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Original article

Colocalization pattern of cocaine- and amphetamine-regulated transcript peptide and parvalbumin immunoreactivity in the hippocampus proper of the chinchilla

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Abstract

This study set out to investigate, for the first time, the distribution and colocalization pattern of cocaine-and amphetamine-regulated transcript (CART) and one of the calcium binding--proteins: parvalbumin (PV) in the chinchilla's hippocampus proper (HP). HP, consisting of Ammon's horn (CA) and the dentate gyrus (DG), is an important component of the limbic system, involved in learning and memory processes. CA showed a higher immunoreactivity of CART (-IR) compared to DG. CART-IR neurons were mainly observed in the molecular layer of DG and in the pyramidal layer of CA. CART-IR fibers were present in the granular layer; in the hilus numerous mossy fibers were detected, while in the molecular layer CART-IR fibers were not found. In all CA fields (CA1-CA3), CART-IR fibers were only present in the lacuno-sum-molecular layer. Immunofluorescence with double- labeling showed that only CART-IR cells stained positive for PV, whereas in CART-IR fibers there was no PV-positive reaction. Our research supplements missing knowledge about the distribution and colocalization pattern of CART with PV in the chinchilla's hippocampus, and also provides a better understanding of the similarities and differences among individuals of the same species and also with other mammals.

Key words: hippocampus, dentate gyrus, CART, PV, $Ca2^{+}$, memory, chinchilla

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Introduction

Cocaine-and amphetamine-regulated transcript (CART) has been analyzed for over 20 years. First, CART mRNA was identified in the rats striatum (Douglass et al. 1995), and further research has shown that CART peptide is present throughout various regions of the central nervous system (CNS) (Douglass et al. 1995, Koylu et al. 1997, Smith et al. 1997, Larsen et al. 2003, Fagergren and Hurd 2007, Hubert et al. 2008), enteric nervous system (ENS) (Zacharko-Siembida and Arciszewski 2014), peripheral nervous system (PNS) (Zacharko-Siembida and Arciszewski 2014, Zacharko-Siembida et al. 2014, Radzimirska et al. 2016) and cells in the urinary bladder (Janiuk and Kasacka 2015). The presence of CART in the dense core vesicles of synaptic terminals implies that this peptide acts as a neurotransmitter and/or neuromodulator (Smith et al. 1997) and coexists with other biologically active substances (Smith et al. 1997, Zacharko-Siembida and Arciszewski 2014, Zacharko-Siembida et al. 2014, Radzimirska et al. 2016). CART seems to be an active peptide which is associated with its many physiological functions. CART peptides are synthesized in the mesolimbic dopamine system of CNS (Smith et al. 1997, Hubert et al. 2008). As recently discovered, functional interactions of CART and stress-related peptides may influence hypothalamic-pituitary-adrenalin axis activity (Koylu et al. 1997, Larsen et al. 2003). This neurotransmitter is a mediator of the leptin regulation of bone mass (McGee-Lawrence et al. 2015). CART peptide attends in the processes involved in feeding and body-weight regulation, leading to the activation of the dopaminergic reward system in the hypothalamus. Studies confirm that the administration of recombinant CART peptide into the right lateral ventricle of the hypothalamus leads to a large decrease in food intake in both lean and obese rats (Larsen et al. 2000). Recent studies show that high blood pressure can be a factor that increases CART secretion (Janiuk and Kasacka 2015). In rats with simultaneously stimulated D1 and D2 dopamine receptors intracucumbal injection of CART reduced the effects of psychostimulants (Moffett et al. 2011). Psychostimulants such as cocaine lead to increased expression of CART peptides and CART mRNA in the striatum, nucleus accumbens, amygdala complex and the entorhinal and piriform cortices. In addition, some research has indicated a neuroprotective action of CART (Bharne et al. 2013). Upadhya et al. (2016) suggested the importance of CART as a therapeutic agent in the treatment of Parkinson's disease (PD).

Calcium-binding proteins (CaBPs) play a role as factors regulating the normal function of the limbic system neurons, mainly by influencing the levels of Ca^{2+} ions in the neuroplasm. Parvalbumin (PV) belongs to a large family of EF-hand calcium binding proteins (Schwaller 2010). PV is a buffering protein which was classified as a calcium slow buffer. Its primary task is to reduce the level of Ca2+ in the cytoplasm (Schwaller 2010). Omnipresent Ca2+ is one of the main messengers that regulate various activities in neurons (Pchitskaya et al. 2018). Neurons require extremely precise control of calcium-dependent processes, since it is believed that calcium ions regulate such vital functions as synaptic plasticity, metabolism, transport information between different organelles and induction of gene expression in the nucleus (Schwaller 2010). There is evidence that PV plays a crucial role in the regulation of Ca2+ level (Schwaller 2010) and that neuronal calcium signaling is abnormal in many neurodegenerative disorders such as Alzheimer's disease (AD), Huntington's disease (HD) and PD (Pchitskaya et al. 2018). The expression patterns of calcium binding proteins in various populations of central neurons and their functions are still being investigated.

