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Original article

Immunocytochemical detection of calretinin in the claustrum and endopiriform nucleus of the chinchilla

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Abstract

Calretinin (CR) as a buffer and sensor protein plays an important role in regulatory processes of Ca^{2+} and anty-apoptotic cellular protection. In the present study, immunohistochemical peroxidase-antiperoxidase (PAP) method was used in order to determine the numbers, morphology, morphometry and distribution pattern of CR in neurons of the chinchilla's claustrum (Cl) and endopiriform nucleus (EN). In Cl and EN the presence of several classes of neurons with different immunoreactivity to CR was found. In Cl, CR-immunoreactive (IR) neurons were predominantly found in close vicinity to insular border while CR-IR neurons were evenly scattered throughout EN. In general, immunoreaction to CR was observed in neuronal cytoplasm, nucleus and in fibres-like nerve extensions. Statistical analysis showed the differences between average large diameter as well as cross-sectional area of CR-IR neurons present in Cl and EN. It is suggested, that CR acting as a calcium binding protein may play a role in neuronal network. Further co-localization studies are necessary to fully elucidate neurophysiology and neuropathology of the chinchilla's Cl and EN neurons.

Key words: calretinin, calcium-binding proteins, central nervous system, claustrum, chinchilla

Introduction

The claustrum is a narrow band of gray matter located along the rhinal sulcus, between the putamen, amygdala and insular cortex. Due to several differences in cellular structure as well as topographical relation to the rhinal sulcus, the claustrum is routinely divided into two parts. The dorsal claustrum (insular or proper claustrum, Cl) is localized above the level of the rhinal sulcus whereas the ventral portion of the claustrum (also called the endopiriform nucleus; EN) lies below the rhinal sulcus (Buchanan and Johnson 2011). Between most neocortical and allocortical areas and the claustrum numerous functional claustro-cortical bidirectional connections are present (Edelstein and Denaro 2004, Druga 2014). Despite many experimental studies the exact physiological role of the claustrum is still under discussion. So far it has been found that during the course of Alzheimer's disease pathological changes in the claustrum occurred (Moryś et al. 1996). Additionally, implication of the claustrum in propagation of kindled seizures from

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limbic sites to the neocortex has been documented (Mohapel et al. 2001).

Previous studies revealed that Cl and EN neurons express a wide array of biologically active substances including calcium-binding proteins (CaBPs). Further immunohistochemical studies clearly showed that both in Cl and EN, CaBPs-containing neurons are predominantly glutamatergic as well as GABA-ergic in nature (Kowiański et al. 2004, Kowiański et al. 2009). Most CaBPs belong to the same protein superfamily and share the common EF-hand calcium-binding motif (Kretsinger and Nockolds 1973). From the functional point of view CaBPs are further divided into two groups which function as calcium buffers or sensors (Schwaller 2010). Since in the central nervous system (CNS), CaBPs are abundantly and exclusively expressed in certain classes of neurons many researchers found these proteins to be a valuable marker useful for the visualization of fast spiking interneurons, regular spiking interneurons or even burst spiking interneurons (Kawaguchi and Kubota 1997, Pawelzik et al. 2002, Freund 2003).

Calretinin (CR) encoded by the CALB2 gene located on chromosome 16 (Parmentier et al. 1991) is a 271 amino acid-long CaBP belonging to the troponin superfamily (Schwaller 2014). Since this protein was originally found by cloning cDNA from the chicken retina, the name „cal-retinin" has been proposed (Ellis et al. 1991). CR is generally considered as a Ca^{2+} buffer protein; however it also regulates intracellular processes as a Ca^{2+} sensory protein (Billing-Marczak and Kuźnicki 1999). Although the physiological role(s) of CR are far from being fully understood, it is generally accepted that it acts as a neuronal modulator and regulator of synaptic plasticity (Billing-Marczak and Kuźnicki 1999, Schwaller et al. 2002). Because of its specific localization in CNS, CR has received growing research attention in recent years (Maskey et al. 2012, Szalak et al. 2013, Bae et al. 2015). Using the immunohistochemical technique the distribution patterns of CR have been described in discrete regions of the mammalian brain including the dolphin (Cozzi et al. 2014), rodents (Edelstein et al. 2010, Druga et al. 2015), rabbit (Wójcik et al. 2004), cat (Rahman and Baizer 2007), monkey (Reynhout and Baizer 1999) and human (Prensa et al. 2003). Several attempts aimed at the characterization of CR-positive neurons in CNS of the chinchilla have been undertaken but these studies focused on the hippocampus (Szalak and Jaworska-Adamu 2011), frontal cortex (Krawczyk et al. 2012) and dorsal raphe nucleus (Jaworska-Adamu and Szalak 2009) only, and there are no reports in the literature regarding expression of CR in the chinchilla claustrum. To broaden neuroanatomical knowledge about CaBPs role(s) in the mammalian brain and particularly to advance our own study, in the current study we further immunohistochemically examine the morphological characteristics and distribution of CR in Cl and EN of the chinchilla. The obtained results may also serve as a basis for further comparative analysis.

