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Transsynaptic Coordination of the Formation of Morphofunctional Contacts between the Brain and the Neurotransplant: an Ulrastructural Study

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We studied the role of neurotransmitter signaling mediated by synaptic vesicles in the formation of aberrant functional connections between fascia dentata grafts and the somatosensory neocortex in adult rats. Quantitative analysis of the different populations of synaptic vesicles in the ectopic giant axonal endings of granular neurons was performed and the results were compared with the normal. Two pools of small clear vesicles (rapidly releasable pool and pool of reserve vesicles circulating in the active zone) and one pool of large dense-core vesicles were analyzed. Significant differences from the control suggest that synaptic integration of the transplants into the recipient brain is coordinated by transsynaptic signaling and mediated by different populations of synaptic vesicles.

Key Words: *neurotransplant; fascia dentata; neocortex; pool of the synaptic vesicles; ultrastructure*

Neurotransplantation is a promising therapeutic approach to repair of the damaged brain in traumas and neurodegenerative diseases. Transplanted neural precursors differentiate, form functional connections in the transplants, and project axons to the surrounding brain tissue of the recipient [1,9,15]. During embryonic and postnatal development, the growth of axons of the immature neurons towards their specific targets is guided by a combination of attractive and repulsive factors. Temporal-spatial pattern of activities of various exogenous morphogenes during the ontogeny is well studied. Transsynaptic protein complexes involved in the initiation of the synaptic connections have been identified [6,11]. However, the cellular and molecular mechanisms modulating the late stages of synaptogenesis and especially on the formation of synaptic contacts between the immature transplanted cells and mature brain remain unknown. The understanding of these mechanisms is essential for understanding brain tissue regeneration after neurotransplantation.

Here we studied organization of synaptic vesicle populations providing transsynaptic signaling and formation of functional connections between hippocampal fascia dentata grafts and neurons of the somatosensory area of rat neocortex.

This donor structure was chosen because axons of granular neuron in the fascia dentata (mossy fibres) form giant synaptic terminals with a peculiar specific intraterminal formation of the synaptic contacts that can be identified by electron microscopy. Along with unique morphological characteristics, these synaptic endings have complex neurochemical composition and contain excitatory and inhibitory transmitters, endogenous neuropeptides, zinc ions, and specific complexes

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of the adhesion molecules [4,8,10,14]. It is important to note, that different metabolites are associated with specific subsynaptic organelles. This allows useing giant synaptic endings of the hippocampal formation as a microscopic object for evaluation of structural and chemical mechanisms involved in adaptation of axons of transplanted neurons to neuronal targets in the recipient brain.

MATERIALS AND METHODS

Experiments on allotransplantation were performed on Wistar rats; all manipulations were performed with strict adherence to Regulations on Animals Experiments (according to GOST R ICO 10993-2-2009). Extraction of 20-day-old fetuses from the uterus of donor animals and transplantation of fascia dentate fragments to recipient mature male rats (n=7) were performed under nembutal anesthesia (40 mg/kg intraperitoneally) and local anesthesia (2.0% novocaine subcutaneously). Embryonic fascia dentata anlage was isolated under a stereomicroscope and transferred in sterile saline until transplantation. Recipient rats were fixed in stereotaxis, the skull over somatosensory area of the neocortex was drilled (d=2-3 mm), the required volume of the brain tissue was aspirated using a vacuum pump and replaced with a fragment of donor brain.

In 5 months, the animals were narcotized with nembutal and the brain was perfused with saline and then with 2.5% glutaraldehyde in phosphate buffer. The fragments containing the transplant and adjacent brain tissue were isolated, postfixed in 1.0% OsO_4, and processed routinely for electron microscopy. The spot for electron microscopy (transplant or recipient neocortex) was preliminary chosen on semithin sections under a light microscope. For comparison of giant synaptic terminals formed after transplantation with those in the contralateral hemisphere *in situ*, a hippocampal layer containing endings of axons of granular neurons was isolated and analyzed. Ultrastructural study was performed under a JEM-100B electron microscope.