Hippocampus proper (HP) comprises Ammon's horn (CA) and the dentate gyrus (DG) (El-Falougy and Benuska 2006). Similarly, as in other areas of the brain, HP neurons are classified into two types i.e. primary neurons and interneurons (El-Falougy and Benuska 2006). HP is the main part of the hippocampal formation and an important component of the so-called extended "hippocampal memory system" (Eichenbaum and Cohen 2008). HP is also the main part of the limbic system, which plays a crucial role in the formation of memory, behavioral reactions and the pathogenesis of neurodegenerative diseases of CNS including AD, HD and PD (Faria et al. 2016, Mendioroz et al. 2016). HP, via numerous connections to various brain structures, participates in many control functions of CNS (El-Falougy and Benuska 2006). Biologically active substances such as CART and PV can potentially be involved in the process of neuronal chemical coding change. Based on the chemical content of biologically active substances, various neuronal populations can be determined and their function predicted (Eguiagaray et al. 2004). Therefore, the aim of the present study was to immunohistochemically investigate the localization of CART and PV in the hippocampus proper of the chinchilla. Taking into account that until now the precise central function(s) of both peptides are still unknown, we believe that a description of CART and PV expression in the chinchilla HP will provide a new insight into this field.

Materials and Methods

Tissue preparation

Animal care protocols, experimental design and methods were reviewed and approved by the II[nd] Local Ethical Committee at the University of Life Sciences in Lublin, Poland. Five (n=5) sexually mature male chinchillas (ca. 1.5 years old) were used in the study. After slaughter the brains were immediately dissected out and immersed in 15% saturated picric acid and 2% paraformaldehyde fixative solution for 24h at room temperature (RT). For cryoprotection the material was placed in a container filled with 18% sucrose solutions and kept for 5-6 days until it sank to the floor of the flask. Finally, frozen brains (-20°C) were cut into 10 µm-thick sections on a cryostat. Each section was mounted onto a glass slide (SuperFrost® Plus, Menzel-Gläser, Thermo Scientific, Braunschweig, German) and stored at -20°C until use.

Immunohistochemistry

For immunohistochemical staining an indirect immunofluorescence method described elsewhere (Zacharko-Siembida et al. 2014) was used. Briefly, sections were dried for 15 min at RT. Potential non-specific binding sites were blocked by section incubation 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 (Sigma-Aldrich, St. Louis, MO, USA). The sections were then placed in a dark humid chamber and incubated overnight (RT) with different combinations of primary antibodies. Firstly, mouse monoclonal Hu C/D antibodies used as a general neuronal marker (1:400; Molecular Probes, Eugene, OR, USA; code A-21271) were combined with CART antibodies raised in rabbit, (1:10000; Phoenix Pharmaceuticals, USA; code H-003-61). In other staining, in order to examine the colocalization of CART with PV, the rabbit CART antibodies were mixed with mouse antibodies raised against PV (1:2000, SWant, Switzerland; code PV 235). Bound primary antibodies were visualized with Texas Red-conjugated anti-rabbit goat IgG (1:400; MP Biomedicals, OH, USA) and FITC-conjugated anti-mouse goat IgG (1:400; MP Biomedicals). After final washes with PBS, the slides were cover-slipped and viewed with a spinning disk confocal microscope (BX-DSU Olympus, Nagano, Japan) equipped with interference filters appropriate for detection of Texas Red (545–580 nm; MWIY2) and FITC (470–490 nm; MNIBA2). The specificity of primary antibodies was also inspected using procedures in which the primary antibodies were either skipped or substituted with non-immunoreactive sera.

Cell counting and statistical analysis

The proportion of neurons immunoreactive (IR) to CART and PV were expressed as a percentage in relation to all Hu CD-IR neurons. The frequencies of CART-expressing neurons co-storing PV were expressed as percentages relative to the total visualized CART-IR nerve cells. Only neurons with a clearly visible nucleus were counted. In each animal at least one hundred neurons in CA1, CA2, CA3 and GD were viewed and counted. The identical method was also used in colocalization studies (co-expression of CART with PV). All numerical data are expressed as mean ± standard deviation (SD). Multiple homologous and non-homologous group comparisons were performed using the non-parametric Kruskal-Wallis test. Probabilities of $p<0.05$ were considered significant.

