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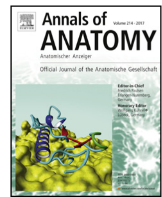

RESEARCH ARTICLE

Calcium-binding proteins expression in the septum and cingulate cortex of the adult guinea pig

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ABSTRACT

For the first time this study demonstrates the distribution pattern and expression of three neuroanatomical markers: calbindin D28k (CB), calretinin (CR) and parvalbumin (PV) in topographically connected brain regions — the septum (SE) and the cingulate cortex (CC). The co-existence among calcium-binding proteins (CaBPs) was also examined. The study was conducted on the adult guinea pig with the use of immunohistochemical and molecular biological techniques. Among the studied CaBPs, which occurred in both examined brain regions at transcript and protein levels, CB was the most expressed in the SE, while CR in the CC. CR, unlike CB and PV, showed higher immunoreactivity in the superficial layers (II–III) of the CC than in the deep ones (V–VI). Most of CB and PV-positive perikarya were detected in the deep layers of the CC. Some CC neurons contained both CB and PV, suggesting cooperation between these CaBPs in the CC. Co-localization between CB and CR in the CC was not observed.

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1. Introduction

The septum (SE) and the cingulate cortex (CC) constitute essential components of the limbic system, a topographically organized network of brain structures, known as the Papez circuit. Originally, the Papez circuit was considered to be the neurological basis of emotions (Papez, 1995), while, in the middle of twentieth century it became widely accepted that this circuit is important for memory (Vann and Nelson, 2015). In mammalian CC, two main regions (anterior and posterior) were distinguished. The anterior part of CC (ACC), specialization of neocortex, is related to modulation of so-called intelligent behaviour such as emotional self-control (Allman et al., 2001). Recent studies (Greicius et al., 2003; Oblak et al., 2011; Bird et al., 2015) have shown that the posterior part of CC (PCC), in regard to its connections and electrophysiological properties, may play a different role than the ACC. The PPC is essential for creating long-lasting memories relating to life episodes as well as face processing and it is thought to contribute to deficits in social behaviour (Oblak et al., 2011; Bird et al., 2015).

The SE, the nodal integrative point of the limbic system is referred as the “path integration” (Tsanov, 2015), which is topographically connected with other brain limbic regions (including the CC). The projections from the SE are proposed to have important roles in learning and memory circuit modulation (Khakpai et al., 2013). It is thought that septohippocampal projections are implicated in episodic memory (Stackman and Walsh, 1995), however, according to Dougherty et al. (1998) the septocingulate pathway is much more engaged in the episodic memory system than the septohippocampal pathway and it is perceived as a critical component of the system. Numerous clinical studies have indicated that disturbances in the functioning of episodic memory are characteristic to some neurodegenerative diseases (Gold and Budson, 2008; Baran et al., 2009; El Haj et al., 2013).

A proper level of calcium-binding proteins (CaBPs), various neurotransmitters and their receptors are essential for physiological cellular processes (Chen et al., 2011; Lu et al., 2004; Schwaller, 2014). Perturbation of calcium homeostasis can lead to neurodegenerative processes (Bezprozvanny and Mattson, 2008). CaBPs, highly abundant within the central and peripheral nervous system, are well known and widely used as valuable markers for distinguishing subpopulations of neurons and pathways in anatomical and developmental studies (Hof et al., 2001; Bogus-Nowakowska

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et al., 2012; Żakowski and Robak, 2013; Najdzion et al., 2014; Równiak et al., 2015; Wasilewska et al., 2016; Hermanowicz-Sobieraj and Robak, 2017). Among CaBPs, the most extensively studied are calbindin D28k (CB), calretinin (CR) and parvalbumin (PV). On the basis of Ca^{2+} binding kinetics, CB and CR act as fast calcium buffers (Faas et al., 2007; Schwaller, 2009), while PV as a slow buffer (Schwaller, 2009). It is thought that CaBPs play a neuroprotective role (Hof et al., 1993a; Lukas and Jones, 1994; Riascos et al., 2011; Yuan et al., 2013). Nonetheless, the role of CR in this regard is still discussed (Schwaller, 2014). CaBPs also mark neurons belonging to principal or non-principal subpopulations of neurons, which differ in bio/electrical activity. The principal neurons are projection neurons which are excitatory in nature whereas non-principal GABAergic interneurons exert inhibition action mainly on principal cells (Freund and Buzsáki, 1996).

Despite numerous papers showing CaBPs occurrence in the CC of mammals (Hof and Nimchinsky, 1992; Hof et al., 1993b; Nimchinsky et al., 1997; Zhao et al., 2013; Xu and Zhang, 2015), none of them refers to the guinea pig. In the SE, the distribution of CB, CR and PV as well as their co-existence pattern have been reported in the guinea pig (Hermanowicz-Sobieraj and Robak, 2017), however, there is no data regarding occurrence of CaBPs at mRNA level in the SE of mammals.

The guinea pig, as rodent species, is unusual as a model in biomedical research, especially compared to other laboratory animals (Hennessy, 1999; Grove-Strawser et al., 2002; Albert et al., 2012; Hennessy et al., 2017). In various functional aspects, the guinea pig has more features in common with human than with usually examined rodents (Mitchell and Taggart, 2009; Dyson et al., 2012; Sharman et al., 2013; Workman et al., 2013). Therefore, the aim of the study was to determine the expression of CB, CR and PV at transcript level (mRNA) and final product level (protein) in the SE and CC. Because the co-localization pattern of CaBPs in the guinea pig SE has been reported recently (Hermanowicz-Sobieraj and Robak, 2017) we investigated co-localization among CaBPs in the guinea pig CC. Due to specific cytoarchitecture of the CC and topographically organized connections between the CC and SE, CaBPs expression presented in this system seems to be worthy of attention.

