionic neurons were commonly found to lie close to TH-IR neurons (Fig. 2A). Interestingly, very numerous CART-IR basket-like formations were found to encircle TH-positive ganglionic neurons (Fig 2B). Double immunohistochemical staining revealed that in PP the vast majority of CART-IR ganglionic neurons simultaneously expressed ChAT (Fig. 2C). Additionally,



Fig. 1. Immunofluorescent microphotographs showing immunoreactivity to CART found in the pelvic plexus of the female pig. Amongst Hu C/D-positive pelvic ganglionic neurons (Fig. 1A) only one (small-sized marked with an arrow) is immunoreactive to CART. (Fig. 2A) depicts very numerous CART-immunoreactive (CART-IR) nerve fibres (arrows) running between and in a close vicinity to Hu C/D-IR pelvic ganglia neurons. Very numerous CART-IR nerve fibres were also found in nerve branches forming pelvic plexus which is shown in Fig. 1C. Fig. 1D shows the presence of CART-IR basket like formations encircling CART-negative neurons.



CART-positive nerve fibres found in both pelvic ganglia and nerve branches of PP were mostly immunopositive to ChAT. Moderate numbers of CART-positive/ChAT-negative nerve terminals running between ChAT-positive and ChAT-negative ganglionic neurons were observed. Both in pelvic ganglia as well as in the PP branches, nerve fibres immunoreactive to CART substantially outnumbered those immunopositive to serotonin (5-HT). In none of CART-IR nerve fibres co-localization with serotonin was found, however, those two types of fibres frequently run in a very close neighborhood. Likewise, none of CART-IR ganglionic neurons simultaneously expressed serotonin. In pelvic ganglia, sparse serotonin-IR nerve cells were surrounded by CART-IR nerve fibres (Fig. 2D). In none of CART-IR neurons found in the left and right pelvic ganglia the co-expression with PACAP was visualized (Fig. 2E). In general, immunoreactivity to PACAP-IR was rarely observed both in nerve fibres present in the PP branches and those terminated around pelvic ganglia neurons. Interestingly, none of PACAP-IR nerve fibres showed immunoreactivity to CART. Very sparse, mainly varicose, PACAP-IR nerve terminals were found to lie in a close vicinity to CART-IR ganglionic neurons (Fig. 2E). In pelvic ganglia neurons the very faint expression of SP was found in a small subset of neurons, however, none of them was immunopositive to CART. Numerous SP-IR basket-like formations encircling CART-IR ganglionic neurons were regularly observed (Fig. 2F). In general, the distribution patterns of SP-IR and CART-IR nerve fibres present in PP were completely different (Fig. 2F). In the present study, no expression of calbindin was detected in both the right and left PP. Therefore no co-localization between CART and calbindin was observed either in neurons or nerve fibres (data not shown).

Discussion

In this study, we were able to demonstrate and describe the expression and localization of CART in PP of the female pig. The results obtained by immunohistochemical analyses clearly show that CART is present in a relatively small subset of pelvic ganglia neurons (approx. 5%), numerous ganglionic nerve terminals and very numerous nerve fibres in nerve branches forming PP. To the best of our knowledge, this is the first report demonstrating the putative neuroactive role of CART in the autonomic porcine PP. Previously, the presence of CART was visualized in both sympathetic and parasympathetic parts of the autonomic nervous system, albeit the CART distribu-

