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Structural Characteristics and Spatial Organization of Parvalbumin-Containing Neurons in Somatosensory Zone SI of the Cerebral Cortex in Rats

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The aim of the present work was to perform layer-by-layer morphometric and immunohistochemical studies of parvalbumin-positive (PA^+) neurons in the somatosensory zone (SI) of the cerebral cortex in white mongrel rats (n = 10). Studies of frontal and tangential sections of thickness 60 and 4 μm revealed significant variation in shape, body size, and process branching among PA^+ neurons in all cortical layers. The greatest proportion of PA^+ neurons (47.1%) was located in cortical layer IV in the barrel formation zone. Studies of tangential sections showed that the greatest proportion of PA^+ neurons was located in barrel septa (43%). These cells in layer IV were distributed most densely in barrel walls, such that their outlines were clearly visible. The quantitative dominance of PA^+ neurons in septa may be linked with the direction of the course of their dendrites in the internal part of the barrel and the formation of dendrodendritic gap junctions, which may in turn provide the morphological basis for individual local pacemaker rhythmogenesis and regulation of the functional state of cortical columns.

Keywords: cerebral cortex, barrels, neurons, parvalbumin, immunohistochemistry.

The inhibitory system of the cerebral cortex plays a dominant role in regulating the functional state of both individual cortical columns and the brain overall [5]. At present 21 types of inhibitory neurons are defined, with different morphological features, origins, and spike activity; there are also various accessory neuropeptides [4]. Parvalbumin (PA) is a calcium-binding intracellular protein typical of almost 40% of all CNS GABAergic interneurons, whose main function is to take part in releasing neurotransmitters and stabilizing the intracellular calcium content after repolarization of the cell. PA-containing neocortical interneurons are fast-spiking and arise from a transient structure present at the embryonic stage of development, the rudiment of the basal ganglia, i.e., the ganglionic eminence [2]. Interest in studies of this group of inhibitory interneurons has increased over recent years from a few reports in 1986–1987 to 1500 publications indexed in PubMed in 2014 alone [8]. Contemporary data indicate that PA-containing neurons in the cerebral cortex are able to form various neural networks connected by both chemical and electrical synapses, though the functional significance of these networks and their relationship with the columnar organization of the neocortex require further investigation [1, 7]. Studies of the structural-functional organization and morphological characteristics of PA-containing, presumptively GABAergic, fast-spiking neurons in cortical columns may promote our understanding of their role in the processes of the inhibition, synchronization, and regulation of rhythmogenesis of both the cortex and subcortical structures. The aim of the present work was to conduct a layer-by-layer morphometric study of PA-containing neurons in somatosensory zone SI in the rat neocortex.

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Materials and Methods

Experiments were performed on mongrel white laboratory rats (n = 10) of both genders, weighing 150–200 g. Animal keeping and experimental procedures were performed in compliance with a protocol approved by the Bioethics Committee of the Southern Federal University on April 18, 2012. Experiments addressed the somatosensory zone of the cortex (SI). Under deep ether anesthesia, i.v. injection of heparin was followed by transcardiac perfusion with phosphate buffer and then 4% paraformaldehyde containing 0.05% glutaraldehyde in phosphate buffer. At 2 h after perfusion was complete, brains were extracted and placed in fixative solution overnight in a refrigerator at 4°C for postfixation. Tangential and frontal sections of thickness 60 μm were cut on a VT 100E vibratome (Leica, Germany). After cryoprotection in 30% sucrose in phosphate buffer, sections were snap-frozen in liquid nitrogen vapor and then thawed in phosphate buffer and incubated for four days at 10°C in primary monoclonal antibodies to PA (Sigma) diluted 1:100 and 1:1000. After washing in phosphate buffer, sections were incubated in RTU Envision Flex/HRP secondary antibodies (Dako, Germany) overnight. Immunohistochemical reactions were performed on thin sections by embedding brain in paraffin and cutting sections of thickness 4 μm; these were placed on glass slices with polylysine coatings and incubated with primary monoclonal anti-PA antibody (Sigma, Sweden) diluted 1:100. Thin sections and 60-μm sections were processed to detect immune complexes using a Dako EnVision System + Peroxidase visualization system (DAB, Dako, Germany). After additional fixation in 1% osmium tetroxide and dehydration, sections of thickness 60 μm were embedded in epoxide resin using a plane parallel method. Sections (thin sections 4 μm thick and thick sections 60 μm thick) were examined under a Leica 2500 light microscope (Leica, Germany) fitted with a Leica DFC 495 digital camera. Numbers of neurons and processes were counted only on sections of thickness 60 μm, as these allowed more cells to be identified than thin (4 μm) sections. Morphometry was performed using the interactive measurement module (LAS Interactive Measurement) of Leica Application Suite 4.3 (Leica, Germany). PA-positive (PA^+) neurons were counted on four frontal sections of thickness 60 μm using an area of 10000 μm^2 in each layer of the cortex. A total of 360 immunopositive cells were counted in all layers. The quantitative distribution of PA^+ neurons was assessed in the zone forming the barrel cortex (layer IV), as our observations show that this layer contains the greatest number of the cells of interest. After identification of the barrel cortex, PA^+ neurons were counted between barrels (in septa), in the walls, and in the internal parts of each barrel. A total of 748 immunopositive cells were counted in 10 barrels on an area of 519,710.3 μm^2.