Materials and Methods

Animal and tissue sampling

Animal care protocols, experimental design and methods were reviewed and approved by the IInd 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. The brains were dissected out immediately after slaughter. The brains were fixed for 12 hours in cold buffered 10% formalin (pH=7.0; +4°C). The material was processed conventionally for paraffin embedding. For further immunohistochemical analyses, paraffin sections of 6 m thickness were cut and collected on SuperFrost Plus (Meznel-Glaser, Braunschweig, Germany) microscopy slides.

Immunohistochemistry and antibodies

The slides were immunohistochemically stained (peroxidase-antiperoxidase method) according to the following protocol. First, in order to remove paraffin the sections were washed in xylene (3 x 15 min). Then the slides were rehydrated by sequential incubation with a graded series of ethyl alcohol and finally washed in distilled water. For the antigen retrieval step, the slides were placed in a container containing citrate buffer (pH=6.0) and heated to 97°C (3 x 7 min) in a microwave oven (800W). After cooling, sections were washed in 3% hydrogen peroxidase (for 20 min) in order to block endogenous peroxidase activity. The slides were then rinsed in PBS (pH=7.4) twice (15 min each) and incubated in 2.5% normal horse serum (S-2012; Vector, USA) at room temperature (RT) for 20 minutes. Excess blocking serum was dried out, and mouse primary antisera raised against calretinin (dilution 1:2000; C7479, Sigma, Germany) were imposed and incubated overnight at 4°C. The next day, the slides were washed in wash buffer (2 x 15 min) and covered by anti-mouse/rabbit Ig (ImPRESS™; MP-7500 Vector, USA), for 1 hour (RT). For the visualization of primary antisera 3,3'-diaminobenzidine (DAB, Vector, USA) chromogen was used. The working solution of DAB was applied on to the slides and the process was monitored under a light

