Praca oryginalna *Original paper*

Cocaine- and amphetamine-regulated transcript-like immunoreactivity (CART-LI) in intramural ganglia of porcine urinary bladder trigone

ANNA ZACHARKO-SIEMBIDA, MARCIN BARTŁOMIEJ ARCISZEWSKI

Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, Akademicka 12, 20-033 Lublin, Poland

Received 18.02.2014 *Accepted 27.03.2014*

Zacharko-Siembida A., Arciszewski M. B.

Cocaine- and amphetamine-regulated transcript-like immunoreactivity (CART-LI) in intramural ganglia of porcine urinary bladder trigone

Summary

Although the expression pattern of cocaine- and amphetamine-regulated transcript (CART) has been studied in several porcine tissues, it is notable that no data are available on CART expression in the lower urinary tract. In order to map and determine the neurochemical code of CART-like immunoreactivity in the intramural ganglia of the porcine urinary bladder trigone, cryostat sections were immunohistochemically double-stained for CART and HuC/D, as well as for substance P (SP), calbindin, somatostatin and pituitary adenylate cyclase-activating peptide (PACAP). In the ganglia of the urinary bladder trigone, immunoreactivity to CART was detected both in numerous nerve fibres and in minor subpopulation of HuC/D-positive neuronal cell bodies (2.7 ± 0.8%). Neither CART-immunoreactive (IR) nerve fibres, nor CART-IR ganglionic neurons showed simultaneous expression of somatostatin, calbindin and SP. In a substantial proportion of CART-IR neurons (but not nerve fibres) co-localization with PACAP was found. This data suggest that CART present in nervous structures of the porcine urinary bladder may have a role in the parasympathetic regulation of several urinary bladder functions.

Keywords: Cocaine- and amphetamine-regulated transcript, immunohistochemistry, urinary bladder trigone, intramural ganglia, pig

Functions of the urinary bladder are controlled by neural circuits in the central nervous system (CNS: brain and spinal cord) as well as peripheral sensory and autonomic ganglia. Both noradrenaline and acetylcholine, major neurotransmitters of the autonomic nervous system, play a substantial role in the promotion of urine storage and bladder emptying (13). A retrograde tracing study with the use of Fast Blue revealed that, in the pig, sympathetic efferent neurons projecting to the urinary bladder are located in the caudal mesenteric ganglion, and in lumbar and sacral sympathetic chain ganglia (15), whereas parasympathetic output is from pelvic ganglia and the intermedio-lateral nucleus along the S3-S4 neuromers of the spinal cord (16). A substantial role in the neural autonomic regulation of the urinary bladder is also played by intramural ganglia scattered throughout the organ wall, including the urinary bladder trigone. Similarly to other peripheral neurons, a wide array of biologically active substances, including neuropeptides, have been found in neurons and nerve fibres of intramural ganglia of the urinary bladder trigone (14).

Recently, a novel neuropeptide, cocaine- and amphetamine-regulated transcript (CART) affecting mainly food intake, locomotion and motivation, was identified in certain subsets of neurons of the central nervous system (for review see 24). Molecular studies in the rat revealed that alternative splicing produces two isoforms of CART (long and short), which may be further posttranslationally processed into two biologically active 55-102 and 62-102 fragments (5). Neuroanatomical and physiological studies have confirmed that CART is a peptide that also functions as peripheral neurotransmitter. The presence of CART in numerous peripheral ganglia was immunohistochemically determined in pigs and other mammals (2, 8, 17, 21, 25). On the basis of functional studies, one could conclude that CART activities at the periphery are substantially different from those observed in the CNS and still not completely understood.

In order to determine whether, and to what extent, CART is present in porcine intramural ganglia of the urinary bladder trigone, we mapped CART expression by immunohistochemical methods. To further explore the putative neuroanatomical properties of CART in urinary bladder trigone ganglia, we looked for evidence of the co-existence of CART with calbindin D-28k, pituitary adenylate cyclase-activating peptide (PACAP), somatostatin and substance P (SP).