For quantitative analysis, at least 50 randomly chosen microphotographs of giant synapses from experimental and control samples were used. Figure 1 (fragment a) shows a scheme of a giant synaptic ending of granular neuron in the fascia dentata with multiple active axonal spiny zones. Small (40 nm in diameter) clear synaptic vesicles concentrated around the active zones were counted. In view of the data on functional and chemical heterogeneity of these ve-

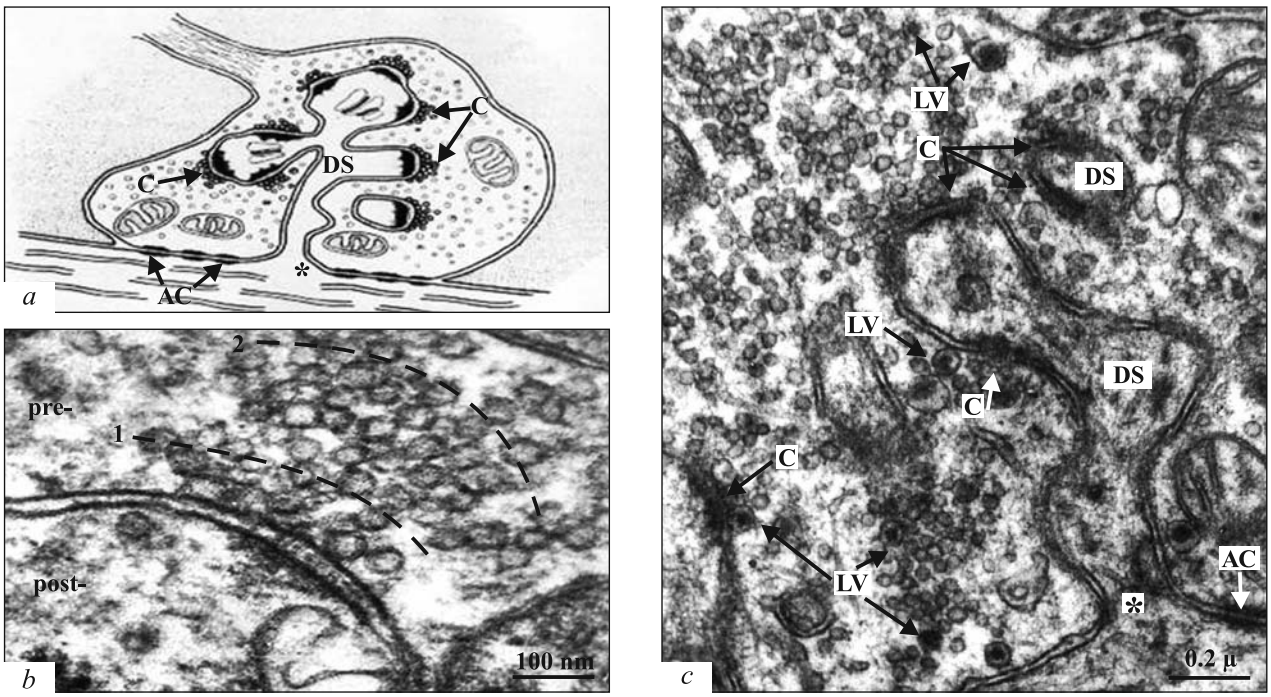

Fig. 1. Giant synaptic terminals of axons of granular neurons of the fascia dentata formed in the recipient brain after transplantation of embryonic fascia dentata anlage into somatosensory cortex of rats. *a*) scheme: giant axonal terminals forms multiple intraterminal active zones with knobs of invaginated dendritic spine and adhesion contacts with dendrite surface. *b*) axon-spine synaptic contact. *1*) Line demarkating pool I rapidly releasable vesicles; *2*) line demarkating reserve pool of vesicles (pool II); *c*) ultrastructure of fragment of giant ectopic synaptic ending containing small clear vesicles and large dense-core vesicles (pool III). Here and in Fig. 2: pre: presynaptic component of the synapse; post: postsynaptic component of the synapse; C: intrateminal asymmetric synaptic contacts with knobs of dendritic spines; AC: adhesion contacts between axonal terminals and dendrite surface; DS: dendritic spines; LV: large vesicles with electron-dense core; asterisks show the sites of derivation of dendritic spine from the dendrite.