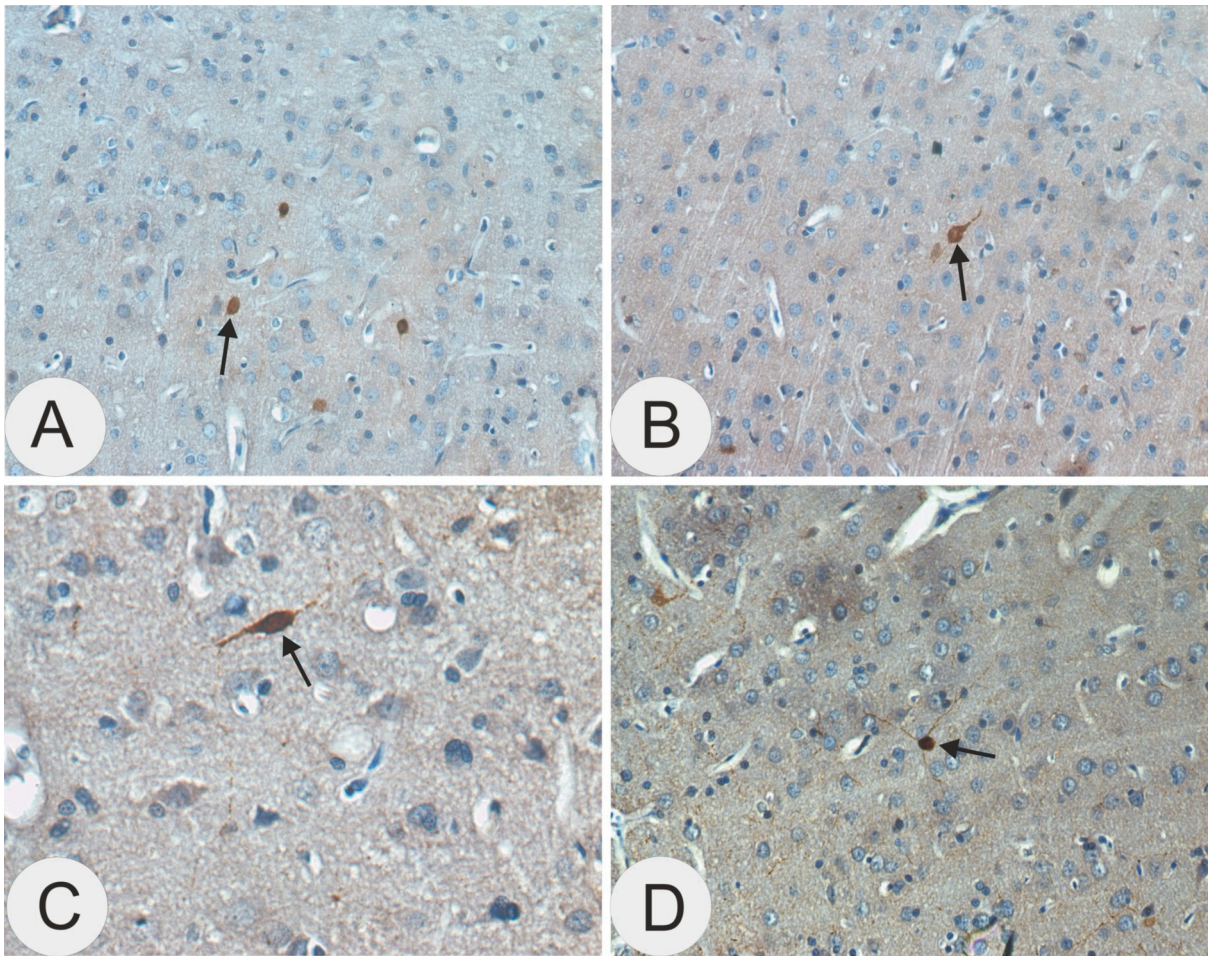

Fig. 1. Immunoreactivity to CR in moderate numbers of neurons of the chinchilla's claustrum are seen in (A-D). Most CR-positive neurons were classified as round (arrow in A) or oval (arrow in B) in shape; note in (A) CR-IR neurons with short protrusions. Scarce fusiform neurons showing intense or weak immunoreaction to CR (arrow in C) as well as single multipolar cells with weak immunoreaction to CR were also found (arrow in D). Magnification x20 in (A), (B), (D) and x40 in (C).

microscope. Finally, the slides were rinsed in distilled water. Counterstaining (for 20 min) with Mayer's hematoxylin was carried out. After washing in distilled water the slides were dehydrated in ethyl alcohol series, cleared in xylene, mounted in Canadian balm and cover slipped. The specificity of the antibodies used was verified by negative control in which primary antibodies were replaced with the same concentrations of appropriate non-immune IgG.

Morphometric and statistical analysis

The slides were viewed under a light microscope (Olympus BX51 light) connected to a digital camera (Olympus Color View III). From each animal approx. 25-30 sections immunostained for CR were analyzed. The intensity of immunoreaction for calretinin was arbitrarily assessed according to the method described elsewhere (Arciszewski 2004). The following semi-quantitative scale: none (-), weak (+), moderate (++) and intense (+++) was used. In each animal (n=5) no less than three hundred CR-IR neurons in Cl and three hundred CR-IR neurons in EN were viewed and counted. Using the Cell D software (Olympus) the distribution of CR-IR neurons in Cl and EN were studied. Additionally, the shape, larger diameter and the average cross-sectional area of the CR-IR neurons were arbitrarily judged and measured. The proportions of CR-IR neurons were presented as percentages of the total number of CR labeled neurons. Results were expressed as a mean ± SEM. The obtained results were statistically analyzed using the one-way analysis of variance test (ANOVA) followed by post-hoc Tukey's test. Data are presented as means ± standard deviation (SD). Probabilities of less than 5% ($p<0.05$) were considered significant.

