DOI 10.1007/s11055-020-00945-8
Neuroscience and Behavioral Physiology, Vol. 50, No. 5, June, 2020

Changes in the Interaction between Astrocyte Processes and Synaptic Terminals in the Generation of Epileptiform Activity

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Translated from Rossiiskii Fiziologicheskii Zhurnal imeni I. M. Sechenova, Vol. 105, No. 6, pp. 707–715, June, 2019. Original article submitted April 26,2018. Revised version received April 1, 2019. Accepted April 3, 2019.

The aim of the present work was to compare the structural organization of excitatory synaptic contacts in intraocular septal transplants showing normal and epileptiform activity. Experiments were performed using Wistar rats. Donor material for transplants was obtained from the septal area of the brain from 17-day-old rat fetuses. The electrophysiological properties of transplants were tested three months after surgery using short-term stimulation with single electrical pulses. Test results were used to divide transplants into two groups: those with normal and those with epileptiform activity. Microscopic examination of neurotransplants of both groups showed that nerve and glial cells, as well as the neuropil, consisting of axons, dendrites, synaptic terminals, and astrocyte processes, were well differentiated. Axodendritic and axospinous synaptic terminals were regarded as three-component structural complexes (tripartite synapses), which included not only pre- and postsynaptic components, but also their surrounding astrocyte processes. Most had the morphological signs of excitatory contacts with clear postsynaptic densities whose sizes were taken as a correlate of the efficiency of nervous transmission. Morphometric analysis of these synapses from functionally different types of transplants revealed no significant differences in the extent of postsynaptic densities or the cross-sectional areas or perimeters of presynaptic boutons. At the same time, large differences were seen in the extent to which synapses were surrounded by astrocyte processes. The proportion of perisynaptic processes was 1.8 times lower in transplants characterized by epileptiform activity than in controls. These data provide evidence that presynaptic astrocyte processes were the first to react to electrical stimulation and initiated the development of epileptiform activity. It is suggested that decreases in astrocyte sheaths of excitatory synapses promoted the propagation of neurotransmitters across intercellular spaces and the involvement of neighboring neurons in synchronized neuron activity.

Keywords: intraocular neurotransplants, septum, epileptiform activity, ultrastructure, synapse, astrocyte process, morphometry.

Changes in the structure and function of nerve and glial cells in epileptic brain activity have been quite well studied. Epilepsy involves the death of neurons, impairments to the domain organization of astrocytes, reactive astrogliosis, and significant increases in intermediate gliofilaments. Surviving neurons show reorganization of nerve processes: aberrant axon branching, loss of dendritic spines, and degeneration of synaptic terminals [1–3]. However, these studies are usually carried out on parts of the brain already profoundly altered by convulsive activity due to the actions of chemical convulsants or electrical stimuli. Questions of submicroscopic abnormalities in nervous tissue during initiation of epileptic states remain unstudied. In addition, in the whole brain it is difficult to identify the epileptogenic foci from which pathological excitation spreads. Many data on the generation and prevention of convulsive activity at the cellular level have therefore been obtained by modeling of pathological foci in vitro [4–7]. In the present study, the model for investigating the cellular and subcellular mechanisms of the development of epileptogenic foci consisted of intraocular neurotransplants developing in the anterior

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chamber of the eye. Neurons in transplants functioning in isolation from the CNS in conditions or a stringent deficit of natural afferent and efferent influences form autosynaptic connections of atypical contacts with neighboring nerve cells. Electrophysiological studies of analogous intraocular neurotransplants of the hippocampus and septal area of the brain show increased excitability and easy provocation of epileptiform discharges [8]. However, in the hippocampus, which has a densely packed layer of pyramidal neurons, epileptiform synchronous activity can be due to connexin-containing gap junctions between nerve cells without involvement of glutamate-mediated excitation [9]. To concentrate attention on synaptic processes and to exclude the possibility of generating pathological activity as a result of electrotonic communications between closely located nerve cells, we selected transplants of the septal area as our experimental model; neurons in this structure are located diffusely and do not form dense layers.

The main excitatory neurotransmitter in the septal area of the brain, as in most other sections of the CNS, is glutamic acid [10], while the morphological correlates of excitation are type I synapses, which have asymmetrical active zones with marked electron densities on the postsynaptic side [11]. Current concepts hold that excitatory synaptic complexes must also include surrounding astrocyte processes, which control the extracellular glutamate level [12–14]. The aim of the present work was to compare the ultrastructural organization of synaptic contacts in intraocular septal transplants displaying normal or epileptiform activity. This was approached by morphometric analysis of the tripartite organization of excitatory synaptic terminals in intraocular neurotransplants of the septum.