sicles [5,7], the population was divided into 2 pools: pool I comprised docked vesicles, *i.e.* group of rapidly releasable vesicles located at a distance of 80 nm from the membrane; pool II consisted of vesicles located farther from the synaptic membrane (80-200 nm) and constituting a reserve or circulating pool (Fig. 1, *b*). The percentage of the large (up to 100 nm in diameter) electron-dense vesicles (pool III) from the total population of synaptic vesicles in the terminal was also estimated (Fig. 1, *c*).

Significance of differences was analyzed using the Student's *t* test.

RESULTS

Neurotransplants anatomically integrated into the recipient brain were found in all operated animals. Preliminary histological analysis showed that they contained differentiated granular neurons, most of them were arranged in a dense layer as in fascia den-

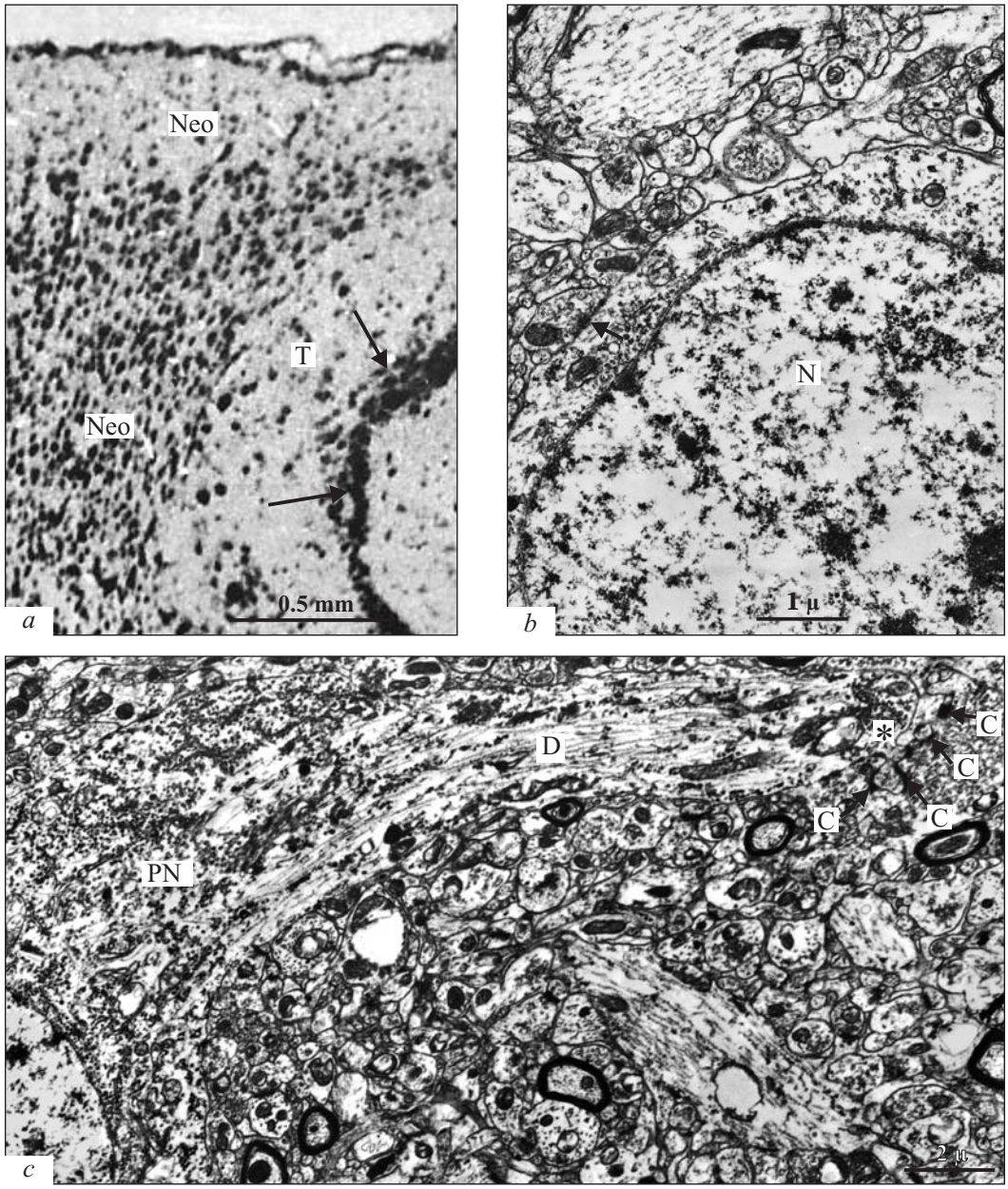

Fig. 2. Fascia dentata transplant in the recipient brain. *a*) Histological section through the transplant (T) in the site of its anatomic contact with recipient neocortex (Neo). Arrows show granular neuron layer in the neurotransplant. *b*) Ultrastructure of the granular neuron and adjacent neuropil in the transplant. N: neuronal nucleus. Axosomatic synapse is shown by an arrow. *c*) Pyramidal neuron (PN) with dendrite (D) in the recipient neocortex. Giant synaptic ending forming multiple intraterminal active zones with the knobs of branched dendritic spine is seen in the upper right part of the photograph.

tata *in situ* (Fig. 2, *a*). Electron microscopy showed that neurons in the transplants looked like granular neurons (d~9 μ) typical for this brain region and had large nucleus, thin cytoplasmic rim, and axosomatic synapses on the surface (Fig. 2, *b*). Ultarstructural features of the transplanted neurons and