Material and methods

The experiments were performed in accordance with the rules approved by the local ethical committee and with Principles of Laboratory Animal Care, NIH publications No. 86-23, revised in 1985. Five (n = 5) 6-week-old female piglets weighing approx. 15 kg were used. The pigs were sedated with azaperone (Stresnil, Pharmacia&Upjohn, Poland, 0.5 mg/kg b.w.) and killed by an overdose of sodium pentobarbital (Morbital, Biowet Pulawy, Poland; 50 mg/kg b.w.). A midline incision of the abdomen was made and the urinary bladder was exposed. Urinary bladder trigones were gently cut off with sharp scissors. The dissected material fixed in Stefanini's fixative (for 24 hours) was placed in an 18% sucrose solution until the tissue sank to the bottom of the flask. Cryosections with a thickness of 10 µm were obtained with a cryostat. Every fifth section was placed on a glass slide (SuperFrost®Plus, Menzel-Gläser, Germany) and stored at –70°C until use. Sections were processed for double-labelling immunohistochemistry according to a method described elsewhere (1). To permeabilize the tissue, and to minimize nonspecific binding, the preparations were washed three times (15 minutes each) in 0.01 M phosphate-buffered saline (PBS; pH = 7.4) containing 0.25% Triton X-100, 10% normal goat serum and 0.25% bovine serum albumin (Sigma-Aldrich, Germany). The sections were incubated overnight (humid atmosphere, room temperature) with a mixture of rabbit antibodies raised against CART combined with one of the antisera listed in table 1. Mouse antibodies raised against HuC/D served as a panneuronal marker. After incubation, an excess of primary antisera was washed off with PBS (3 × 15 minutes). In order to visualize bound primary antibodies, incubation (1 hour, room temperature) with species-specific secondary antibodies conjugated to FITC or Texas Red (Table 1) was applied. After final washing, the sections were cover-slipped with phosphate-buffered glycerol (pH = 8.2) and examined under a spinning disk confocal microscope (BX-DSU Olympus, Nagano, Japan) equipped with interference filters optimized for detection of red and green fluorochromes (545-580 nm and 470-490 nm, respectively). Specificity controls of primary antibodies were carried out by preincubation of the antisera with the corresponding peptide or by omission of the primary or secondary antiserum. When controlled primary antibodies were omitted or replaced with non-immune normal goat serum, no immunoreactions for omitted or replaced antibodies were seen. Also control staining with pre-absorbed primary antisera gave no positive immunoreactions. For cell counts, at least 10 random fields containing urinary bladder trigone ganglia were analyzed for the presence of CART. In every animal, at least 600 randomly selected HuC/D-immunoreactive (IR) neurons were analyzed. The proportion of CART-IR neurons was expressed as a percentage of the total number of HuC/D-IR neurons. Semi-quantitative estimation of CART-IR nerve fibre density was based on the following 5 degree scale: absent (–), single (+), moderate (++), numerous (+++) and very numerous (++++).

Results and Discussion

CART-like immunoreactivity was present in intramural ganglia of the urinary bladder trigone as well as in singular nerve fibres supplying the muscularis propria of the urinary bladder trigone. In intramural ganglia of the urinary bladder trigone, immunoreactivity to CART was predominantly seen in moderate to numerous (++/+++) numbers of mainly varicose nerve fibres (Fig. 1A). In general, CART-IR nerve fibres closely encircled neuronal perikarya, and formed characteristic "basket-like" formations. CART-IR nerve fibres were evenly distributed throughout the ganglia

Tab. 1. Immunoproperties of antibodies used in the study. (*) indicates monoclonals

