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Ultrastructural Immunocytochemistry of GABAergic Cells in Neocortical Neurotransplants

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Neural transplantation is a promising regenerative therapy in the treatment of several neurological diseases. Importantly, transplanted tissue should not become a source of pathological functional activity. To assess the possibility of maintaining the balance between excitatory and inhibitory processes, an electron microscopic immunochemical study of the GABAergic system in rat neocortical transplants was performed. Accumulation of GABA-positive label in astrocytes and a relatively insignificant immune reaction to GABA in neurons and synaptic endings were found. These findings suggest that under conditions of impaired differentiation of GABA-containing neurons that generate phasic inhibition through inhibitory synapses, tonic inhibitory influences predominate in neurotransplants due to GABA released from astrocytes.

Key Words: *neocortical allotransplants; neurons; astrocytes; electron immunocytochemistry; GABA*

Neurotransplantation is a promising neurosurgical method of cellular and functional repair of the damaged brain in neurological diseases. Various transplantation technologies with the use of the suspensions of neural progenitor cells or the fragments of fetal tissue demonstrated positive therapeutic results in both animal models and clinics. The main principles, limitations, and the results of transplantation therapy have been discussed in a number of reviews [7,10]. However, some unsolved issues in this branch of restorative medicine still require further laboratory investigations. In particular, an important condition for the use of neurotransplantation is prevention of the formation of epileptic foci. It is known that a decrease in the number of inhibitory GABAergic neurons plays a central role in epileptogenesis [2]. In our previous experiments, a significant decrease in the differentiation of GABA-containing neurons in neocortical transplants was found [1,15]. At the same time, there is evidence that, in addition to phasic inhibition generated due to the release of the inhibitory neurotransmitter from synaptic vesicles of GABAergic neurons, there are tonic inhibitory currents formed by extracellular GABA [3,13]. GABA that is present in the extracellular space is mainly release from astrocytes [6].

The aim of this study was to assess the possibility of participation of tonic inhibition in the maintenance of the balance of excitatory and inhibitory processes in transplants. To this end, we performed an ultrastructural immunochemical study of GABA in intraneocortical transplants, with particular attention to astrocytic glial cells.

MATERIALS AND METHODS

The study was performed on Wistar rats kept under standard vivarium conditions. The experiments were carried out in compliance with the Directive 2010/63/EU of the European Parliament and the Council (On the Protection of Animals Used for Scientific Purposes; September 22, 2010). The chemical agents and antibodies used in the work were purchased from Sigma–Aldrich. All procedures on animals were carried

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out under Nembutal anesthesia (30-40 mg/kg, intraperitoneally). The neocortex primordium of 17-day-old fetuses was used as a donor material for grafting. Homotopical allotransplantation was performed into the acute cavity of the somatosensory cortex of adult male rats (n=3). The grafts survived for 4 months. After that, the animals were transcardially perfused with a fixative consisting of 2.5% formaldehyde solution and 1% glutaraldehyde solution in 0.1 M PBS (pH 7.4). Next, the brain area containing neurotransplant was isolated and cut into 50 μm coronal sections. Some of them were stained with cresyl violet by the Nissl method for histological study.

For immunocytochemical detection of GABA at the ultrastructural level, previously published methodological guidelines were used [76]. First, the sections were treated with a 10% blocking solution of BSA in Tris-HCL buffer (pH 7.4) for 30 min; then, they were incubated in primary rabbit anti-GABA (1:1000) for 1 day at 4°C. After washing in 0.1% BSA/Tris buffer, the sections were incubated for 4 h with secondary gold-labeled anti-rabbit antibodies (1:100 diluted with 1% BSA/Tris-HCl), washed with 1% BSA/Tris-HCl buffer), treated with avidin–biotin–peroxidase complex and 0.05% solution of 3,3'-diaminobenzidine in 0.1 M PBS (5 min). Then, the sections were additionally fixed with 1% osmic acid solution, dehydrated in a series of alcohols, and embedded in epoxy resin Epon 812. Control samples were treated with the same reagents, except incubation with anti-GABA antibodies. Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined in a JEM-100B electron microscope (Jeol). The brightness, contrast, and nonspecific background staining were adjusted using the Adobe Photoshop software.

