MORPHOFUNCTIONAL PATTERNS OF NEURONAL NETWORK DEVELOPING IN DISSOCIATED HIPPOCAMPAL CELL CULTURES

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The aim of investigation was to study the morphofunctional patterns of neuronal network developing in primary long-term hippocampal cell cultures.

Materials and Methods. The ultrastructural features of developing intercellular contacts in neuronal network formed by cultured hippocampal cells of 18 day-old mouse embryos were studied. The sequence of ultrastructural development of these contacts was compared with dynamics of functional network neuronal activity estimated by parameters of multicellular fluorescent Ca^{2+}-imaging. At the same time the changes of quantitative interrelation and positional relationship of neurons and glial cells were immunocitochemically determined.

Results. In primary hippocampal cell culture at 3rd–4th weeks *in vitro* the gradual formation of mature synaptic contacts correlates with appearence of complex Ca^{2+} neuronal network activity. In this period individual neurons form a uniform monolayer distributed among numerous glial cells. Thus the results obtained reflect morphofunctional patterns of different stages of cultural development as a biological model of neuronal network ontogenesis.

Key words: primary dissociated hippocampal cultures; neuronal networks; synaptogenesis; electron microscopy; Ca^{2+}-imaging.

The formation of neuronal networks is one of the manifestations of plasticity of the developing nervous system in health and disease, therefore the problem of morphofunctional development of brain is essential in both neurobiology and medicine. The failure of intercellular structure in ontogenesis subsequently results in the development of pathological neuronal networks underlying the changes of psychical functions of brain, the formation of epilepsy and many other diseases of the central nervous system.

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There are a number of biological models to study different aspects of cerebral functioning and structure: behavioral models *in vivo*, surviving acute brain slices, cell and tissue cultures of various CNS structures, etc. One of the most adequate models to study the features of cell-to-cell interaction and the formation of normal and/or pathological developing interneuronal and neuroglial relationships are primary dissociated neuronaql cultures, in which the activity processes of neurons and glial cells are accessible for observation and manipulations. Moreover, in low cellular density culture the study of the localization and migration of different cells in developing neuronal network is simplified. In addition, this model enables to carry out chronic experiments for a long-time period (up to a year and longer) [1, 2].

Currently, there have been thoroughly studied the features of developing hippocampal cell cultures: morphological changes of cells [3–5], synaptogenesis [6, 7], expression of specific receptor types [8], the change of patterns of the electrical activity [9]. However, since there have been performed no comprehensive researches of morphofunctional state of neuronal networks formed in long-term embryonal hippocampal cell culture, the correlations of ultrastructural changes of the developing interneuronal contacts and the dynamics of functional network activity still remains unclear.

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Hippocampal cell cultures. The study was performed onlong-term (up to 1 month) cultures of hippocampal cell from 18-day embryos of CBA mice. The basic rules of experimental animal management and care met those represented in the Order of the Ministry of Health of the Russian Federation No.708н, dated August 23, 2010, “Approval of laboratory practice rules”. Hippocampi were mechanically reduced to small fragments and treated by 0.25% trypsin (Invitrogen, USA) for cell dissociation. Cell suspension obtained by enzymatic and mechanical dissociation was applied on cover glasses (40 mcL per a glass) covered by supporting substrate (polyethylenimine, Sigma, USA). The glasses were put into Petri dishes 35 mm in diameter. In each dish there: 1 ml of nutrient medium Neurobasal™ (Invitrogen) was added. This medium contained 2% of biologically active supplement B27 (Invitrogen), 2 мМ of L-glutamine (Invitrogen) and 10% of embryonic calf serum (PanEco, Russia). In 1 day, the medium with less serum content (0.5%) for further cultivation was used and this medium was changed in every 2 days. The cultures were incubated in CO_2-incubator (5% CO_2, 95% air) at 35.5°C. The cultures on 5, 7, 10, 14, 21 and 30 day *in vitro* (DIV) were used in experiments.