Primary antibodies				
Antigen	Raised in	Working dilution	Code	Supplier
Cocaine- and amphetamine-regulated transcript $_{(61\text{-}102)}$ (CART)	rabbit	1: 5000	H-003-61	Phoenix Pharmaceuticals, USA
HuC/D	mouse	1 : 800	A-21271	Molecular Probes, USA
Substance P (SP)	rat*	1 : 300	8450-0505	AbD Serotec, UK
Pituitary adenylate cyclase-activating peptide-27 (PACAP)	guinea-pig	1 : 150	T-5039	Bachem, Switzerland
Calbindin D-28k (CALB)	mouse*	1 : 1500	300	SWant, Switzerland
Somatostatin	rat	1 : 300	8330-0009	Biogenesis UK
Secondary antibodies				
Antigen	Conjugate	Raised in	Working dilution	Code	Supplier
Rabbit IgG	Texas Red	goat	1 : 400	55675	MP Biomedicals, USA
Mouse IgG	FITC	goat	1 : 300	55493	MP Biomedicals, USA
Guinea-pig IgG	FITC	goat	1 : 300	55000	MP Biomedicals, USA
Rat IgG	FITC	goat	1 : 400	55745	MP Biomedicals, USA

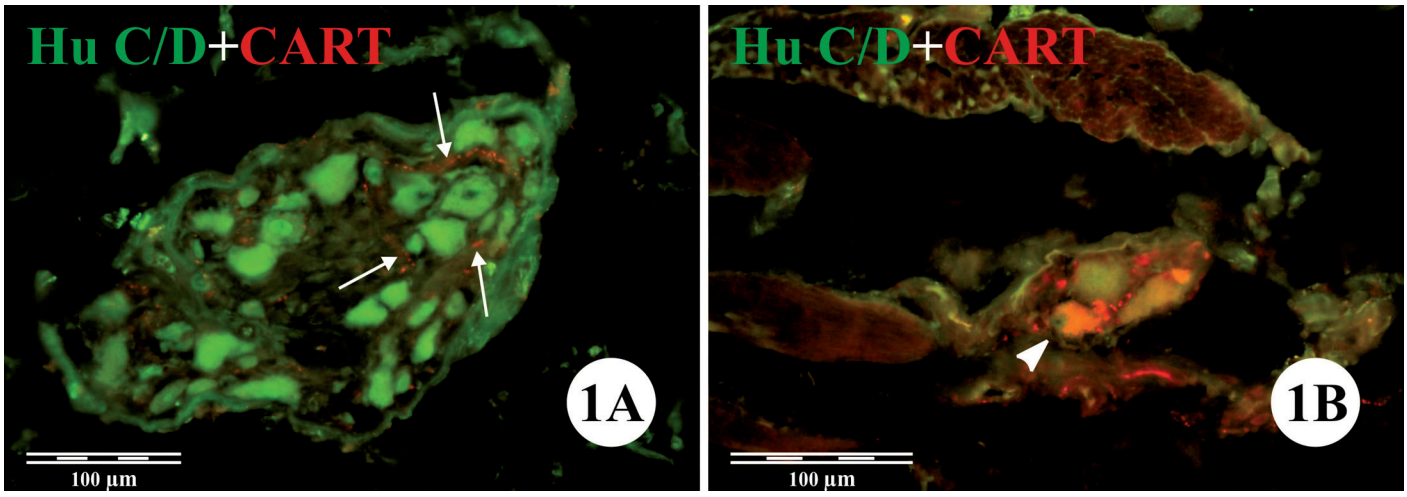

Fig. 1. In the intramural ganglia of the porcine urinary bladder trigone both varicose CART-IR nerve fibres (arrows in 1A) and single CART-expressing neurons (arrowhead in 1B) are seen