Results

In the chinchilla hippocampus, DG and CA have been recognized as layered structures. DG consists of the inner granular layer and outer molecular layer. Below these two layers the hilus is located. CA is composed of three cellular layers: the inner lacunosum (molecular) stratum, the middle pyramidal stratum, and the outer stratum oriens.

Distribution pattern and morphology of CART-IR structures in DG and CA of the chinchilla

Nerve cells expressing CART were detected in all studied fields of CA as well as in DG of the chinchilla (Fig. 4, 1A-4A). In general, in the particular layers of CA and DG different CART distribution patterns were found. Amongst nerve cells of DG (all layers) as many as 3.4 ± 0.6% showed immunoreactivity to CART (Fig. 1). CART-IR nerve cells were mainly observed in the molecular layer, while in the granular layer and hilus, single CART-IR neurons were noted. The vast majority of CART-IR neurons present in the granular layer of DG were round and oval in shape, whereas CART-IR nerve cells found in the hilus and molecular layer were mainly fusiform, triangular and sometimes pyramidal in shape. In the granular layer CART-IR neurons were frequently encircled by CART-positive nerve fibres. Likewise, in the hilus numerous CART-IR mossy nervous fibres were detected. In the molecular layer CART-IR nerve fibres were not observed (Figs. 4, 4A). In CART-IR neurons present in all fields of CA, the expression of the neuropeptide was localized in the neuronal cell body (cytoplasm and nucleus) as well as in fibre-like nerve exten-