the possibility of growth of their axons into the surrounding neocortex were previously studied by us [1-3,15]. Here we studied synaptic connections of the transplanted neurons with unusual postsynaptic targets in the recipient brain. Among synapses of the standard size, giant (up to 4-5 μ) synaptic terminals of mossy fibers growing from the transplants were found. The axons of transplanted neurons induced the formation of branched dendrite spines with multiple active zones (Figs. 1-2, *c*). Aberrant giant synapses, similar to those *in situ*, were characterized by two types of membrane specializations: asymmetric active zones with dendrite spines and symmetric adhesion contacts with dendrite surface. Generally, the synaptic chimeras whose presynaptic structure came from transplanted neurons and postsynaptic from cell elements of the recipient neocortex were similar to those in normal brain (Fig. 1, *a*, *c*).

Presynaptic profiles of ectopic mossy fiber terminals contained numerous synaptic vesicles, though looked less densely packed in visual comparison with normal terminals. Active zones of the synapses were identified by the presence of asymmetric axon-spine contacts and compact clusters of clear small synaptic vesicles with insignificant number of large (up to 100 nm in diameter) electron-dense vesicles (Fig. 1, *c*). In the axonal system of hippocampal mossy fibers, small vesicles contain the main neurotransmitter, glutamate, while large vesicles with electron-dense core contain neuropeptide co-transmitters [4,14]. For evaluation ofthe role of different populations of synaptic vesicles in adaptation of giant synaptic terminals to their atypical targets, we compared spatial distribution of vesicles in ectopic and control synapses. The results of quantitative analysis are shown in Table 1.