Results

Light microscopy studies of both thick and thin frontal sections after the immunohistochemical reaction revealed many intensely staining cells in virtually all layers of somatosensory cortex zone SI, with processes running in different directions and expressing the antigen of interest, i.e., PA (PA^+ neurons). Reaction product was located in the cytoplasm and nucleus, as well as in the axons and dendrites, of the neurons of interest. Cortical layer IV included a zone with a greater PA density, which was more clearly visible on thick sections (Fig. 1, *a*, *c*). On examination at greater magnification, this effect could be seen to result from the grouping of PA^+ neuron bodies in barrel-forming areas, with numerous crisscrossing processes from these cells in the neuropil (see Fig. 1, *d*). A dark rim formed around PA-negative cells – probably because of the formation of dendrosomatic and axosomatic synaptic contacts between the processes of PA^+ neurons and the bodies of other neurons.

Examination of both thin and thick sections identified the morphological features of PA^+ neuron bodies and the way their processes branched (see Fig. 1, *b*, *e–h*). Body shape discriminated PA^+ neurons in different cortical layers to stellate, fusiform, round, and triangular types, while the nature of their processes divided them into unipolar, bipolar, and multipolar with branching dendrites (see Fig. 1, *b*). In layer I of the cortex, the most characteristic neurons were those with body cross-sectional areas of no more than 60 μm^2. Layer II contained PA^+ neurons with a wider range of body areas than in layer I, though it was dominated by cells with a range of body areas of 60–90 μm^2. Both layer III and layer IV of the cortex contained structurally diverse PA^+ neurons with areas ranging from 60 to 180 μm^2. Layers V and VI contained neurons with the greatest range of body areas: half of the PA^+ neurons in these layers had large bodies – of 150–360 μm^2, while other cells were smaller, at 30–120 μm^2. Overall, studies of the body areas of PA^+ neurons in different cortical layers demonstrated a tendency to an increase in cell size with increasing depth.

Layer-by-layer counting of PA^+ neurons on frontal sections of thickness 60 μm on areas of 10000 μm^2 showed that of all the 360 immunopositive cells counted, 47.1% were located in layer IV, 8.3% in layer II, 10.9% in layer III, 17.1% in layer V, and 14% in layer VI. The smallest proportion of PA^+ neurons was seen in molecular layer I of the cortex – 2.6% of all PA^+ neurons counted.

On examination of an area of 519,701.3 μm^2 on tangential sections of thickness 60 μm, most PA^+ neurons counted in the barrel zone (total 748 cells counted) were located between barrels, i.e., in septa (43% of cells), with 26% in the walls of barrels and 31% in the internal parts of barrels (see Fig. 1, *i*). PA^+ neurons were distributed more densely in barrel walls (12 ± 8 cells/10000 μm^2) than in the internal parts of barrels (5.0 ± 2.2 cells) and septa (4.2 ± 1.7 cells).