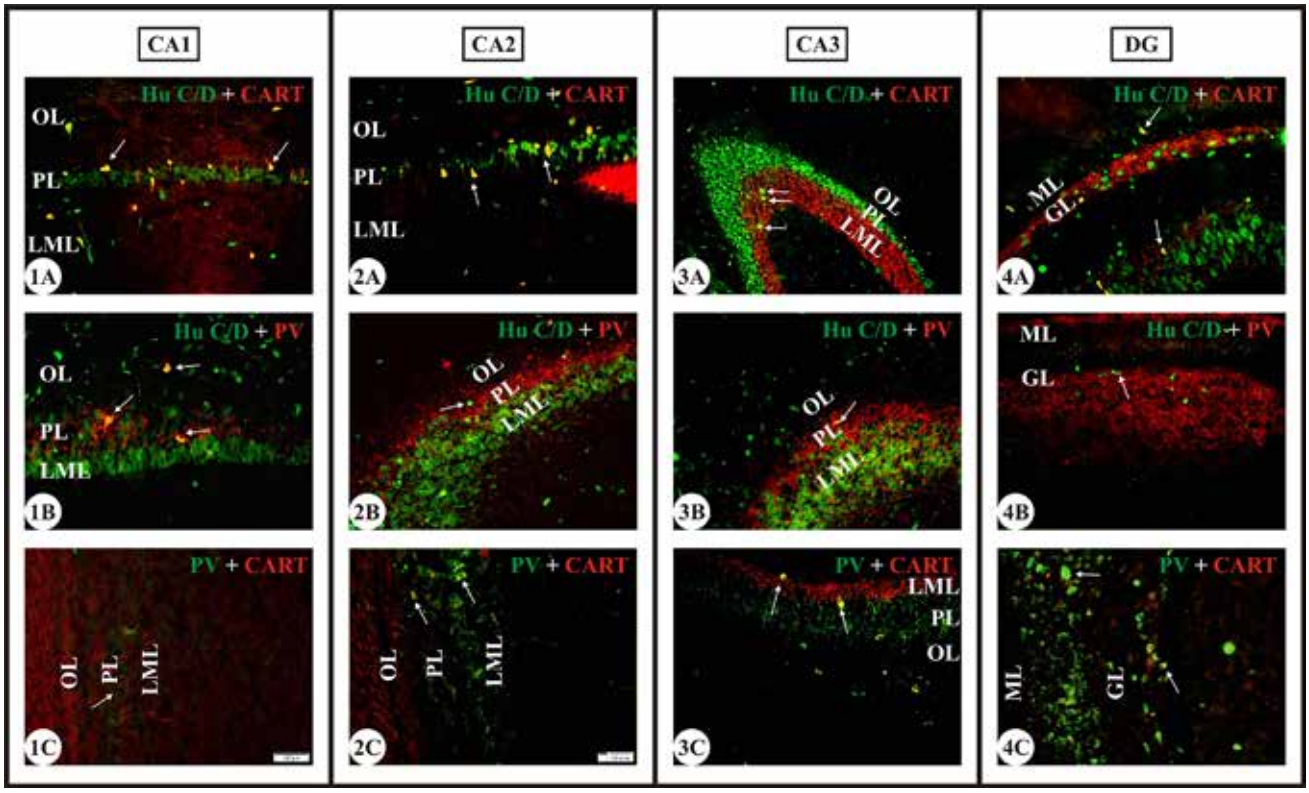

Fig.4. Double-labeling immunofluorescence in CA1 (1), CA2 (2), CA3 (3) fields and DG (4). 1-4A immunoreactivity to cocaine- and amphetamine-regulated transcript CART is demonstrated in CA fields and DG. 1-4B immunoreactivity to parvalbumin PV is demonstrated in CA fields and DG. 1-4C co-expression patterns of CART with PV. OL – oriens layer, PL – pyramidal layer, LML – lacunosum (molecular) layer, ML – molecular layer, GL – granular layer. The arrows indicate colocalization of the neurons in the CA1-CA3 fields and DG. Scale bar = 100 μm.

sions (Fig. 4, 1A-3A). In the CA1 field, 6.4 ± 0.9% of mainly pyramidal and fusiform in shape neurons were CART-IR. The vast majority of CA1 field CART-positive neurons were localized in the pyramidal layer (Fig. 1) whereas in the remaining layers only single CART-IR neurons were visualized. Numerous thin, long CART-positive nerve fibres were detected, especially in the lacunosum (molecular) layer. In comparison to the CA1 field, in the CA2 field less numerous CART-IR neurons (5.6 ± 0.6%; n=5) were observed (Fig. 1). The triangular, fusiform and pyramidal CART-IR neurons were mainly located in the pyramidal layer of CA2 fields. Similarly to the CA1 field, numerous CART-IR long nerve fibres were detected in the lacunosum (molecular) layer. The smallest population of CART-positive neurons (4.2 ± 0.4%; n=5) in comparison to the CA1 and CA2 fields, was found in the CA3 field (Fig. 1). In the CA3 field, CART-IR neurons were mainly located in the pyramidal layer, whereas in both the oriens and lacunosum (molecular) layers only single CART-positive neurons were observed. Very numerous CART-IR nerve fibres in the CA3 field formed a strand that stretched the entire length of the lacunosum (molecular) layer of the CA3 field.

Distribution pattern and morphology of PV-IR structures in the DG and CA of the chinchilla

As many as 3.2 ± 0.4% (n=5) of DG neurons were immunopositive to PV (Fig. 2). PV-IR cells were evenly scattered throughout all layers of the DG. Most of the PV-IR DG neurons were triangular, round or oval in shape, whereas in the hilus CART-IR fusiform cells were additionally detected. Single PV-IR nerve fibres were found throughout the DG. In general, PV-IR neurons found in all layers were moderately stained (Fig. 4, 4B). In turn, an intensive immunoreactivity to PV was observed in the neuronal cytoplasm, nucleus and nerve fibres in neurons located in all CA fields (Fig 4, 1B-3B). A small proportion of PV-IR neurons was found in the CA1 field and amounted to 3.4 ± 0.6% (Fig. 2). No presence of PV-IR neurons was detected in the lacunosum (molecular) layer of the CA1 field. PV-IR nerve cells were predominantly oval and pyramidal in shape. Numerous PV-IR nerve fibres were mostly observed in the oriens layer of the CA1 field. A significantly higher population of PV-IR neurons (4.2 ± 0.5%, n=5, $p<0.05$; vs. CA1 field), was noted in the CA2 field (Fig. 2). PV-IR cells of the CA2 field were mainly pyramidal, fusiform or triangular in shape. Abundant PV-IR