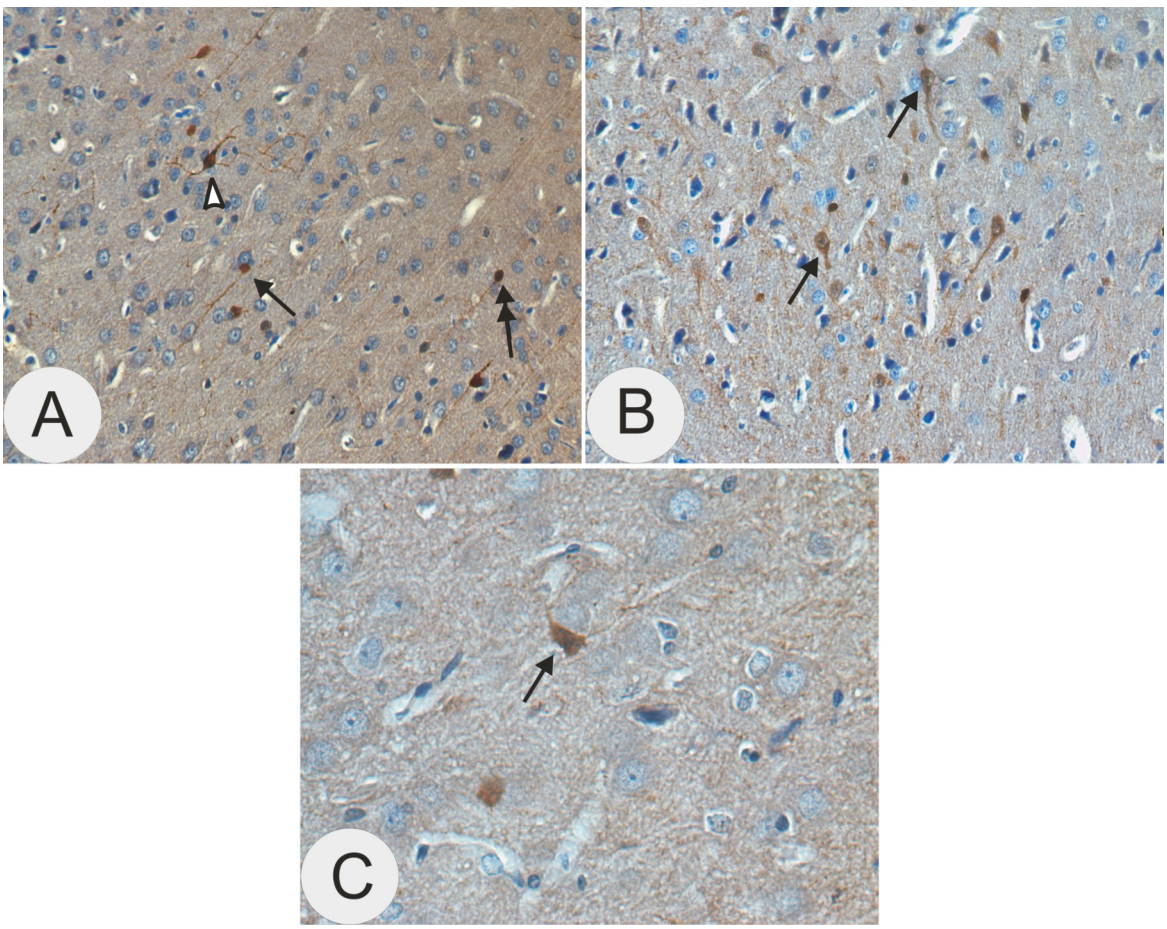

Fig. 2. Expression of CR in EN neurons. Numerous CR-IR neurons predominantly belonged to the group of round (arrow in A), oval (double arrow in A), multipolar (arrowhead in A) and fusiform cells and showed intense to weak immunoreaction to CR (A-B). Singular triangular neurons showed moderate immunoreaction to CR (arrow in C). Note in (A) and (B) the presence of long processes in CR-IR neurons. Magnification x20 in (A), (B) and x40 in (C).