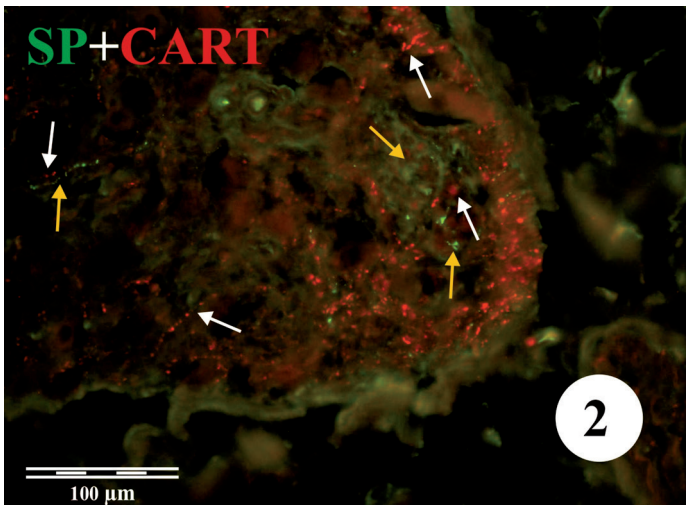

Fig. 2. CART-like immunoreactivity (white arrows) was not observed in SP-IR nerve fibres (yellow arrows) supplying the ganglia of the urinary bladder trigone

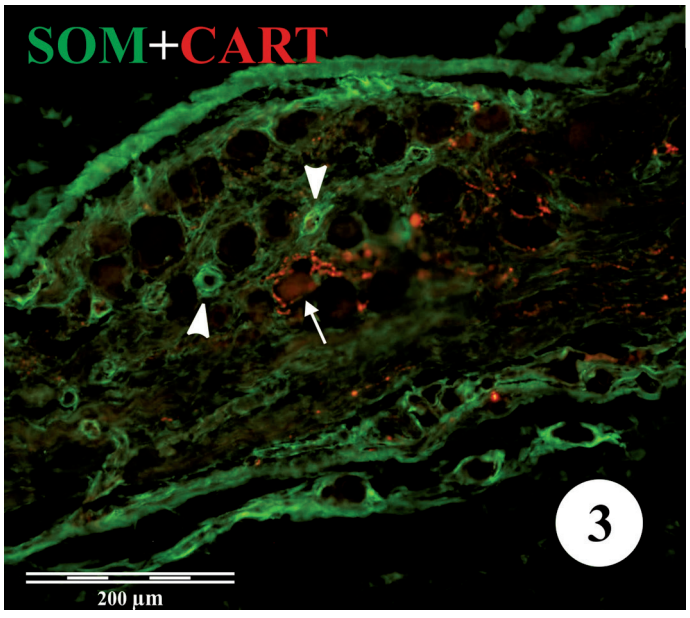

Fig. 3. The transverse section through an intramural ganglion of the porcine urinary bladder trigone illustrates that no expression of CART is seen (arrow marks CART-IR neuron) in somatostatin-positive structures (arrowheads)

of the urinary bladder trigone (the average numbers of CART-IR nerve fibres observed in the central region and in the peripheral region of ganglia were relatively similar). By immunostaining to HuC/D, it is possible to visualize precisely neuronal perikarya of the intramural ganglia of the urinary bladder trigone. Amongst HuC/D-positive neurons, perikarya that were additionally immunopositive to CART (Fig. 1B) were rarely seen (2.7 ± 0.8% of overall neuronal population). Virtually all immunostained HuC/D-IR/CART-IR neurons were classified as middle-sized (with diameters ranging between 28 and 41 µm) and oval cells. CART-IR neurons were predominantly seen at the ganglion periphery. Neither CART-IR nerve fibres nor CART-positive neurons displayed simultaneous immunoreactivity to SP (Fig. 2). Single CART-IR nerve fibres frequently ran in close vicinity to SP-IR neurons as well as to SP-IR nerve fibres present in the ganglia. No co-localization of CART and somatostatin was found in either neurons or nerve fibres of intramural ganglia of the urinary bladder trigone (Fig. 3). Only sparse CART--IR nerve fibres were found in close neighbourhood of somatostatin-positive structures. The vast majority of CART-IR neurons were found to co-express PACAP; however PACAP-positive neurons lacking CART were commonly found (Fig. 4). Immunoreactivity to PACAP was seen only in the neuronal cytoplasm of neurons, which prevents us from determining the co-localization pattern of CART and PACAP in nerve fibres. CART-IR nerve fibres frequently encircled PACAP-positive neurons, forming characteristic "basket-like formations". In none of CART-IR ganglionic neurons and CART-IR nerve fibres did we find a simultaneous expression of calbindin (Fig. 5). Calbindin-positive elements (most likely of non-neuronal origin) were frequently distributed in a close neighbourhood of CART-IR neurons.