tion patterns described seem to be species-specific and vary between particular autonomic centers/ganglia. The presence of dense pericellular baskets of varicose CART-IR terminals around and between sympathetic neurons of the superior cervical ganglion (Gonsalvez et al. 2010), stellate ganglion (Dun et al. 2000, Richardson et al. 2006), coeliac-superior mesenteric ganglion (Palus and Calka 2016) or male major pelvic ganglion (Fenwick et al. 2006) has been previously reported. With the use of the retrograde tracer cholera toxin unit it has been demonstrated that these CART-IR nerve terminals originate from CART-IR sympathetic preganglionic neurons (SPN) of the thoracolumbar medullary intermediolateral cell column and in case of major pelvic ganglia also from the central autonomic area and intercalated nucleus (Fenwick et al. 2006). Interestingly, co-localization studies allowed to reveal that nearly all CART-expressing SPN additionally expressed ChAT (Parker et al. 2013). These findings are generally in line with our results presented here, because in porcine pelvic ganglia numerous CART-IR/ChAT-IR nerve terminals were commonly found to form basket-like formations and CART-IR nerve terminals regularly encircled TH-positive/CART-negative neurons. Likewise, very numerous CART-IR/ChAT-IR nerve fibres present in the plexus nerve strands were observed. Taken together, and based on the previous findings, we speculate that CART-IR/ChAT-IR nerve fibres in the porcine PP are axons of medullar SPN which may reach the pelvic plexus via the lumbar splanchnic and hypogastric nerves. It is worth to remember that in the hypogastric nerve noradrenergic and cholinergic fibres coexist (Alsaïd et al. 2009), however, to verify the latter hypothesis more extensive studies utilizing retrograde tracing technique are needed. The possibility that CART-IR nerve fibres in the rat major pelvic ganglion also originate from parasympathetic preganglionic neurons (PPN) of the medullary L₅-S₁ segment has been previously tested. However, none of the major pelvic ganglion-projecting SPN was found to express CART (Fenwick et al. 2006), which suggests that pelvic splanchnic nerves do not participate in CART-like innervation to the porcine PP. Because in rats, experimental exposure to hypoxia induced the expression of *c-fos* in CART-IR SPN the concept that CART is exclusively present in vasoconstrictor neurons and mediates cardiovascular functions has been considered (Gonsalvez et al. 2010). But in other studies, the treatment with hypotensive hydralazine evoked expression of *c-fos* in only 40% of CART-IR SPN which led the authors to conclude that CART is not an exclusive marker for cardiovascular SPN and may be also present in a subset of spinal interneurons (Fenwick et al. 2006). The latter finding may be easily