Methods. Intraocular transplantation into the anterior chamber of the eye was carried out using Wistar laboratory rats kept in standard institute animal house conditions. Experiments were performed in compliance with the requirements for working with animals (directive 2010/63/EU) and the recommendations of the Bioethics Committee of the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences. Donor material was obtained using females on day 17 of pregnancy, which underwent Caesarian section under deep Nembutal anesthesia and additional local anesthesia with 2.0% novocaine solution, with harvesting of fetuses, which were placed in sterile physiological saline. A binocular microscope was used to collect fragments of the septal area of the brain, and these were kept in Eagle's medium prior to implantation into the anterior chamber of the eye. Recipients were male rats of the same strain, anesthetized with ether vapor. Atropine (1.0%) drops were applied to recipients' eyes 15 min before surgery to dilate the pupil; 1–2 drops of dicaine were also applied for local anesthesia. Implantation of fragments of donor tissue into the anterior chamber of the eye was performed through small incisions in the cornea using a Microman pipette.

At three months after surgery, transplants, along with pieces of iris, were extracted from the anterior chamber of the eye and placed in the experimental chamber with a flow of Ringer–Krebs solution for testing electrophysiological properties. Electrical stimulation was applied using single (no more than 10) pulses, using nichrome electrodes at the junction of the transplant and the iris. Local potentials and multicellular activity were recorded using tungsten microelectrodes. Test results were used to divide transplants into two groups depending on the type of activity – normal (controls) and epileptiform. In the control group (n = 3), responses consisted of single evoked potentials with latent periods of 3–7 msec; in the experimental group (n = 3), responses consisted of repeated multiple discharges following with intervals of 5–20 msec. Each experiment lasted no more than 5–7 min. electrophysiological testing of transplants was performed by A. G. Bragin.

Microscopic studies were performed after fixation of transplants in 2.5% glutaraldehyde. General studies were carried out on histological sections stained with cresyl violet by the Nissl method. Transplants for electron microscopy were cut into pieces of no more than 0.8–1.0 mm^3, post-fixed in 1.0% osmium tetroxide, and embedded in Epon blocks; ultrathin sections were cut on an LKB ultratome (Switzerland). Sample preparation has been described in detail elsewhere [15, 16]. Ultrastructural studies were conducted using a JEOL JEM 100B electron microscope (Japan). Morphometric analysis was performed by selecting from each group at least 100 microimages of synaptic terminals, mainly located on dendritic spines and having clear ultrastructural signs of excitatory contacts: asymmetrical active zones with clear densities on the postsynaptic side [11]. Microphotographs were digitized and stored as computer files and analyzed using UTHSCSA Image Tool software. Synapses were compared using the following parameters: total perimeter and cross-sectional areas of presynaptic terminals (T), extent of astrocyte dendrites adjacent to terminals (A), and length of postsynaptic density (PSD). The extent of development of the perisynaptic glia (Ka) was computed as the ratio of the length of the astrocyte process (A) to the perimeter of the terminal (T). significant differences were identified using Student's t test.

Results and discussion. Histological sections of intraocular septal transplants consisted of cellular formations located in the anterior chamber of the eye between the cornea and the iris. Large blood vessels grew from the iris into the neurotransplant, with wide perivascular spaces (Fig. 1). Previous studies have shown that in the depth of the transplanted tissue the vessel walls become thinner and gradually acquire the morphological features typical of the CNS capillaries forming the blood-brain barrier [15]. Nerve and glial cells in septal transplants were distributed diffusely, without any particular orientation. Neurons were completely differentiated, had large, light nuclei with intensely stain-

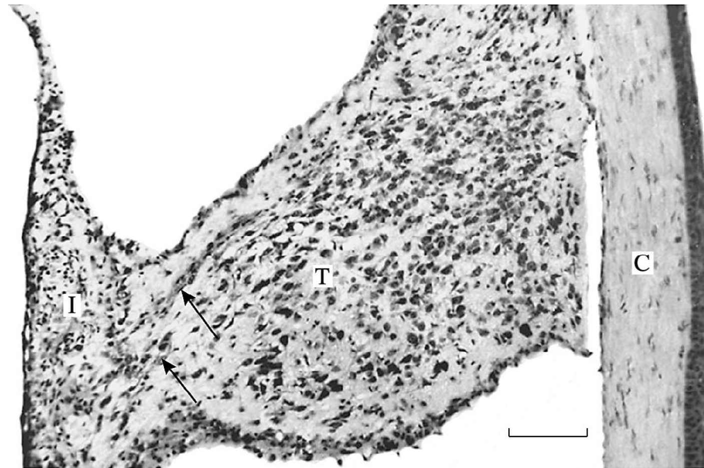

Fig. 1. General view of intraocular transplant of septal area of the rat brain. Nissl staining. T – transplant; I –iris; C – cornea. Scale bar: 1.0 mm.

