Immunoreactivity of the calbindin D28k in the parahippocampal gyrus of chinchilla

Radosław Szalak, Jadwiga Jaworska-Adamu, Karol Rycerz, Paweł Kulik, Marcin Bartłomiej Arciszewski

Department of Animal Anatomy and Histology, Faculty of Veterinary Medicine, University of Life Sciences, 20-950 Lublin, Poland
radoslaw.szalak@up.lublin.pl

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Abstract

Ten adult male chinchillas were used. The localisation of calbindin D28k (CB) was examined with the use of two types of reactions: immunocytochemical peroxidase-antiperoxidase and immunofluorescence staining with a specific monoclonal antibody against CB. Immunocytochemical examination demonstrated the presence of CB-positive neurons in the following layers of all parts the parahippocampal gyrus (PG): marginal, external cellular, middle cellular, and internal cellular, i.e. in entorhinal area, parasubiculum, and presubiculum. Immunofluorescence staining revealed the presence of CB in both Hu C/D-immunoreactive (IR) neurons and nervous fibers of the PG. CB-IR neuronal cell bodies were moderately numerous (ca. 10% of Hu C/D-IR neurons) and clearly distinguished from the background. Each layer of the brain area consisted of two types of neurons: pyramidal and multiform. Among the second type of neurons, four kinds of morphologically different neuronal subclasses were observed: multipolar, bipolar, round, and Cajal-Retzius cells. It is concluded that the expression of CB in the PG of the chinchilla is species specific and limited to several subclasses of neurons.

Key words: chinchilla, parahippocampal gyrus, calbindin D28k, memory.

Introduction

The presence of many structures of the limbic system affects mutual connections with neocortex. Some of them form a unit called hippocampal formation, which consists of rhinencephalon included in archicortex (19). This complex formation belonging to the limbic system includes: entorhinal area, parasubiculum, presubiculum, and these two form parahippocampal gyrus (PG), as well as subiculum, proper hippocampus, and dentate gyrus. Entorhinal area, parasubiculum, and presubiculum belong to periarichicortex i.e. transitional form between phylogenetically old archicortex, and neocortex (16, 25). PG consists of four layers: marginal layer, cellular layer external, middle, and internal, observed e.g. in sheep, chinchilla, guinea pig, monkey, and macaque (11, 22, 23, 27, 29). The individual layers are composed of multiform nervous cells: pyramidal, fusiform, stellate, round, and oval. Similarly as in other areas of the brain, PG neurons are classified into two types: primary neurons and interneurons. These cells functionally differ from each other, that is associated with the presence of a number of neuropeptides as well as calcium-binding proteins. The presence of glutamic acid as an excitatory neurotransmitter in the primary cells is particularly important for memorising process and learning. Gamma-aminobutyric acid (GABA), present in interneurons, is responsible for the inhibitory effect on other interneurons as well as on primary cells, which form a network involved in the control of neuronal activity (8, 9, 14, 27). As it was shown in guinea pig, a specific topographical localisation of PG participates in bidirectional communication between the hippocampus and neocortex (23). Afferent and efferent neuronal pathways of limbic system e.g. between the rhinencephalon’s structures belonging to the limbic lobe and neocortex, nucleus accumbens, striatum, hypothalamus, and brainstem affect the plurality of functions in the central nervous system (CNS). These connections play a crucial role in autobiographical-declarative memory, spatial memory, as well as in memory formation and consolidation and optimisation during sleep (24, 29). The afferent and efferent hippocampal formation pathways take part in the autonomic regulation in such processes as reproduction, food intake, in some endocrine responses, in extrapyramidal motor activities (6, 12, 14, 16, 18–
In the current literature, two kinds of calcium-binding proteins, belonging to the „EF-hand” family, are described: sensory and buffer. The calcium ions’ buffering proteins include: parvalbumin (PV), calbindin D28k (CB), calretinin (CR), calcineurin, and many others. CB as a buffering protein is considered as an excellent neurochemical marker in the CNS. In contrast to other „EF-hand” family proteins, it appears in an early embryonic period. Its expression maintains at a constant level in adults and decreases in aging individuals. CB, like most buffering proteins, locates in CNS neurons, protecting them against the calcium ions’ neurotoxic effect. At the cellular level, it is responsible for pre- and postsynaptic calcium signals regulation and synchronisation (5, 16). The knowledge on the regulatory role of calcium binding proteins in pivotal processes is far from being fully understood. Important research is conducted to specify distribution and co-localisation of this protein in order to explain functions and processes occurring in neurons in the pathogenesis of many CNS diseases. Until now, the immunoreactivity to CB have been localised in neurons of the mammalian enteric nervous system (3) and CNS (1, 4–6, 12, 22, 28), including GP in human (7), monkey (17), and mouse (25). In our previous research, CB expression in some CNS areas of chinchilla was presented (12, 13, 28). Considering that calcium-binding proteins are valuable markers of different neuronal classes and play important role in the CNS (15, 21), immunohistochemistry was applied to examine the distribution pattern of CB in GP of chinchilla.