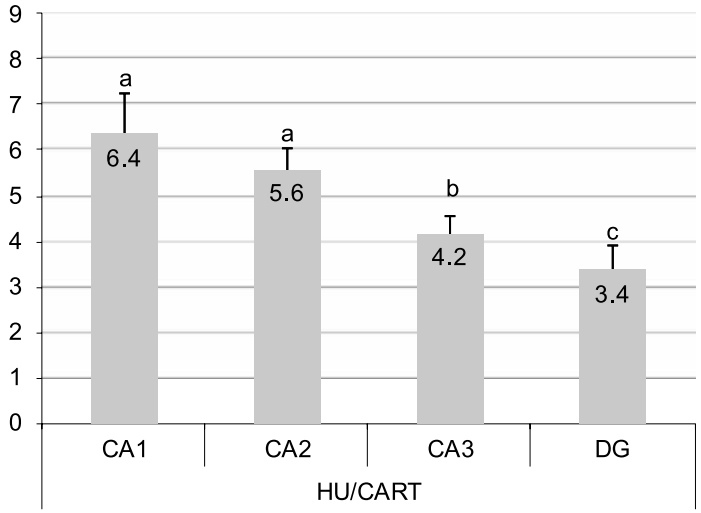

Fig. 1. Percentage of HU/CART positive cells in hippocampal CA1, CA2, CA3 fields and DG with standard deviation bars. Different letters (a-c) indicate statistically significant differences (Kruskal-Wallis $p<0.05$).

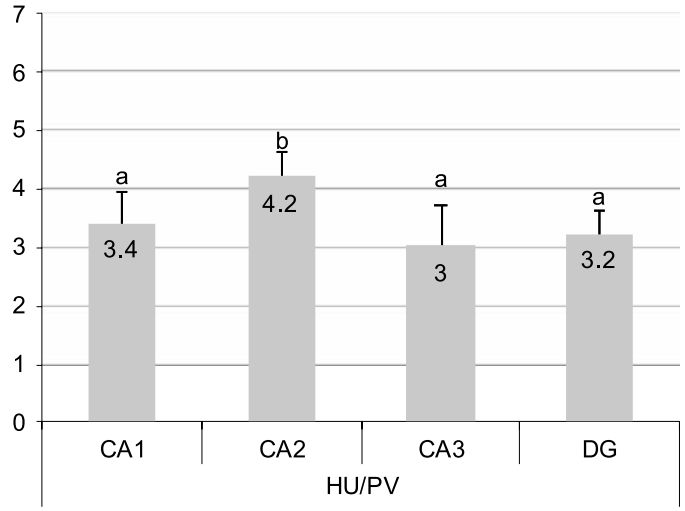

Fig. 2. Percentage of HU/PV positive cells in hippocampal CA1, CA2, CA3 fields and DG with standard deviation bars. Different letters (a, b) indicate statistically significant differences (Kruskal-Wallis $p<0.05$).

nerve fibres were found mostly in the oriens layer and in the pyramidal layer. In the latter layer, PV-IR nerve fibres frequently surrounded the cell bodies of neurons. In the CA3 field, the population of PV-IR neurons was calculated as 3.0 ± 0.7% and was statistically lower when compared to the CA1 and CA2 fields (Fig. 2). Similarly to the CA1 field, PV-IR nerve fibres were mainly located in the oriens and pyramidal layer and surrounded the cell bodies of neurons. Additionally, in the lacunosum (molecular) layer of the CA3 field, numerous nerve fibres showed immunoreactivity to PV (Fig. 4, 1B-3B).

Co-localization pattern of CART-IR peptide and PV-IR

The co-localization pattern of CART and PV was observed in both the DG and CA (Fig. 4, 1C-4C). In the granular and molecular layer of the DG, most of the CART-IR neurons (70.0 ± 11.1%; n=5) were additionally positive to PV (Fig. 3). No co-localization of CART and PV was detected in the hilus. The highest proportion of CART-IR/PV-IR neurons was found in the CA2 field, while a lower proportion was noted in the CA3 field (73.0 ± 8.3% and 52.0 ± 4.5%, respectively) (Fig. 3). In the CA1 field, the vast majority of CART-IR neurons did not show the presence of PV. Double immunofluorescence staining revealed that in neither DG nor CA did CART- IR nerve fibers show immunoreaction of PV.

Discussion

As far as we are aware, this is the first study analyzing the distribution of CART-IR and PV-IR nervous structures as well as the co-localization patterns of these two substances in the hippocampus of the chinchilla.