The present study demonstrated for the first time the presence of CART-like immunoreactivity in intramural ganglia of the porcine urinary bladder trigone. The expression of CART was predominantly seen in nerve fibres within the ganglia, whereas the population

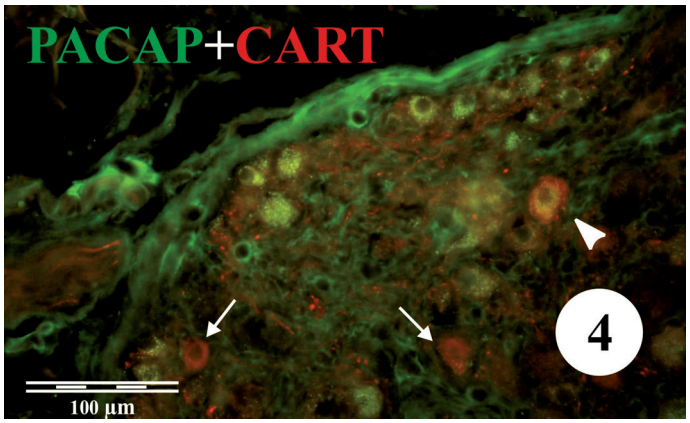

Fig. 4. The micrograph presents two different subpopulations of CART-expressing neurons found in the intramural ganglia of the urinary bladder trigone. A CART-IR/PACAP-IR neuron is indicated with an arrowhead whereas two CART-positive/ PACAP-negative neurons are marked with arrows

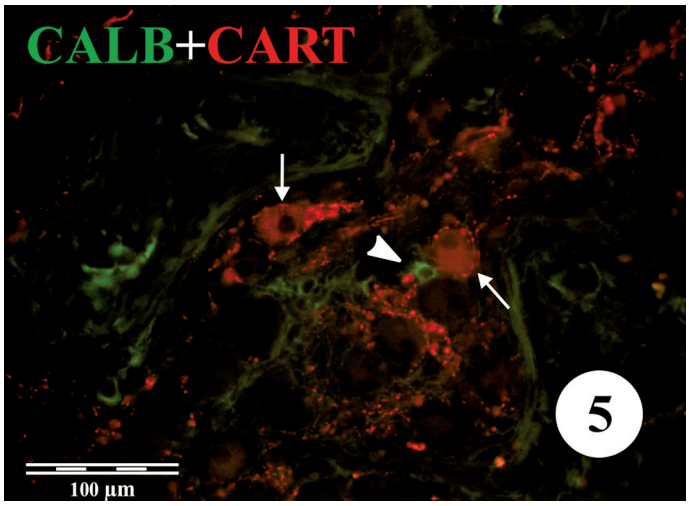

Fig. 5. In the transverse section through a urinary bladder trigone ganglion, no expression of CALB (arrowhead) is seen either in CART-IR neurons (arrows) or in CART-IR nerve fibres