2. Material and methods

2.1. Animals and tissue collection

The experiment was performed in accordance with the European Union Directive for animal experiments (2010/63/EU) and was approved by the Local Animal Ethics Committee of the University of Warmia and Mazury in Olsztyn, Poland. The study was conducted on female adult Dunkin-Hartley guinea pigs (0.8 kg, 3 months-old) from Nofer Institute Occupational Medicine in Łódź, Poland. The animals were kept under standard feeding and lightning conditions (12:12 h lights dark cycles). All guinea pigs (n = 9) were euthanized by an intraperitoneal injection of Morbital (Biowet, Poland; 2 ml/kg body weight). Brains for real-time PCR were removed from the skulls, transferred to RNALater (Ambion, USA), and kept at −80 °C. Then, brains were cut into 300 μm slices using a vibratome. From each slice encompassing the septum and the cigulate cortex, these both structures were dissected out using a stainless steel punch tool. Next the tissues were placed into separate sterile tubes with RNALater (Ambion, USA) and stored at −80 °C for further processing. The procedure of the tissue preparation for immunohistochemical analyses was described in detail in our previous manuscript (Hermanowicz-Sobieraj and Robak, 2017).

2.2. Total RNA isolation, cDNA synthesis

Total RNA from the guinea pig brains (n = 6) was isolated with the Total RNA Mini Plus Kit (A&A Biotechnology, Poland). RNA quantity and quality were specified with spectrophotometry (NanoDrop ND-1000, Thermo Scientific, USA). Reverse transcription to cDNA was performed with 2.5 μg of total RNA using Maxima cDNA Synthesis Kit (Life Technologies, USA) in a total volume of 20 μl at 25 °C for 10 min, 50 °C for 15 min and 85 °C for 5 min.

2.3. Quantitative real-time PCR (qPCR)

Real-time PCR analysis was performed on a 7500 Fast real-time PCR system (Applied Biosystems, USA) using Sybr Green JumpStart^{TM} Taq ReadyMix^{TM} (Sigma-Aldrich, USA). Real-time PCR mix consisted of: 10 μl SYBR Green JumpStart Taq ReadyMix, 1 μl cDNA, 0.2 μl reference dye, 2 μl 5 mM starter mix (reverse and forward, Sigma-Aldrich) and RNase free water to a total volume of 20 μl. From each sample of cDNA real-time PCR was conducted for CB, CR, PV and GAPDH as a reference gene. All samples were run in triplicate. Negative controls were performed with the use of water as a cDNA substitute. The PCR cycling conditions were as follows: initial denaturation at 95 °C/10 min, denaturation at 95 °C/15 s, and annealing at 60 °C/1 min for 40 cycles.

The sequences of the primers were as follows: for CB: forward GGCAGAGAGAAGATGGCAAGA, reverse ATCGGAAGAGCAGCAGGAAAT, for CR: forward TGAGAATGAACTGGATGCCCT, reverse CCTTTCGGTAGAGCTTGCCT, for PV: forward TCCCAAGAGAGGTGAAGGAAC, reverse GCTATGGAATGCTGCCGAAC.

2.4. Immunohistochemistry (IHC)

Frozen sections from the guinea pig brains (n = 3) containing the CC were processed for single- and double-immunofluorescence staining. A solution of rabbit antibodies raised against calbindin D28k (1:2000, code CB-38, Swant, Switzerland), mouse antibodies raised against calretinin (1:2000, code $6B_3$, Swant, Switzerland) and mouse antibodies raised against parvalbumin (1:2000, code P3088, Sigma Aldrich, USA) were used for single immunofluorescence staining and a mixture of calbindin D28k and calretinin or parvalbumin for double immunofluoresence staining. In order to show the binding sites of antibodies, the sections were incubated with Alexa Fluor 488 (1:800, code A-21202, Molecular Probes, USA) anti-mouse and Alexa Fluor 555 (1:800, code A-31572, Molecular Probes, USA) anti-rabbit antibodies. All details of immuohistochemical method have been described in our previous manuscripts (Hermanowicz-Sobieraj and Robak, 2017; Wasilewska et al., 2016).

The sections were viewed with an Olympus BX51 microscope equipped with a CCD camera connected to a PC. The images were acquired with Cell-F software (Olympus GmbH, Germany).

2.5. Controls

The specificity of the primary antisera has been established by various researchers (Airaksinen et al., 1997; Mészár et al., 2012; Zimmermann and Schwaller, 2002), as well as in our recent studies (Hermanowicz-Sobieraj and Robak, 2017; Wasilewska et al., 2016; Równiak et al., 2015; Najdzion et al., 2014). Also, the specificity of the primary antisera was tested by the manufacturers. The rabbit anti-CB (CB-38) and mouse anti-CR ($6B_3$) antisera were tested by immunoblot analysis. The guinea pig brain extracts were specifically labelled by these primary antibodies, exhibiting band at 28 and 29 kDa for CB and CR, respectively. To check the secondary antibodies specificity, the replacement of all primary antisera by

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