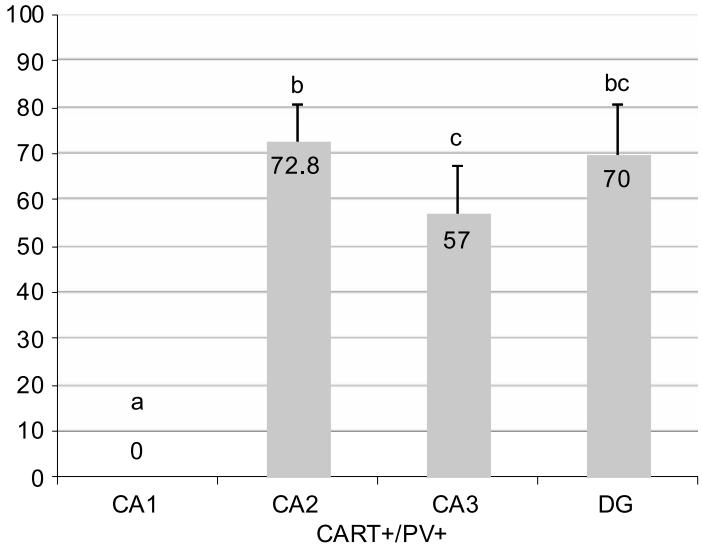

Fig. 3. Percentage of CART-IR neurons co-expressing with PV in hippocampal CA1, CA2, CA3 fields and DG with standard deviation bars. Different letters (a-c) indicate statistically significant differences (Kruskal-Wallis $p<0.05$).

The chinchilla, as a rodent, is often used as a model laboratory animal (Ren at al. 2019), but it is also well known as a companion animal. Based on the results obtained in the present study, CART peptides were found to be present in neurons, cytoplasm and nerve fibers throughout the DG and in all fields of the CA of the chinchilla hippocampus, although the percentages of CART-positive neurons were lower in the DG when compared to particular CA fields. CART-IR neurons prevailed in the molecular layer of the DG, whereas in the granular layer and in the hilus, only single CART-positive cells were found. Additionally, numerous CART-IR nerve fibers were detected in the granular layer, but many mossy nerve fibers were found in the hilus. In the CA, numerous CART-positive neurons were observed in the pyramidal layer, and CART-IR nerve fibers ha detected in the lacunosum (molecular) layer. As previously reported, CART-positive neurons and nerve fibers have been observed in the hippocampus in various mammalian species including human. Consistently with our study, in the rat DG (Seress et al. 2004) single CART-IR neurons were found, although in this animal species CART-positive cells were mainly located on the border between the molecular and granular layer, whereas in the chinchilla CART-IR neurons were present in both layers. CART-IR nerve cells in the granular layer were also observed in the guinea pig and the domestic pig (Kolenkiewicz et al. 2009). Additionally, in the guinea pig DG, CART-positive neurons were also present in the molecular layer. We observed single CART-positive neurons in the hilus of the chinchilla DG which is in line with previous reports obtained from the guinea pig but not in in the rat and domestic pig (Kolenkiewicz et al. 2009). Interestingly, in human DG (Seress et al. 2004), the immunoreactivity pattern to CART was similar to that observed in the chinchilla and guinea pig (CART-IR neurons were present in the molecular layer and in the hilus). The hilus of the rat (Seress et al. 2004), guinea pig and domestic pig DG (Kolenkiewicz et al. 2009) contains numerous mossy CART-IR nerve fibers, which resembles the pattern found here in the chinchilla. It is well known that these hippocampal nerve fibres extend from the DG to the CA3 field, the region responsible for the short-term memory encoding. Based on the findings of Saress et al. (2004), in the rat CA3 field CART-IR cells form mossy fiber bundles. A similar strand of nerve fibres, extending from the hilus to the CA3 field was observed in the chinchilla. Mossy fibres and cells are important structures engaged in learning and memory processes. These fibres are sensitive and are known to die quickly during stress and memory-related diseases (Viana da Silva et al. 2019). CART-positive neurons were also detected in various CA layers in mammals. In the human, Seress et al. (2004) described the presence of CART-expressing neurons in all fields of the CA. In the domestic pig CART-IR neurons were found only in the CA3 field, whereas in the guinea pig they were present in both the CA1 and CA3 fields (Kolenkiewicz et al. 2009). In the rat, Seress et al. (2004) described no presence of CART-positive neurons in the hippocampus proper, which is in contrast to other studies showing a loose network of CART-positive nerve fibers in all layers of the CA1-CA3 field in the human (Seress et al. 2004).

In most animals, CaBPs are known to be expressed in interneurons, projecting neurons (and emerging nerve fibres) of the hippocampal formation. Ludkiewicz et al. (2002) described PV-IR neurons in the DG and in the CA1 and CA3 fields of the rat hippocampus,

and numerous nerve fibres which frequently formed "baskets" of PV-IR surrounding cells. Likewise, in the chinchilla (present studies) PV-IR nerve fibres also surrounded the cell bodies of PV-IR neurons, but the PV-positive neurons were visualized also in the CA2 field. Pitkänen and Amaral (1993) found PV-IR neurons in all fields of the hippocampus (CA1-CA3) of the monkey, but they noted differences in PV staining between these three fields. Several previous reports investigated the co-localization of CART with CaBPs in different animal brain structures (Najdzion et al. 2014, Wasilewska et al. 2016, Najdzion 2017, Najdzion 2018). However to date, there are no studies that have shown the correlation between CART and PV in hippocampal structures. In the chinchilla (present studies) the co-expression of CART and PV was present in neurons of the DG (except the hilus) and in the CA (except the CA1 field). However, the co-localization of CART with PV in nerve fibres of the DG and CA has not been shown.