Discussion

There are several classifications of GABAergic interneurons in the CNS. They can be differentiated on the basis of functional electrophysiological characteristics, in terms of the type of synaptic contacts formed with the bodies and

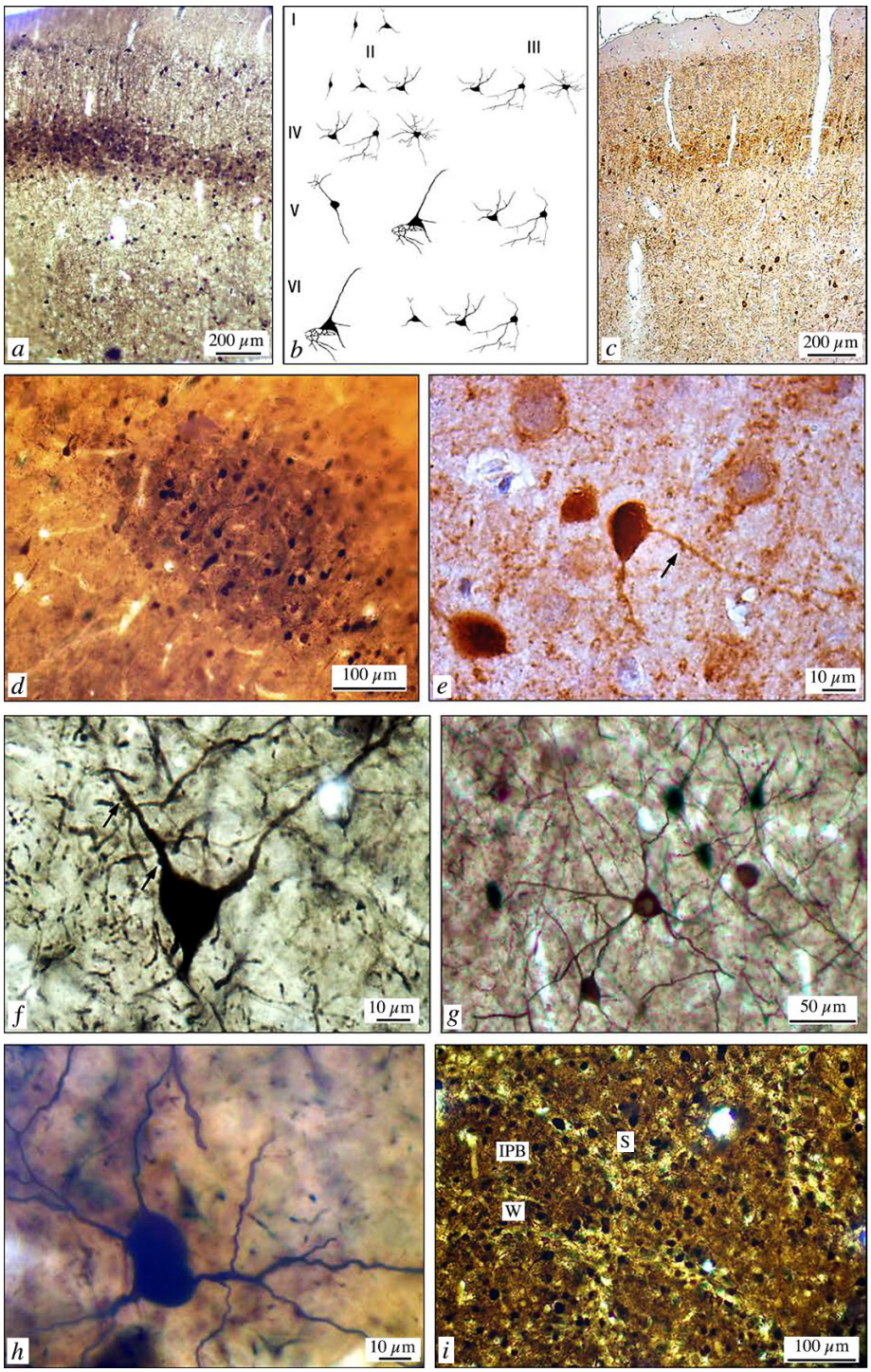

Fig. 1. Layer-by-layer distribution and morphological characteristics of parvalbumin-containing (PA^+) neurons in the somatosensory zone SI of the rat cerebral cortex. *a*) Distribution of PA^+ neurons in a frontal Epon section of thickness 60 μm – cortical layer IV appears dark; *b*) different morphological shapes of PA^+ neurons (drawn from original photographs); *c*) distribution of PA^+ neurons on a frontal section of thickness 4 μm; *d*) locations of PA^+ neurons within a single barrel of size 300 μm^2 in layer IV on a frontal section of thickness 60 μm; *e*) a bipolar neuron expressing PA on a section of thickness 4 μm, showing a dendritic spine (arrow); *f*) a PA^+ neuron with a triangular body in layer V, with dendritic spines (arrows); *g*) a "classical" stellate PA^+ neuron in cortical layer IV; *h*) two contacting large PA^+ neurons with round bodies and branching processes; *i*) distribution of PA^+ neurons in the barrel cortex on a tangential section of thickness 60 μm. S – barrel septum; W – barrel wall; IPB – internal part of barrel; I–IV – cortical layers. Immunohistochemical reaction.