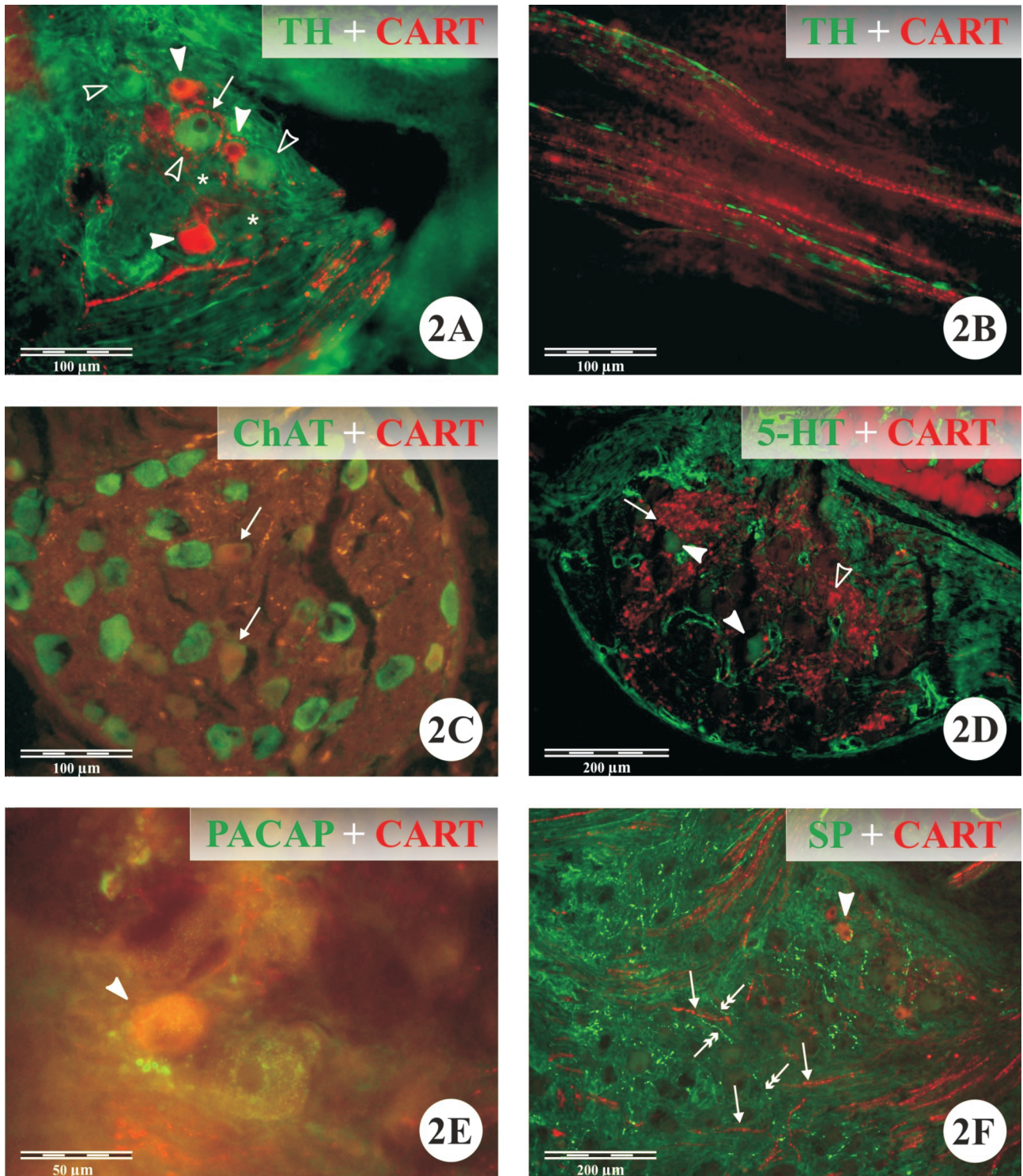


Fig. 2. Multicolor photographs illustrating double labelling for CART and biologically active substances in the porcine female pelvic plexus. In Fig. 2A three different subpopulations of pelvic ganglia neurons are presented; neurons IR to CART but TH-negative are marked with arrowheads, neurons expressing TH but CART-negative are indicated with empty arrowheads whereas asterisks mark perikarya negative both to CART and TH. Note the presence of pericellular CART-IR varicosities around TH-IR neurons (arrow). The distribution pattern of TH-IR and CART-IR nerve fibres are shown in Fig. 2B. Cholinergic ganglionic neurons (immunopositive to ChAT) also expressing CART are marked with arrows in Fig. 2C. Fig. 2D shows the expression of CART and 5-HT in different ganglionic nerve cells (empty arrowhead and arrowheads, respectively) and that CART-IR nerve terminals are located in a close vicinity to 5-HT-IR neurons (arrow). Double-labeling (Fig. 2E) revealed that CART and PACAP were not co-expressed in the same neurons (arrowhead). Fig. 2F presents CART-IR/SP-negative neurons encircled by SP-IR basket-like formations (arrowhead). Completely different distribution patterns of CART-IR (arrows) or SP-IR (double arrows) nerve fibres in the pelvic ganglia are depicted.

correlated to our research, because it must be kept in mind that although vasoconstrictors innervating female pelvic viscera are predominantly located in sympathetic paravertebral ganglia (Jänig 1988) some of them are also present in pelvic ganglia. Electrophysiological *in vitro* study shown that neurons in pelvic ganglia receiving preganglionic input from lumbar spinal SPN are part of pelvic vasodilator pathways causing vasodilatation of uterine arteries in guinea-pig (Morris et al. 2005). Another possibility is that the CART-IR basket like formations surrounding TH-positive (predominantly) neurons are endings of CART-IR pelvic ganglia interneurons. This may be in turn supported by the finding that in the present study we visualized nearly 5% population of small CART-expressing PP ganglionic neurons. So far, noradrenergic neurons in the female PP have been considered as innervating the musculature of the uterus, uterine cervix (Houdeau et al. 1998) and urinary bladder (de Groat et al. 2015), which suggests that CART may be involved in modulation of the activity of motor neurons. Although CART-IR nerve fibres were commonly found in smooth musculature of the hindgut, no direct contractile or relaxatory effects have been observed (Ekblad et al. 2003). Nevertheless, at the moment the precise functional role of porcine ganglionic pelvic neurons supplied by CART-IR axonal terminals is a matter of speculation only. In a subset of CART-IR pelvic ganglia neurons immunoreactivity to ChAT has been revealed which leads us to postulate cholinergic character of these neurons. Previous immunohistochemical study performed in the rat parasympathetic sphenopalatine and otic ganglia have disclosed a small (approx. 5%) populations of CART-IR neurons (Ivanusic et al. 2012). Moreover, in the rat intracardiac ganglia, approx. 46% of neuronal profiles showed the presence of CART (Richardson et al. 2006). Recently, a minor population of CART-IR neurons in intramural ganglia of the porcine urinary bladder trigone has been described and characterized (Zacharko-Siembida and Arciszewski 2014). Taken together, these findings suggest that CART-IR parasympathetic neurons may play certain, however till now largely unknown, function(s). As mentioned above CART-IR neurons found in PP may serve as interneurons and such kind of activity has been previously proposed in relation to CART-containing amygdala's neurons (Równiak et al. 2010), dorsal root ganglia neurons (Kozsurek et al. 2007), enteric neurons (Ellis and Mawe 2003) or spinal SPN (Fenwick et al. 2006). Another possibility is that CART-IR/ChAT-IR pelvic ganglionic neurons project to pelvic viscera or urogenital organs' intramural ganglia and such neuronal pathways have been studied with the use of retrograde tracers (Li and

Masuko 2001). Noteworthy, the presence of CART-IR nerve fibres have been found in the wall of the urinary bladder (Zvarova et al. 2014) or rectum (Gonkowski et al. 2009).