In conclusion, in the present work we have described for the first time CART-immunoreactivity in the hippocampal formation of the chinchilla. We postulate that CART co-stored in PV-IR hippocampal neurons may be involved in maintaining intracellular calcium homeostasis. It cannot be excluded that both peptides taken together are involved in neuromodulatory central processes, but this hypothesis needs to be experimentally verified. Nevertheless, further studies explaining whether CART is co-localized with other neurotransmitters in the chinchilla brain would be helpful in understanding their possible neurotrophic or neuroprotective role.

References

Bharne AP, Upadhya MA, Shelkar GP, Singru PS, Subhedar NK, Kokare DM (**2013**) Neuroprotective effect of cocaine- and amphetamine-regulated transcript peptide in spinal cord injury in mice. Neuropharmacology 67: 126-135.

Douglass J, McKinzie AA, Couceyro P (**1995**) PCR differential display identifies a rat brain mRNA that is transcriptionally regulated by cocaine and amphetamine. J Neurosci 15: 2471-2481.

Eguiagaray JG, Egea J, Bravo-Cordero JJ, García AG (**2004**) Neurotransmitters, calcium signaling and neuronal communication. Neurocirugia 15: 109-118.

Eichenbaum H, Cohen NJ (2008) From Conditioning to Conscious Recollection: Memory systems of the brain. Oxford Scholarship Online, DOI:10.1093/acprof:oso/9780195178043.001.0001

El-Falougy H, Benuska J (**2006**) History, anatomical nomenclature, comparative anatomy and functions of the hippocampal formation. Bratisl Lek Listy 107: 103-106.

Faria AV, Ratnanather JT, Tward DJ, Lee DS, van den Noort F, Wu D, Brown T, Johnson H, Paulsen JS, Ross CA, Younes L, Miller MI (**2016**) Linking white matter and deep gray matter alterations in premanifest Huntington disease. Neuroimage Clin 11: 450-460.

Fagergren P, Hurd Y (**2007**) CART mRNA expression in rat monkey and human brain: relevance to cocaine abuse. Physiol Behav 92: 218-225.

Hubert GW, Jones DC, Moffett MC, Rogger G, Kuhar MJ (**2008**) CART peptides as modulators of dopamine and psychostimulants and interactions with the mesolimbic dopaminergic system. Biochem Pharmacol 75: 57-62.

Janiuk I, Kasacka I (**2015**) Quantitative evaluation of CART-containing cells in urinary bladder of rats with renovascular hypertension. Eur J Histochem 59: 2446. doi: 10.4081/ejh.2015.2446.

Kolenkiewicz M, Robak A, Równiak M, Bogus-Nowakowska K, Całka J, Majewski M (**2009**) Distribution of cocaine- and amphetamine-regulated transcript in the hippocampal formation of the guinea pig and domestic pig. Folia Morphol (Warsz) 68: 23-31.

Koylu EO, Couceyro PR, Lambert PD, Ling NC, DeSouza EB, Kuhar MJ (**1997**) Immunohistochemical localization of novel CART peptides in rat hypothalamus, pituitary and adrenal gland. J Neuroendocrinol 9: 823-833.

Larsen PJ, Vrang N, Petersen PC, Kristensen P (**2000**) Chronic intracerebroventricular administration of recombinant CART (42-89) peptide inhibits and causes weight loss in lean and obese Zucker (fa/fa) rats. Obes Res 8: 590-596.

Larsen PJ, Seier V, Fink-Jensen A, Holst JJ, Warberg J, Vrang NJ (**2003**) Cocaine- and amphetamine-regulated transcript is present in hypothalamic neuroendocrine neurones and is released to the hypothalamic-pituitary portal circuit. J Neuroendocrinol 15: 219-226.

Ludkiewicz B, Wójcik S, Spodnik E, Domaradzka-Pytel B, Klejbor I, Moryś J (**2002**) Cholinergic innervation of parvalbumin- and calbindin-containing neurons in the hippocampus during postnatal development of the rat brain. Folia Morphol (Warsz) 61: 89-96.

McGee-Lawrence M, Buckendahl P, Carpenter C, Henriksen K, Vaughan M, Donahue S (**2015**) Suppressed bone remodeling in black bears conserves energy and bone mass during hibernation. J Exp Biol 218: 2067-2074.