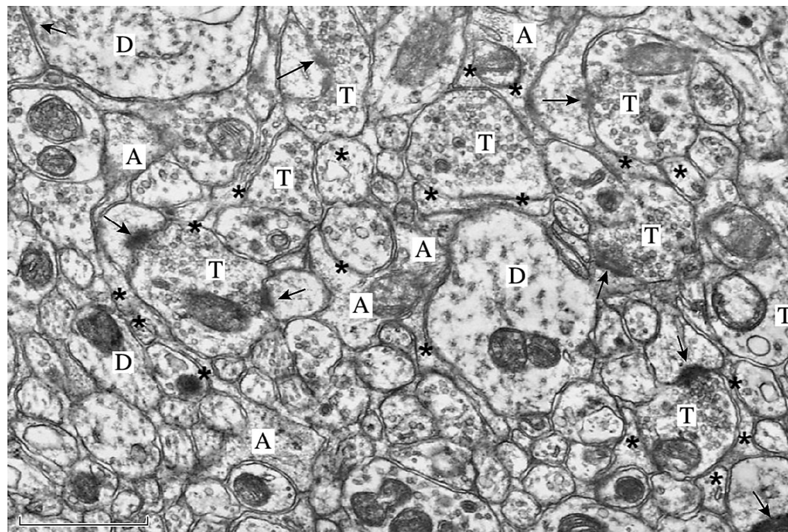

Fig. 2. The neuropil area of an intraocular septal transplant. Excitatory synaptic contacts are shown by arrows; fine perisynaptic astrocyte process are shown by asterisks. A – Larger astrocyte processes; D – dendrites; T – synaptic terminals. Scale bar: 0.5 μm.

ing nucleoli and extensive cytoplasm containing clumps of Nissl substance.

In terms of ultrastructural characteristics, neurons and gliocytes also corresponded to fully fledged mature cells, whose cytoplasm contained all the necessary organelles. The neuropil areas of transplants, with their complex organization, consisted of mutually interconnected glial processes, axons, dendrites, and synaptic terminals. The glial component consisted of astrocyte processes, some containing gliofilaments. Most synaptic junctions in the neuropil had clear signs of excitatory contacts: light synaptic vesicles of diameter 35–40 nm were present on the presynaptic side, while marked accumulations of electron-dense material were present on the postsynaptic side beneath the plasma membrane (Fig. 2). Starting with the classical studies of Gray [11], carried out using neocortex, these structural characteristics of synapses in other CNS organs were also associated with glutamatergic neurotransmission [17, 18]. Synaptic contacts of this type were seen in intraocular septal neurotransplants from both experimental groups, both on dendritic spines and on fine dendrite branches. At the visual level, there were no fundamental structural differences between the numerous synaptic connections in transplants with the normal and pathological types of functional activity. Both groups of neurotransplants contained synapses with relatively large and quite small presynaptic boutons. At the same time, epitransplants often showed numerous active zones where 2–3 terminals abutted onto a single spine and, conversely, 2–3 dendrite branches formed synaptic connections with a single axonal bouton or the head of a dendritic spine. In addition,

TABLE 1. Morphometric Data for Excitatory Synapses in Intraocular Neurotransplants with Different Types of Functional Activity ($M \pm m$)

Synaptic terminal parameters	Normal activity (control), $n = 100$	Epileptiform activity ($n = 112$)	Significance
Extent of position density, μm	0.366 ± 0.014	0.402 ± 0.018	Not significant
Area of presynaptic bouton, μm^2	0.52 ± 0.03	0.49 ± 0.03	
Perimeter of presynaptic bouton, μm	2.14 ± 0.09	2.13 ± 0.07	
Astrocyte processes in contact with presynaptic bouton, μm	0.96 ± 0.06	0.55 ± 0.05	$p < 0.001$
Coefficient Ka	0.45 ± 0.03	0.25 ± 0.02	

many synaptic contacts within them had active zones of the perforated type. These distinguishing features of transplanted tissue characterized by pathological activity are consistent with electrophysiological data showing hyperactivity in neurons developing in the anterior chamber of the eye [8]. Other investigators have also shown that active zones with perforations of the postsynaptic density expressed more excitatory receptors and were more efficient [19, 20].