To shed some light on the putative neuroactive role of CART-IR neurons in PP, in the present study we analyzed chemical content of CART-expressing perikarya. We figured out, using immunohistochemistry as a tool, that in PP of the female pig nerve structures immunoreactive to CART co-store neither PACAP, 5-HT, calbindin nor SP. However, we also were able to show that CART-IR pelvic ganglia neurons are frequently surrounded by SP-IR or PACAP-IR nerve fibres, and in reverse SP-IR and 5-HT-IR pelvic ganglia cells were encircled by CART-IR nerve terminals, which suggest the existence of functional co-operation(s) between these neuropeptides. Despite the fact that great efforts have been made to understand the relationships between CART and other peptides involved in neuromodulation and/or neurotransmission these processes are far to be clarified. Noteworthy, co-localization between CART and PACAP has been found in a substantial population of intramural neurons (but not nerve fibres) supplying the porcine urinary bladder (Zacharko-Siembida and Arciszewski 2014b) but the significance of this finding is largely unknown. At the central level, PACAP attenuates CART stimulatory effect on feeding and acts as a CART receptor antagonist (Burgos et al. 2013). Using *in vitro* models it has been shown that stimulation of PC12 cells with CART resulted in an increased level of phosphor-ERK and this effect was antagonized by PACAP (Lin et al. 2011). Whether such or similar activity is also present in PP is a matter of speculation only. It must be remembered that in the mouse, application of PACAP on isolated MPG increased neuronal excitability and shortened the duration of the afterhyperpolarization (Tompkins et al. 2010) and the level of PACAP increased after culturing of MPG neurons (Girard et al. 2010). Since an increased number of peripheral neuronal CART-IR structures after mycotoxin application has been documented (Makowska et al. 2017) the possibility that both CART and PACAP exert neuroprotective effects should be considered. The significance of the finding that CART-IR nerve fibres run in close contact to 5-HT-expressing cells has to be elucidated, as well. Previous study has revealed that in the rat MPG, most of 5-HT-IR cells were also TH-positive what suggests that they are small intensely fluorescent (SIF) cells (Karhula et al. 1990). In peripheral autonomic ganglia, SIF cells are considered as interneurons involved in endocrine secretion including releasing of biologically active substances into intercellular spaces (Matthews 1989). To offer the full per-

spective on CART-serotonin interactions, it must be mentioned that anti-CART peptide IgG changes the density of hippocampal 5-HT_{2A} receptor (Rothman et al. 2003) and anorexia-associated activation of 5-HT₄ receptor increases the level of CART in the nucleus accumbens (Jean et al. 2007). The presence of SP-IR varicose basket-like formations around CART-IR pelvic ganglia neurons suggests the direct neuromodulatory role of SP in passing signals through CART-ergic neurons. Noteworthy, previously the co-localization of CART and SP was found only in neurons of the central nuclei (Hubert and Kuhar 2005), and in small subsets of neurons in porcine dorsal root ganglia (Zacharko-Siembida et al. 2014) or enteric ganglia (Gonkowski et al. 2009). In male rodents, which are known not to express SP in MPG neurons, pericellular SP-IR nerve fibres are provided to the PP both via the hypogastric and pelvic nerves (Dhami and Mitchell 1991). Because it is well known that dorsal root ganglia project to pelvic viscera via pelvic nerves (de Groat et al. 1983) it is also likely that some SP-IR sensory neurons make functional synapses with CART-IR pelvic ganglia neurons. Interestingly, in the central nervous system SP was found to mediate the activity of both cholinergic (Vlasova and Dolgopolskii 2000) and noradrenergic neurons (Ebner and Singewald 2007), but whether such action is also present in relation to peripheral PP is a matter of speculation only. In conclusion, in the present work we have identified and described for the first time CART-like immunoreactivity in elements of PP in the female pig. The small population of CART-ergic ganglionic neurons may serve as an additional source of CART-positive nerve fibres supplying pelvic viscera. The presence of CART-IR nerve terminals surrounding noradrenergic and presumably SIF cells suggests the neuromodulatory role of the peptide in PP. We also have provided morphological data suggesting that certain neuropeptides like SP or PACAP can co-modulate the release of CART in pelvic ganglia neurons, however the exact character of this action still remains unclear.

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