**Material and Methods**

**Animals.** Ten sexually mature male chinchillas (ca. 1.5 year old) were used in the study. Their brains were dissected out immediately after slaughter. The brains of five animals were subjected for immunocytotoxicological studies, whereas brains from other five animals were used for immunofluorescence staining.

**Immunocytotoxicity.** After the dissection, the whole brain (n = 5) was fixed in buffered 10% formalin (pH 7.0) for 12 h at 4°C. After the fixation, the material was processed through a graded series of alcohols, cleared in xylene, embedded in paraffin, and cut to 6 μm-thick transverse sections. Deparaffinised sections were processed according to peroxidase anti-peroxidase (PAP) immunocytotoxicological technique. Endogenous peroxidase activity was eliminated from the sections by incubation with 0.3% H2O2 for 10 min. In order to block unspecific binding, an incubation with 1% normal goat serum (NGS; Sigma-Aldrich, USA) was performed. Afterwards, the sections were incubated overnight with specific antibodies against rabbit calbindin D-28k (1:3000; Sigma-Aldrich). The slides were then incubated with monoclonal goat anti-rabbit IgG (1:50, Sigma-Aldrich). Localisation of the antiserum complexes was visualised with the chromogen 3,3’-diaminobenzidin tetrahydrochloride (DAB; Sigma-Aldrich). In the stained sections, reaction products formed as water-insoluble brown precipitates of different intensity were found. Finally, the sections were washed in distilled H2O, dehydrated in alcohol, cleared, and mounted in DPX (Fluka). The slides were examined under a light microscope (Axiolab, Zeiss, Germany) connected to a digital camera. In order to test the specificity of antibodies used, control staining in which the primary antibodies were omitted or replaced with non-immunoreactive sera were made.

**Immunofluorescence.** For double immunohistocytotoxicological staining, an indirect immunofluorescence method described elsewhere (2) was used. Briefly, after the dissection, the brain was immersed in Stefanini’s solution of paraformaldehyde and picric acid for 24 h (4°C). After the fixation, it was rinsed for several days in Tyrode’s solution containing sucrose (one change per 1 d), and then embedded in O.C.T compound and cut with a cryostat into 10 μm-thick sections. Prior to the staining, the selected sections were dried at room temperature for 30 min. Potential non-specific binding sites in the tissue were blocked by incubation with PBS (3×15 min) supplemented with 10% NGS, 0.25% bovine serum albumin, and 0.25% Triton X-100 (Sigma-Aldrich, USA). The sections were incubated at room temperature overnight with a combination of mouse monoclonal Hu C/D antibodies (1:400; Molecular Probes, USA) and rabbit calbindin D-28k (1:2000; SWant, Switzerland) antisera. Bound primary antibodies were visualised with Texas Red-conjugated anti-rabbit goat IgG (1:400; MP Biomedicals, USA) and FITC-conjugated anti-mouse goat IgG (1:400; MP Biomedicals). After final washes, the specimens were coverslipped and viewed with a spinning disk confocal microscope (BX-DSU Olympus, Japan) equipped with interference filters appropriate for Texas Red (545–580 nm) and FITC (470–490 nm). The specificity of the primary antibodies was previously demonstrated (3) by the pre-absorption experiments. The specificity of immunohistochemical reaction was also tested by the experiments in which the primary antibodies were either omitted or replaced with non-immunoreactive sera. The frequency of CB-IR GP neurons was expressed as a percentage relative to the total number of Hu C/D positive perikarya. In each animal a random sample of a minimum two hundred of Hu C/D-IR GP neurons was examined. Only neurons with well-visible nuclei were counted.
Fig. 1. Differentiated CB immunoreactivity in neurons of the PG external cellular layer

Fig. 2. Intensive CB immunoreactivity in multipolar neurons’ cell bodies and processes of the internal cellular layer

Fig. 3. Moderate CB immunoreactivity in multipolar neurons’ cell bodies and processes of the PG middle cellular layer

Fig. 4. Intensive CB immunoreactivity in round neuron of the external cellular layer

Fig. 5. Moderate CB immunoreactivity in bipolar neuron of the PG external cellular layer

Fig. 6. Moderate CB immunoreactivity in Cajal-Retzius neuron of the PG marginal layer

Fig. 7. Transverse section of the chinchilla PG showing the immunoreactivity to CB in the parasubiculum
Results

Immunocytochemistry. CB immunoreactive neurons were observed in marginal, external cellular, middle cellular, and internal cellular layers of all parts of PG, i.e. in entorhinal area, parasubiculum (Fig. 7), and presubiculum.