RESULTS

Histological analysis of transplants showed that they did not reproduce the layered cytoarchitectonics typical for the neocortex; nervous and glial cells were diffusely distributed and only sometimes formed small clusters. There was a clear-cut border between the graft and the host brain and only somewhere full tissue fusion was seen. More often, the border was formed by an accumulation of glial cells, but sometimes large blood vessels from the meninges penetrated into the interphase (Fig. 1). Although we have previously shown that the interphase organized by glial cells does not prevent the growth of nerve processes through it, the zones filled with wide perivascular spaces and connective tissue elements undoubtedly limited functional integration of the neurotransplants with the brain [15].

The immunochemical analysis showed preserved ultrastructure of neurografts, the neurons, glial cells,

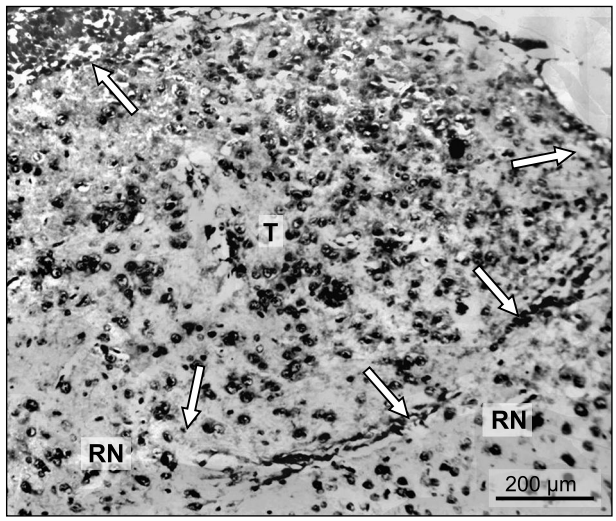

Fig 1. Histological structure of homotopical intraneocortical transplant, general view. The border between the transplant (T) and recipient neocortex (RN) is shown by arrows. Nissl staining with cresyl violet.

their processes, and synaptic contacts can be easily identified. The ratio of nerve and glial elements in the neuropil corresponded to that in a normal healthy brain. In parenchyma of the grafts, there were no morphological signs of neuroinflammation, reactive forms of astrocytes, which, according to the literature [12], are characterized by hypertrophied sizes and the presence of intermediate microfilaments.

When accessing the immune reaction for GABA in different types of neurotransplant cells, intense label was found in protoplasmic astrocytes of a typical size. $GABA^+$ astrocytes had light cytoplasm and low number of cell organelles. The immunolabel in the form of electron-dense globules from 20 to 60-80 nm in size was evenly distributed in the cytoplasm. The majority of particles were not clearly bound to membranes or organelles; however, accumulations of up to 8-10 granules were observed above some mitochondria. At the same time, only rare granules were located in the region of astrocytic nuclei (Fig. 2). In cytoplasmic processes of astrocytes, the distribution of the reaction product was similar to that in the cell bodies. In addition, the immunolabeled profiles of transected astrocytic processes were observed everywhere throughout the neuropil. They, like perikaryons of $GABA^+$ astrocytes, showed no signs of a reactive state and contained no intermediate microfilaments. Astrocyte end-feet ensheathing blood capillaries in the neurotransplants also demonstrated active reaction to GABA staining.

The neurons containing GABA were rare; labeling in form of electron-dense globules similar to those in astrocytes was present only in perikaryons above the mitochondria and in the region of the Golgi apparatus

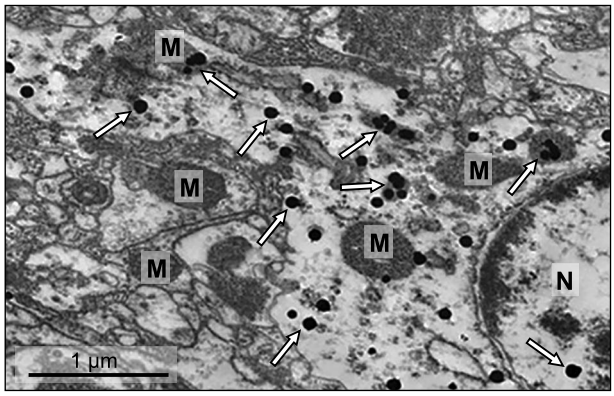

Fig. 2. Ultrastructural immunolocalization of GABA in astrocyte of the intraneocortical transplant. N: astrocyte nucleus; M: mitochondria. Arrows show GABA$^+$ granules.