of ganglionic CART-IR neurons was relatively small (approx. 3%). Such a distribution pattern suggests that CART influences the activities of ganglionic neurons of the urinary bladder trigone, and, at least to some degree, may participate in the regulation of urinary bladder functions (urine outflow). So far, immunoreactivity to CART in the lower urinary tract was reported only in ganglia and nerve fibres of the muscular layer and urothelium of rat ureter and urinary bladder (9, 27). Age-dependent distribution patterns of CART-IR structures in the rat urinary bladder were noted, which may suggest a role of this peptide in ontogenesis (27). Since no more descriptive studies of CART expression in urinary bladder trigone ganglia have been conducted in other mammals, references to other peripheral ganglia would be of interest. In the rat sensory proximal ganglion of the vagus, as many as 40-50% of afferent neurons were found to express CART (25). It is noteworthy that approx. 1% of sensory neurons of the rat trigeminal ganglion and 10% of neurons located in the lumbar segment of rat spinal ganglia contained CART (8, 12). It should be emphasized that CART is not widely distributed in autonomic peripheral neurons in rodents. In general, rat sympathetic ganglia (cervicothoracic and cranial cervical) contain a dense network of CART-IR nerve fibres, but no CART-positive cell bodies (6). Another distribution patterns of CART is noted in rat parasympathetic sphenopalatine and otic ganglia, where 5.25% and 4.32% (respectively) populations of CART-IR neurons are present (8). From the anatomical and functional point of view, intramural ganglia of the urinary bladder trigone are part of the parasympathetic efferent pathway conveying impulses from pelvic ganglia (19). In this light, the CART-like innervation patterns presented here closely resemble those previously described in other parasympathetic ganglia. The presence of numerous CART-IR nerve terminals around ganglionic nervous cell bodies raises the question about their origin. At least two possible sources should be considered. First, it is likely that CART-IR nerve terminals could have derived from pelvic ganglia (or the major pelvic ganglion in the case of rodents) which contain postganglionic neurons supplying the urinary bladder (11). No neuroanatomical studies showing the expression of CART in pelvic ganglia have been conducted, but one would expect a small subpopulation of CART-IR neurons to be present in the parasympathetic component, since pelvic ganglia are mixed ganglia of both sympathetic and parasympathetic character (10). Another possibility is that the observed CART-IR nerve fibres were collaterals of extrinsic CART-IR primary afferent neurons located in dorsal root ganglia (12). It must be pointed out, however, that CART-IR primary sensory neurons usually co-express calcitonin gene-related peptide and/or SP (12), and in the present study we found no co-localization between CART and SP or between CART and somatostatin (also known to be stored in primary sensory neurons). Nevertheless, in order to precisely determine the origin and specific pathways of CART-IR neurons, more comprehensive studies with the use of the retrograde tracing technique are necessary.

The hypothetic role of CART in the urinary bladder parasympathetic voiding reflex is a matter of speculation at the moment. Given the specific distribution pattern described here, it is possible that CART present in ganglia of the urinary bladder trigone may function as the principal neurotransmitter/neuromodulator and/or neuroprotective agent. The role of CART as a neurotransmitter mediating short-term satiety has been previously demonstrated in relation to peripheral neurons of rat nodose ganglia (7), whereas a neuromodulatory action of CART has been widely reported in peripheral intrapancreatic ganglia (for review see 22). It has been found that the numbers of CART-immunoreactive structures of the urinary bladder significantly increase in rats with experimentally induced nephrogenic hypertension, which may

suggest that this peptide in involved in the regulation of neuroplastic processes as a result of hypertension--associated urinary bladder tissue damage (9). Our finding that numerous CART-IR neurons additionally co-expressed PACAP further supports the hypothesis that CART in porcine urinary bladder trigone ganglia may play a role in neuronal transmission, survival and adaptive processes. Vasoactive intestinal peptide and PACAP, belonging to the same peptide family, have been identified as multipotent factors influencing numerous parasympathetic-related urinary bladder functions, including excitation of neurons in urinary bladder intramural ganglia (23). In both central and peripheral neurons, PACAP, acting via PAC1 receptors, exhibits strong neuroprotective properties, promoting neuronal survival and neurite outgrowth (18). It is worth noting that marked changes in PACAP expression have been observed in micturition reflex pathways (spinal cord and dorsal root ganglia) after chronic cystitis (20), spinal cord injury (26) and urinary bladder instillation with tetrodotoxin (3). On the other hand, it must be kept in mind that in the CNS, the PACAP 6-38 fragment has been identified as a functional antagonist of CART (4). In the present study we have not found co-localization between CART and somatostatin or calbindin. The presence of calbindin (but not somatostatin) was reported in CART-IR neurons of rat intracardiac ganglia, which allows the authors to distinguish two functionally different cholinergic inputs to intracardiac neurons (17). The absence of somatostatin and calbindin in CART-positive structures of the porcine urinary bladder suggests that neither of the neuropeptides plays a significant role(s) in the CART-dependent regulation of the urinary bladder activity.