Results

Claustrum. 6.6 ± 2.7% (n=5) of Cl neurons showed immunoreaction to CR. In the vast majority of analyzed neurons strong or moderate immunoreaction to CR was found; however, a small subset of neurons showing weak or very weak immunoreaction to CR was also detected (Fig. 1A). In Cl, CR-immunoreactive (IR) neurons were predominantly found in close vicinity to the insular border. CR-positive nerve fibres were usually short and projected either to the insula or striatum (Fig. 1A). In general, immunoreaction to CR was observed in neuronal cytoplasm, nucleus and incidentally in fibre-like nerve extensions (Fig. 1B, Fig. 1C). CR-IR neurons, round in shape, (Fig. 1A) constituted approx. 45% of the whole subpopulation of CR-positive perikarya, whereas approx. 35% of CR-expressing neurons were categorized as oval in shape (Fig. 1A). Additionally, in Cl of the chinchilla fusiform (approx. 10%) and multipolar (approx. 10%) CR-IR neurons were also found (Fig. 1C; Fig. 1D). The average larger diameter of CR-positive Cl neurons was 8.4 ± 1.2 μm and average cross-sectional area was 99.3 ± 3.5 μm^2.

Endopiriform nucleus. In the chinchilla EN, a statistically larger (vs. Cl; $p<0.05$) subpopulation of neurons showing CR-expression (15.1 ± 3.8%; n=5) was found. CR-IR neurons were evenly scattered throughout EN. In CR-positive neurons immunoreaction to CR was found in the neuroplasm and nucleus as well as in numerous nerve protrusions (Fig. 2A). Similarly to Cl, neurons of EN showed intense, moderate and weak immunoreaction to CR (Fig. 2B). The majority of CR-IR nerve fibres lay in perpendicularly directed toward the striatum or insula. In general, the observed CR-IR nerve fibres were relatively long (Fig. 2A, Fig. 2B). In EN of the chinchilla, the most numerous subpopulations of CR-IR neurons consisted of cells round (approx. 40%) and oval (approx. 30%) in shape, whereas fusiform (approx. 15%), multipolar (approx. 10%) and triangular (approx. 5%) neurons were less numerous (Fig. 2A, Fig. 2B, Fig. 2C).When

compared to Cl, the average large diameter of CR-IR neurons (11.0 ± 1.1 μm) found in EN was statically larger ($P<0.05$). Mean cross-sectional area of CR-IR neurons present in EN (99.6 ± 4.3 μm^2) was statistically similar in relation to CR-expressing Cl neurons ($P<0.05$).

