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RESEARCH ARTICLE

Cocaine- and amphetamine-regulated transcript and calcium binding proteins immunoreactivity in the subicular complex of the guinea pig

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ABSTRACT

In this study we present the distribution and colocalization pattern of cocaine- and amphetamine-regulated transcript (CART) and three calcium-binding proteins: calbindin (CB), calretinin (CR) and parvalbumin (PV) in the subicular complex (SC) of the guinea pig. The subiculum (S) and presubiculum (PrS) showed higher CART-immunoreactivity (-IR) than the parasubiculum (PaS) as far as the perikarya and neuropil were concerned. CART- IR cells were mainly observed in the pyramidal layer and occasionally in the molecular layer of the S. In the PrS and PaS, single CART-IR perikarya were dispersed, however with a tendency to be found only in superficial layers. CART-IR fibers were observed throughout the entire guinea pig subicular neuropil. Double-labeling immunofluorescence showed that CART-IR perikarya, as well as fibers, did not stain positively for any of the three CaBPs. CART-IR fibers were only located near the CB-, CR-, PV-IR perikarya, whereas CART-IR fibers occasionally intersected fibers containing one of the three CaBPs. The distribution pattern of CART was more similar to that of CB and CR than to that of PV. In the PrS, the CART, CB and CR immunoreactivity showed a laminar distribution pattern. In the case of the PV, this distribution pattern in the PrS was much less prominent than that of CART, CB and CR. We conclude that a heterogeneous distribution of the CART and CaBPs in the guinea pig SC is in keeping with findings from other mammals, however species specific differences have been observed.

1. Introduction

The subicular complex (SC) consists of the subiculum (S), presubiculum (PrS) and parasubiculum (PaS). The PrS is generally subdivided into the dorsal and ventral portions. The dorsal portion has been named the postsubiculum (Taube et al., 1990).

The SC is one of the central portions of the limbic structure; e.g. the S is now considered to constitute the major output station of the hippocampal formation (Swanson and Cowan, 1977; Witter and Groenewegen, 1990). There is evidence that the S, PrS and PaS contribute to learning and memory as well as other cognitive functions, e.g. object recognition, spatial processing and affective functions (Broussard et al., 2006; Liu et al., 2001; O'Mara, 2006). It has been suggested that the S may have a functional segregation of its dorsal and ventral sections (O'Mara, 2006). The dorsal S plays a particularly important role in the processing of spatial, mnemonic and movement information, while the ventral S plays a role in the mediation of the hippocampal formation inhibitory control of the hypothalamic-pituitary-adrenal (HPA) axis (O'Mara, 2005). It has been proposed that the ventral S is used to gate information flow within the nucleus accumbens, a factor that is disrupted in models of schizophrenia (Belujon and Grace, 2008). Moreover, clinical and experimental evidence suggests that the S plays an important role in the initiation and maintenance of epileptic discharges in temporal lobe epilepsy (TLE; Stafstrom, 2005). The PrS is also an area of interest in the study of TLE because of the projections it sends to seizure-sensitive cells in layer III of medial entorhinal areas (Eid et al., 1996), which have been implicated in the generation of

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epileptiform discharges during TLE (Tolner et al., 2005; Tolner et al., 2007). Thus, the PaS plays an important role within the brain navigational system (Boccara et al., 2010; Hargreaves, 2005; Solstad et al., 2008).

CART (cocaine- and amphetamine-regulated transcript) is a peptide neurotransmitter involved in reward and reinforcement, stress, anxiety, bone remodeling, endocrine regulation, sensory processing, appetite regulation, body weight homeostasis and energy balance (Gozen et al., 2007; Hunter et al., 2004; Kuhar et al., 2000; Larsen and Hunter, 2006; Vicentic and Jones, 2006). CART may activate the HPA axis during the stress response (Gozen et al., 2007). Endogenous CART may also have a role in epileptogenesis (Keating et al., 2008). CART is present in many regions of the brain. For example, it may be found in the hypothalamus (Koylu et al., 1997), hippocampus proper (Koylu et al., 1998), amygdala (Równiak et al., 2010), preoptic area (Bogus-Nowakowska et al., 2011), mammillary body and anterior thalamic nuclei (Żakowski et al., 2014) or in the mesolimbic dopamine system (Philpot and Smith, 2006). Little is known about CART distribution in the SC, which is strictly connected with TLE (Stafstrom, 2005). There are only a few papers concerning CART peptide occurrence in the SC or S of the rat (Ábrahám et al., 2009; Koylu et al., 1998; Seress et al., 2004) and the neuroanatomical distribution of CART in the SC has only been studied in detail in the tree shrew (Ábrahám et al., 2005).