In summary, the presence of CART in both neurons and nerve fibres of intramural ganglia of the porcine urinary bladder trigone emphasizes its importance as a neuronal messenger. The co-existence of PACAP in CART-IR ganglionic neurons raises new questions about a neuroprotective function of CART in the lower urinary tract. Further experimental research should be done to investigate the exact role of CART in the regulation of urinary bladder activity under normal and pathological conditions.

References

1. *Arciszewski M. B.*: Distribution of calcitonin gene-related peptide (CGRP), substance P (SP) and galanin (GAL) immunoreactive nerve fibers in the seminal vesicle and prostate of the male sheep. Ann. Anat. 2004, 186, 83-87.
2. *Arciszewski M. B., Barabasz S., Skobowiat C., Maksymowicz W., Majewski M.*: Immunodetection of cocaine- and amphetamine-regulated transcript in the rumen, reticulum, omasum and abomasum of the sheep. Anat. Histol. Embryol. 2009, 38, 62-67.
3. *Bossowska A., Majewski M.*: Tetrodotoxin induced changes in the chemical coding of dorsal root ganglion neurons supplying the porcine urinary bladder. Pol. J. Vet. Sci. 2012, 15, 355-363.
4. *Burgos J. R., Iresjö B. M., Smedh U.*: Pituitary adenylate cyclase-activating polypeptide 6-38 blocks cocaine- and amphetamine-regulated transcript peptide-induced hypophagia in rats. PLoS One 2013, 8, e72347.
5. *Dey A., Xhu X., Carroll R., Turck C. W., Stein J., Steiner D. F.*: Biological processing of the cocaine and amphetamine-regulated transcript precursors by prohormone convertases, PC2 and PC1/3. J. Biol. Chem. 2003, 278, 15007--15014.
6. *Gonsalvez D. G., Kerman I. A., McAllen R. M., Anderson C. R.*: Chemical coding for cardiovascular sympathetic preganglionic neurons in rats. J. Neurosci. 2010, 30, 11781-11791.
7. *Heldsinger A., Lu Y., Zhou S. Y., Wu X., Grabauskas G., Song I., Owyang C.*: Cocaine- and amphetamine-regulated transcript is the neurotransmitter regulating the action of cholecystokinin and leptin on short-term satiety in rats. Am. J. Physiol. Gastrointest. Liver Physiol. 2012, 303, G1042-G1051.
8. *Ivanusic J. J., Goulding K. E., Kwok M. M., Jennings E. A.*: Neurochemical classification and projection targets of CART peptide immunoreactive neurons in sensory and parasympathetic ganglia of the head. Neuropeptides 2012, 46, 55-60.
9. *Janiuk I., Kasacka I.*: Distribution of cocaine and amphetamine regulated transcript in ureters and urinary bladder of hypertensive rats. J. Biol. Regul. Homeost. Agents 2013, 27, 409-416.
10. *Keast J. R.*: Plasticity of pelvic autonomic ganglia and urogenital innervation. Int. Rev. Cytol. 2006, 248, 141-208.
11. *Keast J. R., Booth A. M., de Groat W. C.*: Distribution of neurons in the major pelvic ganglion of the rat which supply the bladder, colon or penis. Cell Tissue Res. 1989, 256, 105-112.
12. *Kozsurek M., Lukácsi E., Fekete C., Wittmann G., Réthelyi M., Puskár Z.*: Cocaine- and amphetamine-regulated transcript peptide (CART) is present in peptidergic C primary afferents and axons of excitatory interneurons with a possible role in nociception in the superficial laminae of the rat spinal cord. Eur. J. Neurosci. 2007, 26, 1624-1631.