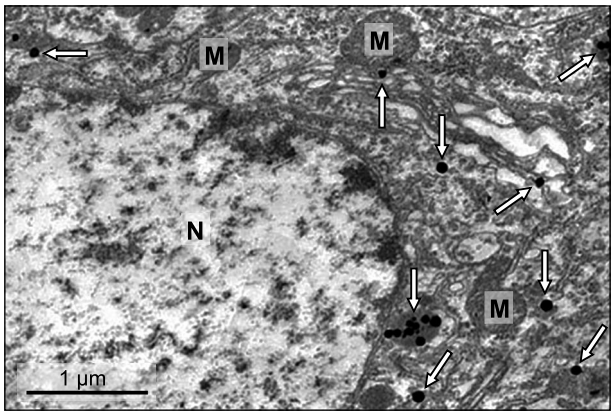

Fig. 3. Ultrastructural immunolocalization of GABA in neuron of the intraneocortical transplant. N: astrocyte nucleus; M: mitochondria. Arrows show GABA$^+$ granules.

and cisterns of the endoplasmic reticulum (Fig. 3). Immunopositive synaptic endings were even more rarely. To some extent, the small number of GABA-containing neurons in the grafts may reflect the known data that inhibitory interneurons in the brain constitute only 10-20% of all nerve cells [8]. In addition, our ultrastructural observations agree with our previous light microscopy data about the lower content of GABAergic neurons in neurotransplants compared to the surrounding native neocortical tissue [15].

Predominant detection of the immunolabel in astrocytes in comparison with neurons and synaptic terminals, can be explained by the fact that the cytochemical reaction protocol used by us was more conducive to the finding of the metabolic, rather than the neurotransmitter GABA pool. It is known that the brain has two GABA pools synthesized by different isoforms of glutamic acid decarboxylase (GAD). GABA synthesized by GAD65 is considered to be a synaptic pool and is associated with the exocytosis of the neurotransmitter from synaptic vesicles and the phasic inhibition of neurons, whereas GABA synthesized by GAD67 belongs to the metabolic pool, is located in the cytoplasm, and is associated with tonic inhibition [5,11]. Apart from the classical GABA synthesis through glutamate decarboxylation, other pathways of GABA accumulation in astrocytic glial cells are also possible. GABA can enter astrocytes from extracellular spaces through transporters located on cytoplasmic membranes [13]. At the same time, our ultrastructural observations of multiple immunopositive granules above mitochondria suggest that neurotransplants have an additional pathway of GABA synthesis through the degradation of mitochondrial putrescine. This alternative pathway was observed in the subventricular zone during the early stages of neuroblast differentiation, when GAD activity was not detected [14]. Furthermore, this mechanism of the GABA synthesis was also found in astrocytes of adult animals with modeled Alzheimer's disease and temporal lobe epilepsy [4,9]. The authors noted that under pathological conditions, GABAergic astroglial cells had pronounced signs of a reactive state and contained glial fibrillary acidic protein. However, in the neurografts studied by us, GABA$^+$ astrocytes had the characteristics of normal healthy cells. Presumably, astrocytic GABA in neocortical transplants, like in early ontogenesis, supports the differentiation and maturation of the implanted tissue. In addition, another important function of astrocytic GABA in neurotransplants can consist in potentiation of tonic inhibitory processes for maintaining the balance between excitation and inhibition.

Thus, our findings suggest that transplanted neuronal tissue is characterized by high morphofunctional plasticity, and if the differentiation of inhibitory neurons is impaired, a lack of the synaptic inhibition can be compensated for by tonic inhibition mediated by GABAergic astrocytes.

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