Discussion

In the present work, we present for the first time the detailed distribution patterns of CR expression in Cl and EN of the chinchilla. Based on the results obtained it becomes apparent that the numbers and distribution patterns of CR-immunoreactive (IR) neurons in the chinchilla slightly differ from previously described animal species. As outlined above, an apparent disproportion in numbers of CR-IR neurons was found between the chinchilla's Cl and EN. In the chinchilla EN, CR-expressing neurons statistically outnumbered those present in Cl, which is in line with previous reports obtained in the mouse (Real et al 2003) and rat (Edelstein et al. 2010). Interestingly, in other species including the rabbit (Wójcik et al. 2004), monkey (Reynhout and Baizer 1999) and humans (Prensa et al. 2003), a higher percentage of CR-IR neurons was detected in Cl than EN. Only in the cat, did both parts of the claustrum contain relatively low and proportional numbers of CR-expressing neurons (Rahman and Baizer 2007). Similarly to previous results presented in the mouse (Real et al. 2003), rat (Druga 2014) and rabbit (Wójcik et al. 2004) single CR-IR neurons found in the chinchilla's Cl were localized in the marginal zone of the insula. On the other hand in the cat (Rahman and Baizer 2007) and monkey (Reynhout and Baizer 1999), CR-expressing neurons were evenly scattered throughout Cl. So far, at least several distribution patterns of CR-expression in EN neurons have been noted in different animal species. In the mouse Cl, CR-expressing neurons were evenly distributed throughout the nucleus (Real et al. 2003), which closely resembles the pattern presented in this study in the chinchilla. However, in the rat CR-IR neurons detected both in Cl and EN were predominantly localized in close neighborhood to the insular cortex (Druga 2014). The topographical localization of CR-IR neurons in the EN of the rabbit is hard to determine because in this nucleus only incidental CR-positive neurons were found (Wójcik et al. 2004). Taking into account the shapes of CR-IR neurons found in Cl of the chinchilla it must be pointed out that similar types of neurons (the majority of round and oval shape, and only scarce numbers of fusiform) were observed in Cl of the rabbit, cat and monkey (Reynhout and Baizer 1999, Wójcik et al. 2004, Rahman and Baizer 2007). For comparative purposes, in Cl of the dolphin small mono and bipolar CR-IR neurons, generally round or fusiform in shape, were predominantly found (Cozzi et al. 2014). In general, the chinchilla's EN contained, besides CR-IR round and oval neurons, also substantial numbers of CR-positive fusiform and multipolar nervous cells as well as less numerous triangular CR-IR neurons. In general, the pattern presented above is in line with results obtained in most animal species studied, excluding the mouse and rat. In Cl of the latter animal species, CR-IR neurons were predominantly categorized as medium-sized multipolar, oval and fusiform in shape (Druga 2014, Real et al. 2003). In the rat Cl and EN, the larger diameter of CR-IR neurons present in the rat Cl and EN is substantially larger when compared to the moderate parameters measured in the chinchilla (respectively 13.7 ± 0.3 μm and 8.4 ± 1.2 μm in Cl and 15.6 ± 0.3 μm and 11.0 ± 1.1 μm in EN). However, further comparative analysis revealed that in EN cross-sectional area of CR-IR neurons is comparable in the chinchilla and rat (99.6 ± 4.3 μm^2 and 94.6 ± 2.0 μm^2; respectively) but differs in Cl (93.3 ± 3.5 μm^2 and 77.2 ± 2.1 μm^2; respectively; Druga et al. 2015).

In general, CNS neurons can be functionally divided into two major types: main glutaminergic neurons and GABA-ergic interneurons (Clements et al. 2008, Kowiański et al. 2004, Kowiański et al. 2009, Schwaller et al. 2002). In the mammals, the majority of Cl and EN neurons belong to the group of main neurons whereas only 6-12% consisted of interneurons (Gómez-Urquijo et al. 2000). Neuron chemical code is determined by numerous neurotransmitters as well as CaBP including CR. It is well known that CR is a useful marker of GABA-ergic neurons (Molgaard et al. 2014). At the central level, CR controls concentration of Ca^{2+} ions and modifies regulation, excitability and activity of nervous cells (Schwaller 2014). During the initial phase of neurotransmission, CR accumulates in the vicinity of the cell membrane and controls Ca^{2+} ion influx (Hack et al. 2000). Because of it is relatively low molecular weight, CR is able to penetrate the cell nucleus (utilizing the passive transport mechanism) and therefore modulates gene expression (Schwaller 2014). Some reports suggest a neuroprotective action of CR-IR neurons mainly due to fact that during numerous neurological and psychiatric disorders CR immunoreactivity is unchanged (Schwaller et al. 2002, Schwaller 2014). Taking into account the above, it has been proposed that CR-positive neurons are resistant a range of functional damages which may confirm the neuroprotective role of CR in CNS (Fonseca and Soriano 1995, Clements et al. 2008). The expression of CR in differ-

ent types on neoplasm may further suggest the role of CR in pathogenesis and cell death processes (Gander et al. 1996). Moreover, immunoreactivity to CR in neoplastic cells is routinely used for differentiation of different types of tumors such as malignant mesothelioma (Doglioni et al. 1996) and in differentiating it from adenocarcinoma (Lugli et al. 2003, Billing-Marczak et al. 2004, Barak et al. 2012).

In summary, in the present work we have described for the first time the distribution pattern of CR in the claustrum of the chinchilla. Different amounts of CR-IR neurons and their specific topography have been outlined. The presence of CR in a substantial number of Cl and EN neurons may support the hypothesis that this CaBP participates the in local neuronal network. The results presented in this study may be the anatomical basis for a further co-localization study determining the exact chemical code of the chinchilla's claustrum neurons.

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