The calcium-binding proteins (CaBPs) are particularly interesting since they occur only in certain subpopulations of nerve cells in the central and peripheral nervous system (Celio, 1990; Rogers et al., 1990). The CaBPs are known to play a crucial role in maintaining constant intracellular levels of calcium ions (Andressen et al., 1993). Moreover, these proteins can be differentiated into fast and slow Ca^{2+} buffers, which have different biophysical properties and mechanisms of actions (Schwaller, 2009; Schwaller et al., 2002). Alternatively, some of the CaBPs could also have specific target ligands in the membrane, suggesting that they could act as a sensor (Schwaller et al., 1997). According to several authors, CaBPs are useful chemical markers of neurons in various brain structures (Barinka and Druga, 2010; Sarnat, 2013; Schwaller et al., 2002). This fact was successfully used in studies of the SC (Fujise et al., 1995). It has been proposed that PV- and CR-IR interneurons play a crucial role in epileptogenesis and that the SC is one of the main centers involved in this process. In epilepsy, the S is affected by a substantial loss of PV-IR and CR-IR interneurons (Knopp et al., 2008).

Uva et al. (2004) described only the general (architectonic) organization of the guinea pig parahippocampal region that had been stained with either the Nissl and Thimm methods or with antibodies to PV and CB. Therefore, the aim of this study was to describe the distribution and morphology of CART-IR structures and selected CaBPs in the guinea pig SC. We hope that the results of this work may be useful in interpreting studies on the effects of neurodegenerative diseases and other experimental manipulations on the subicular neurons containing CART, CB, CR and PV. Furthermore, this study will also provide a clearer understanding of the parallels and differences among species. The study seems to be particularly valuable since the guinea pig is still used as a model in neuroscience and medical research (Joosen et al., 2011).

2. Material and methods

2.1. Animals and tissue processing

Surgical procedures were followed according to guidelines established by the Animal Care and Use Ethical Committee of the University of Warmia and Mazury. All procedures were carried out in accordance with EU Directive 2010/63/EU for animal experiments. Six sexually mature female guinea pigs (strain: Dunkin-Hartley, approximately 12 weeks old, obtained from Polish Mother Memorial Hospital Research Institute in Łódź, Poland were used in the present study. All procedures concerning tissue preparation have been described in detail in our previous publications (Najdzion et al., 2014; Wasilewska et al., 2011). Frozen brains were cut into 20 μm coronal and horizontal plane sections on the cryostat.

2.2. Immunohistochemical procedures

The sections were processed for routine single- and double-labeling immunofluorescence studies. For single labeling, a rabbit polyclonal antibody to $CART_{61–102}$ (1:8000; code H-003-61, Phoenix Pharmaceuticalis, USA) and rabbit antisera against CB with molecular weight 28 kDa (1:2000; code CB-38a, Swant, Switzerland) were visualized by Cy3-conjugated donkey anti-rabbit antibodies (1:8000; code 711-165-152, Jackson ImmunoLabs, USA). Furthermore, a mouse monoclonal antibody to CR (1:2000; code 6B-3, Swant, Switzerland) and PV (1:2000; code P3088, Sigma, USA) were visualized by Cy3-conjugated donkey anti-mouse antibodies (1:8000; code 715-165-150, Jackson ImmunoLabs, USA). For double labeling, a mouse monoclonal antibody to CART (1:6000; code MAB 163, R&D Systems, USA) was combined with rabbit antisera to CB (1:2000). Also a rabbit polyclonal antibody to CART (1:8000) was combined with a mouse monoclonal antibody to CR (1:2000) and PV (1:2000). To visualize the antigen–antisera binding sites, the sections were incubated (1 h, at room temperature) with a mixture of Cy3-conjugated donkey anti-mouse (1:8000) combined with FITC-conjugated donkey anti-rabbit (1:400, code 711-095-152, Jackson ImmunoLabs, USA) and Cy3-conjugated donkey anti-rabbit with FITC-conjugated donkey anti-mouse (1:400, code 715-095-150, Jackson ImmunoLabs, USA). All antibodies were diluted in PBS containing Triton X-100 (0.3–0.5%) and 1% normal donkey serum. Following antibody incubations, sections were then washed 3 times in 0.1 M PBS and were coverslipped in buffered carboxyglycerol (pH 7.8).

2.3. Controls

In the preliminary studies, the primary antibodies that were produced in different species (mouse and rabbit anti-CB, PV, CR, and CART) and provided by different manufacturers (Sigma, Swant, Phoenix Pharmaceuticals, R&D) were tested on the guinea pig tissue. The staining patterns were identical for all variants of the antibodies used, and therefore proved that their specificities were similar. Moreover, the specificity of some primary antisera has been tested by the manufacturers. For example, product descriptions of rabbit anti-calbindin (Swant, CB-38a) and mouse anti-calretinin (Swant, 6B3) antisera include immunoblots of the guinea pig brain homogenates, which were specifically stained by these antibodies, showing bands at 28 kDa and 29 kDa, respectively. The same primary antibodies were applied in our previous studies (Kolenkiewicz et al., 2009; Żakowski et al., 2013, 2014) have also been used by other authors as well (Airaksinen et al., 1997; Ellis and Mawe, 2003; Hunter et al., 2005; Mészár et al., 2012; Solbach and Celio, 1991; Zimmermann and Schwaller, 2002).

To test the specificity of the secondary antibodies, the omission and/or replacement of all primary antisera by non-immune sera or PBS was performed. A lack of any immunoreactions indicated specificity.

2.4. Data analysis

The sections were viewed under an Olympus BX51 microscope. Images were acquired and cell measurements were made with the Cell F software (Olympus GmbH, GER). The lengths of the