Individual layers consisted of two types of neurons: pyramidal and multiform. Among the second type of neurons, four kinds of them were observed: multipolar, bipolar, round, and Cajal-Retzius cells. PG neurons were characterised by different brownish colour of immunocytochemical reaction, whose intensity varied from weak via moderate to strong (Figs 1 and 2). CB immunostaining was localised in cell bodies, and in dendrites, and axons (Fig. 3). Pyramidal cells were characterised by triangular shape and weak CB immunoreactivity (Fig. 1). Multiform, large sized neurons were localised mainly in cellular layers of PG. Most of these cells were characterised by moderate and intensive immunostaining (Figs 1 and 4). Bipolar, fusiform average sized neurons were observed in all layers of the brain area. Their cell bodies with moderate brownish CB immunostaining were arranged vertically with the exception of the marginal layer, in which there were fusiform Cajal-Retzius cells (Figs 5 and 6). These cells’ nervous processes, similarly as their bodies, were arranged along the marginal layer, and were moderately CB-stained (Fig. 6). A small population of neurons was round shaped with an intensive reaction of the studied protein (Fig. 4).

Immunofluorescence. Results of immunofluorescent staining revealed the presence of CB in Hu C/D-positive neurons, as well as in nervous processes of PG. CB-IR neurons’ cell bodies were moderately numerous (accounted for 10% of all Hu C/D-IR neurons), clearly distinguishable from the background. Predominantly, CB-IR/Hu and Hu C/D-IR neurons were classified as pyramidal, round, multiform, and bipolar cells. CB-IR nerve fibers communicated with neighbouring CB-positive and CB-negative neurons (Fig. 8).

Discussion

In the present study the CB distribution in PG was described in chinchilla. The CB immunoreactivity was observed in pyramidal and non-pyramidal neurons of all areas of the examined structure in chinchilla, similarly as in human (7), macaque (17), and mouse (25). CB-positive reaction was observed in multiform neurons like in human (7), macaque (17), and mouse (25). Weak immunoreactivity was demonstrated in pyramidal shaped cells, and moderate and intensive immunoreactivity in multiform, bipolar, Cajal-Retzius, as well as in round neurons similarly as in human (7) and macaque (17). Distribution of pyramidal neurons with weak CB immunoreactivity in PG in the chinchilla, human (7), and mouse (25) may suggest that they are primary projection neurons. Moderate and intensive reaction of the studied protein in other multiform, non-pyramidal: multipolar, bipolar, round, and Cajal-Retzius cells may indicate that they are interneurons. In studies of many CNS structures, co-localisation of the calcium-binding proteins with other neurotransmitters in primary cells and interneurons has been observed. In the PG and hippocampus proper CB is a marker of GABA-ergic interneurons’ population. These cells are characterised by short axons that do not go beyond the studied area. Their connections to each other, as well as to the primary cells take part in neuronal activity control. Interneurons form inhibitory synapses in the form of gap junctions (26). It was demonstrated that the interneurons expressing CB form numerous connections, which can reach out cell bodies, axons, and further dendrite segments of primary cells. In consequence, interneurons’ inhibitory effect on primary neurons causes their disinhibition (disinhibitory cells). As regards the functions, interneurons form two separate cell networks with different contents of neuropeptides (26, 29). The first of them is the fast responding to stimulation interneurons (fast spiking-FS). The second network is divided into three subclasses: the first class is characterised by regular (regular spiking-RS), and the second by rapidly responding to stimulation interneurons. Both of these subclasses reveal the presence of CB-positive reaction. The third subclass consists of regularly communicating interneurons that demonstrate the presence of other calcium-binding proteins (29). The appropriate concentration of calcium ions in both primary cells and interneurons affects their connections, as well as their proper functioning. Therefore calcium-binding proteins regulate metabolic processes affecting the vital functions of cells. The release of neurotransmitters and
neuromodulators to synaptic cleft is associated with the synaptic plasticity, which also depends on the appropriate concentration of calcium ions in the cell (8, 10). Varied distribution of this protein in different classes of PG neurons requires further studies. Defining the distribution of neurons and the presence of CB in the analysis is an introduction to further studies of this protein’s co-localisation with other calcium buffers and neurotransmitters.

References