Mendioroz M, Celarain N, Altuna M, Sánchez-Ruiz de Gordoa J, Zelaya MV, Roldán M, Rubio I, Larumbe R, Erro ME, Méndez I, Echávarri C (**2016**) CRTC1 gene is differentially methylated in the human hippocampus in Alzheimer's disease. Alzheimers Res Ther 8: 15. doi: 10.1186/s13195-016-0183-0.

Moffet MC, Song J, Kuhar MJ (2011) CART peptide inhibits locomotor activity induced by simultaneous stimulation of D1 and D2 receptors, but not by stimulation of individual dopamine receptors. Synapse 65: 1-7.

Najdzion J, Wasilewska B, Bogus-Nowakowska K, Robak A (**2014**) The cocaine- and amphetamine-regulated transcript, calbindin, calretinin and parvalbumin immunoreactivity in the medial geniculate body of the guinea pig. J Chem Neuroanat 59-60: 17-28.

Najdzion J (**2017**) Cocaine- and amphetamine-regulated transcript peptide and calcium binding proteins immunoreactivity in the superficial layers of the superior colliculus in the guinea pig: Implications for visual sensory processing. J Chem Neuroanat 79: 66-79.

Najdzion J (**2018**) Cocaine- and amphetamine-regulated transcript peptide and calcium binding proteins immunoreactivity in the deep layers of the superior colliculus of the guinea pig: Implications for multisensory and visuomotor processing. J Chem Neuroanat 88: 55-69.

Pchitskaya E, Popugaeva E, Bezprozvanny I (**2018**) Calcium signaling and molecular mechanisms underlying neurodegenerative diseases. Cell Calcium 70: 87-94.

Pitkänen A, Amaral DG (**1993**) Distribution of parvalbumin-immunoreactive cells and fibers in the monkey temporal lobe: the hippocampal formation. J Comp Neurol 331: 37–74.

Radzimirska M, Bogus-Nowakowska K, Kuder T, Robak A (**2016**) Distribution of cocaine- and amphetamine-regulated transcript (CART), neuropeptide Y (NPY) and galanin (GAL) in the pterygopalatine ganglion of the domestic duck (Anas platyrhynchos f. domestica). Folia Histochem Cytobiol 54: 25-31.

Ren PY, Li BW, Dong SY, Wu M, Han P (**2019**) Improvement of Mechanical Stability for Single Unit Recording Based on Skull Cap in Living Chinchilla. Curr Med Sci 39: 166-172.

Saress L, Abraham H, Doczi T, Lazar G, Kozicz T (**2004**) Cocaine and amphetamine-regulated transcript peptide (CART) is a selective marker of rat granule cells and of human mossy cells in the hipopocampal denate gyrus. Neuroscience 125: 13-24.

Smith Y, Koylu EO, Couceyro P, Kuhar MJ (**1997**) Ultrastructural localization of CART (cocaine- and amphetamine--regulated transcript) peptides in the nucleus accumbens of monkeys. Synapse 27: 90-94.

Schwaller B (**2010**) Cytosolic Ca2+ buffers. Cold Spring Harb Perspect Biol 2: a004051.

Upadhya MA, Shelkar GP, Subhedar NK, Kokare DM (**2016**) CART modulates the effects of levodopa in rat model of Parkinson's disease. Behav Brain Res 301: 262-272.

Viana da Silva S, Zhang P, Haberl MG, Labrousse V, Grosjean N, Blanchet C, Frick A, Mulle C (**2019**) Hippocampal mossy fibers synapses in CA3 pyramidal cells are altered at an early stage in a mouse model of Alzheimer's disease. J Neurosci 39: 4193-4205.

Wasilewska B, Najdzion J, Równiak M, Bogus-Nowakowska K, Hermanowicz B, Kolenkiewicz M, Żakowski W, Robak A (**2016**) Cocaine- and amphetamine-regulated transcript and calcium binding proteins immunoreactivity in the subicular complex of the guinea pig. Ann Anat 204: 51-62.

Zacharko-Siembida A, Arciszewski MB (**2014**) Immunoreactivity to cocaine-and amphetamine-regulated transcript in the enteric nervous system of the pig and wild boar stomach. Anat Histol Embryol 43: 48-55.

Zacharko-Siembida A, Kulik P, Szalak R, Lalak R, Arciszewski MB (**2014**) Co- expression patterns of cocaine- and amphetamine-regulated transcript (CART) with neuropeptides in dorsal root ganglia of the pig. Acta Histochem 116: 390-398.