The quantitative parameters of both pools of small clear vesicles, especially docked vesicles, in ectopic synapses were significantly lower than in the control. This attested to reduced glutamatergic activity of aberrant axonal connections in granular neurons of the dentate fascia. At the same time, comparison of the proportions of individual populations in the total pool of synaptic vesicles concentrated around active zones implies different functional contribution of the pools of synaptic vesicles in integration with brain transplants. The fraction of rapidly releasable vesicles in ectopic synapses was lower than in the control, which confirms lower level of synaptic transmission in aberrant nerve fibers. At the same time, the relative fraction of reserve pool vesicles in ectopic synapses was significantly higher than in the control, which indicates increasing role of reserve vesicles in adaptive restructuring of terminals to alien targets. It is known that the two pools of glutamatergic small light vesicles differ by not only readiness to transmitter release into the synaptic cleft, but also the presence of specific proteins on the membrane involved in synaptic vesicle trafficking and modulation of synaptic transmission [5,7]. Presynaptic vesicles of the reserve pool carrying synapsins on vesicular membrane differentiate first during maturation of hippocampal synapses in culture, regulate neurotransmitter release, and control short-term synaptic plasticity. The results of our quantitative analysis attest to participation of the vesicular pool in synaptogenesis and stabilization of the synaptic contacts after neurotransplantation. This assumption agrees with previously published data on instability and plasticity of functional connections of the fascia dentata transplants with the neocortex manifested in long-term restructuring of ectopic synaptic complexes [3].

Microscopic and quantitative analysis of peptide-containing large granular vesicles in the giant synaptic

TABLE 1. Quantitative Analysis of Synaptic Vesicles in Giant Axonal Terminals of Granular Neurons Formed in the Neocortex of the Recipient after Transplantation and in Control Hippocampus (*M*±*m*)

Parameter	Control hippocampus	Recipient neocortex
Number of small rapidly releasable vesicles (pool I)	15.5±1.0	10.9±0.2**
Number of reserve vesicles (pool II)	20.6±1.4	16.0±1.1*
Ratio of pool I vesicles to all vesicles in the active zone	0.432±0.005	0.402±0.007**
Ratio of pool II vesicles to all vesicles in the active zone	0.568±0.005	0.598±0.007**
Percentage of large dense-core vesicles (pool III) in the total population of vesicles	3.3±0.6	5.8±0.6*
Number of active zones containing large dense-core vesicles, %	7.9±1.6	62.3±3.4**

Note. *$p \le 0.01$, **$p \le 0.001$ in comparison with the control.

terminals revealed their involvement in the regulation of formation of the brain–transplant communications. In synaptic terminals of aberrant mossy fibers detected in the somatosensory cortex of the recipient, the percentage of peptide-containing granules from the total population of synaptic vesicles was by 1.8 times higher than in intact synapses (Table 1). Taking into account the data on the inhibitory effects of neuropeptide co-transmitters on synaptic transmission [4], this result agrees with the assumption on reduced efficiency of ectopic synapses based on the analysis of glutamatergic vesicles. Comparison of the distribution of neuropeptide-containing vesicles within the presynaptic terminal showed that the differences between the control and "chimeric" synaptic endings were even more pronounced. In axonal terminals *in situ* they were distributed more or less homogeneously and far from active zones, while in ectopic endings they tended to concentrate around the synaptic contacts. The quantitative analysis of synaptic active zones containing large vesicles with electron-dense core showed that they were more numerous (by 7.9 times) in the experimental samples than *in situ*. These data suggest that predominantly extrasynaptic neuropeptide modulation of synaptic transmission in terminals of mossy fibers *in situ* during the formation of aberrant connections is transformed into transsynaptic effects on alien receptive areas in the neurons of the recipient.

Thus, the quantitative analysis of different types of synaptic vesicles in aberrant synaptic terminals of mossy fibers revealed morphological evidence of reduced functional activity in comparison with the normal. Moreover, our findings suggest that integration of the transplants into the recipient brain is coordinated by transplanted neurons through transsynaptic neurotransmitter signaling by synaptic vesicles.

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