Presynaptic glial processes surrounding synaptic profiles to different extents had tortuous outlines and seemed to fill the space between nerve elements, not reaching synaptic clefts. Fine processes did not contain cytological organelles but were filled with some kind of filamentous material. The distances between synapse and astrocyte membranes varied from short nearby parts to areas widened to several tens of nanometers (Fig. 2). On the presynapse side, the area of contact with the astrocyte process often showed micropinocytic figures and empty vesicles. These observations provide grounds for the view that synapses in intraocular transplants, as in the brain in situ, correspond to the current concept of the tripartite organization of synaptic contacts. The numbers of synapses in different parts of the brain making tight contact with astrocyte processes are known to differ [21]. Our comparison of the two groups of intraocular neurotransplants showed a decrease in the number of synapses associated with astrocyte processes. Thus, while these were seen in the control material in 94.0 ± 2.5% of cases, the proportion in tissue displaying epileptiform activity was only 72.3 ± 4.2% (significant difference, $p < 0.001$). This is evidence of the important role of astrocytes in maintaining the normal level of functional activity in transplanted tissue.

Comparative morphometric study of synaptic complexes in neurotransplants with normal and epileptiform activity addressed all three structural components of tripartite synapses. Assuming that the increased excitability of nervous tissue must be reflected in the sizes of synapse active zones, the extents of postsynaptic densities were measured, these being key loci containing neurotransmitter receptor molecules. The sizes of postsynaptic densities were very variable in both experimental groups (from 0.1 to 1.28 μm) and generally showed a positive correlation with the sizes of presynaptic boutons. However, comparison of the mean values of these indicators in transplants with normal and pathological activity revealed significant differences, though there was also a minor tendency to an increase in postsynaptic densities in epileptized tissues (Table 1). In combination with the ultrastructural signs of greater efficiency described above for some synaptic contacts (numerous active zones, perforated PSD), epitransplants nonetheless contained morphological grounds for the generation of anomalous activity. Morphometric analysis of presynaptic boutons in terms of cross-sectional area and perimeter in both experimental groups revealed no significant differences. In addition, larger differences were seen in the third, i.e., astrocyte, component of synaptic complexes. In material with epiactivity, the proportion of astrocyte processes in the immediate vicinity of presynaptic boutons (Ka) was 1.8 times lower than in controls (Table 1).

The numerical data obtained here indicate that the action of short-term electrical stimulation, which induces pathological discharges in some intraocular transplants, had virtually no effect on the morphometric parameters of the presynaptic compartments or sizes of postsynaptic densities of synaptic terminals. In addition, data on the significantly reduced surrounding axon terminals of astrocyte processes in transplants with epileptiform activity was very impressive. Fine terminal branches of astroglial processes constitute an important component of the neuropil and were in close contact with synaptic terminals and take part in modulating glutamatergic synaptic transmission. Only 20% of glutamate released from synaptic vesicles reaches postsynaptic neurons, the remaining 80% of neurotransmitter being returned to the astrocyte cytoplasm by highly specific astroglial membrane transporters to be converted into inactive glutamine. This molecular-cellular mechanism for the uptake of excess glutamic acid from synaptic clefts prevents hyperexcitation and excitotoxicity of neurons [22, 23]. Astrocytes also control ion homeostasis close to functional contacts. They utilize membrane transporters to clear the extrasynaptic space of potassium ions and redistribute them to sites with low concentrations [24, 25]. Astrocytic perisynaptic processes are very plastic and react to changes in neuron activity. Thus, sensory stimulation and long-term potentiation lead to significant increases in the perisynaptic glia on activated synapses in the neocortex and hippocampus [26, 27]. Our experiments, conversely, demonstrated weakening of the astrocyte component around synapses in

transplants with epileptiform activity. As compared with controls, decreases in the astrocyte sheath of excitatory synapses allow neurotransmitter freer diffusion from the synaptic cleft into the intercellular space, with activation of receptors on neighboring synapses. This leads to synchronization of the activity of groups of neurons and further propagation of the focus of epileptiform activity. This suggestion is consistent with current views that astrocyte regulation of synaptic transmission occurs not only on individual synapses, but also leads to changes in activity at the level of neural networks [28]. The important role of diffuse (volumic) propagation of signal molecules across intercellular spaces and the modulation of information processes in the brain have been addressed in other studies. [29, 30].

Finally, additional factors promoting the generation of pathological discharges operate in intraocular nervous tissue neurotransplants. For example, our previous experiments revealed significant decreases in the numbers of inhibitory GABAergic neurons in in oculo neocortex [31]. The transplants from the septal area of the brain studied here may also have abnormalities in the phenotypic differentiation of nerve cells, though in this case the pathological imbalance between excitation and inhibition should be seen both in controls and epitransplants. Considering the short duration of exposure of the transplanted tissue to electrical pulses, we suggest that perisynaptic astrocyte processes are the first to react to increases in neuronal excitation and initiate the development of epileptiform activity.

The authors would like to thank A. G. Bragin for electrophysiological testing of the type of functional activity in intraocular neurotransplants.

This study was supported by the Russian Foundation for Basic Research (Project No. 17-04-00786).

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