13. *Ochodnicky P., Uvelius B., Andersson K. E., Michel M. C.*: Autonomic nervous control of the urinary bladder. Acta Physiol. (Oxf) 2013, 207, 16-33.
14. *Pidsudko Z.*: Distribution and chemical coding of neurons in intramural ganglia of the porcine urinary bladder trigone. Folia Histochem. Cytobiol. 2004, 42, 3-11.
15. *Pidsudko Z.*: Immunohistochemical characteristics and distribution of neurons in the paravertebral, prevertebral and pelvic ganglia supplying the urinary bladder in the male pig. J. Mol. Neurosci. 2014, 52, 56-70.
16. *Ragionieri L., Botti M., Gazza F., Sorteni C., Chiocchetti R., Clavenzani P., Minelli L. B., Panu R.*: Localization of peripheral autonomic neurons innervating the boar urinary bladder trigone and neurochemical features of the sympathetic component. Eur. J. Histochem. 2013, 57, e16.
17. *Richardson R. J., Grkovic I., Anderson C. R.*: Cocaine- and amphetamine--related transcript peptide and somatostatin in rat intracardiac ganglia. Cell Tissue Res. 2006, 324, 17-24.
18. *Tamas A., Reglodi D., Farkas O., Kovesdi E., Pal J., Povlishock J. T., Schwarcz A., Czeiter E., Szanto Z., Doczi T., Buki A., Bukovics P.*: Effect of PACAP in central and peripheral nerve injuries. Int. J. Mol. Sci. 2012, 13, 8430-8448.
19. *Uvelius B., Gabella G.*: The distribution of intramural nerves in urinary bladder after partial denervation in the female rat. Urol. Res. 1998, 26, 291-297.
20. *Vizzard M. A.*: Up-regulation of pituitary adenylate cyclase-activating polypeptide in urinary bladder pathways after chronic cystitis. J. Comp. Neurol. 2000, 420, 335-348.
21. *Wierup N., Gunnarsdóttir A., Ekblad E., Sundler F.*: Characterisation of CART-containing neurons and cells in the porcine pancreas, gastro-intestinal tract, adrenal and thyroid glands. BMC Neurosci. 2007, 8, 51.
22. *Wierup N., Sundler F.*: CART is a novel islet regulatory peptide. Peptides 2006, 27, 2031-2036.
23. *Yoshiyama M., de Groat W. C.*: The role of vasoactive intestinal polypeptide and pituitary adenylate cyclase-activating polypeptide in the neural pathways controlling the lower urinary tract. J. Mol. Neurosci. 2008, 36, 227-240.
24. *Zhang M., Han L., Xu Y.*: Roles of cocaine- and amphetamine-regulated transcript in the central nervous system. Clin. Exp. Pharmacol. Physiol. 2012, 39, 586-592.
25. *Zheng H., Patterson L. M., Berthoud H. R.*: CART in the dorsal vagal complex: sources of immunoreactivity and effects on Fos expression and food intake. Brain Res. 2002, 957, 298-310.
26. *Zvarova K., Dunleavy J. D., Vizzard M. A.*: Changes in pituitary adenylate cyclase activating polypeptide expression in urinary bladder pathways after spinal cord injury. Exp. Neurol. 2005, 192, 46-59.
27. *Zvarova K., Vizzard M. A.*: Distribution and fate of cocaine- and amphetamine--regulated transcript peptide (CARTp)-expressing cells in rat urinary bladder: a developmental study. J. Comp. Neurol. 2005, 489, 501-517.

Corresponding author: Assistant professor Anna Zacharko-Siembida, Akademicka 12, 20-033 Lublin, Poland; e-mail: asiembida13@wp.pl