processes of other cells, in terms of the timing and location of the origins from the ganglionic eminence, in terms of the type of interneuron axon growth, etc. [4]. The most widely accepted classifications of inhibitory interneurons are those based on the expression of molecular markers such as somatostatin, cholecystokinin, and neuropeptide Y, as well as the expression of Ca^{2+}-binding proteins including calbindin, calretinin, and PA [5, 13]. Existing data indicate that PA-containing neurons form two main morphological groups: axoaxonal or "chandelier" cells, responsible for presynaptic inhibition at the initial segments of the axons of pyramidal cells, and multipolar basket cells forming synaptic contacts on the proximal parts of dendrites [6]. However, immunohistochemical studies using antibodies to PA demonstrated that PA^+ neuron bodies have more diverse shapes and process branching patterns, which may provide grounds for identifying additional subtypes on the basis of the morphological characteristics of these neurons. The results of our morphometric studies showed that in terms of body area, PA^+ neurons could be divided into small (less than 60 μm^2), medium (up to 150 μm^2), and large (up to 360 μm^2) cells.

The heterogeneity of these morphological properties may be associated with the different functional assignments of PA^+ interneurons, as well as with the differential expression of genes between the dorsal and medial parts of the ganglionic eminence from which the various subpopulations of neurons of interest arose during ontogeny [15].

Morphometric studies of PA^+ neurons in the different layers of the somatosensory zone of the cortex SI showed that the proportion in layer I was the smallest, while published data on the presence or absence of PA^+ neurons in this layer are quite contradictory [3, 10]. Our data indicate that PA^+ neurons in molecular layer I have sizes comparable to those of PA^+ neurons in the outer granular layer (II) and may be incorrectly assigned on counting. According to our data, the number of PA^+ neurons has a weak tendency to increase from the supragranular layers II–III to the infragranular layers V–VI. The largest proportion of PA^+ neurons was located in cortical layer IV, in the zone forming modular groups of barrels. Studies of tangential sections showed that the largest proportion of PA^+ neurons was located in barrel septa (43%), with the greatest density in the barrel walls, such that clear outlines of barrels could be seen in layer IV. The predominance of PA^+ neurons in the septal area may be related to the direction of the course of the dendrites of PA^+ neurons in the internal parts of barrels and the formation of dendrodendritic gap junctions, which may presumptively be the morphological basis of individual local pacemaker rhythmogenesis in barrels and columns of cortical zone SI [18]. Data have been obtained showing that increases in hyperpolarization lead to activation of voltage-dependent potassium H channels on the membranes of PA^+ neuron bodies and axons, producing waves of endogenous pacemaker activity and demonstrating high threshold activation and inactivation [9, 11, 12, 14]. Gap junctions connecting networks of inhibitory PA^+ neurons facilitate synchronization of the oscillator activity of pacemaker potassium channels and signal conduction with minimal synaptic delays [1].

Thus, the studies reported here established the pattern of quantitative layer-by-layer distribution of PA^+ neurons, which may determine their functional role in organizing intracolumnar rhythmic activity in the cerebral cortex. Morphological variety was seen in the shapes and sizes of PA^+ neuron bodies and the nature of the branching of their processes in each layer, as well as in the cortical zones of the SI, helping our understanding of the role of neurons of different sizes in the structural-functional organization of columns in each layer of the SI zone. PA^+ neurons were seen in the internal part, walls, and septa of barrels, with a significant predominance in septa. Variation of interneurons may provide the structural basis for PA^+ neurons to fulfil such functions as controlling normal neuron activity, generating the oscillator activity of neural networks, buffering calcium, regulating synaptic plasticity, supporting neuroprotective functions, and maintaining the balance between excitation and inhibition in the somatic cortex. However, despite the fact that in terms of morphological characteristics, PA^+ neurons can be separated quite clearly, further studies of the differential expression of genes in them will, in our view, promote the creation of a genomic classifier significantly supplementing and refining the morphofunctional classification of interneurons.

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