Calcium imaging. The functional activity was monitoring by measuring the changes of intracellular calcium concentration ($[Ca^{2+}]_i$) using specific calcium probe Oregon Green BAPTA-1 AM (Invitrogen, USA) and confocal laser microscope Zeiss LSM 510 (Germany). The changes of fluorescence intensivity in response to $[Ca^{2+}]_i$ increase were recorded at image frequency of 4 s^{-1}. According to the obtained image series, we constructed graphs of time dependence of fluorescence intensity for every cell in field of vision (Fig. 1), determined the frequency and duration of Ca^{2+}-oscillations, as well as the number of spontaneously oscillating cells. We revealed the nature of oscillating cells by the configurations of Ca^{2+}-oscillations using MATlab-based software that enabled to analyze further dynamics of intracellular calcium in neurons only.

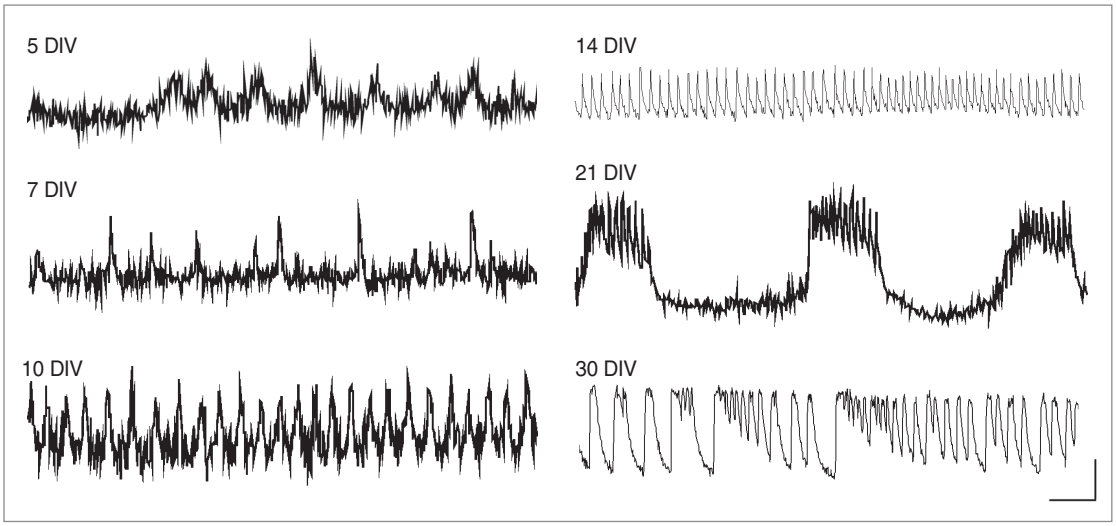

Fig. 1. Graphs of absolute fluorescence intensity changes of Oregon Green BARTA-1 AM in time at different stages of development; horizontal scale — 20 s, vertical scale — 20 relative units

Immunocytochemistry. For cytological characteristics and determination of qualitative and quantitative culture cell composition, the cultures were fixed in ice methanol, and glial cells were traced immunocytochemically by glial fibrillary acidic protein (GFAP), whereas neurons were identified by nuclear protein NeuN (both Invitrogen). Primary antibodies (Abcam) with GFAP were visualized using secondary antichicken antibodies bound to fluorescent marker Cy5 (Millipor, USA) (maximum value of absorption factor at wavelength of 650 nm, fluorescence 670 nm). To visualize primary antibodies to nuclear protein NeuN we used secondary antimouse antibodies with fluorescent marker Alexa 430 (Invitrogen) (maximum value of absorption factor at wavelength of 434 nm, fluorescence 539 nm). The study were carried out using confocal microscope Zeiss LSM 510. The neuron-glia ratio was calculated as the ratio between the number of neurons and the percentage of glial area. The values were represented in relative units per mm^2.

Electron microscopy. For ultrastructural investigations, the cultures were successively fixed in glutar aldehyde (2.5%) on phosphate buffer (pH=7.4) and in osmium tetroxide (1%), dehydrated in absolute alcohol, treated by the mixture of absolute alcohol and EPON-812 without catalyst (1:1) within 1 h, and placed into EPON-812 without catalyst for 10 h and, finally, in EPON-812 with catalyst (42.7% Epon-812, 43.2% DDSA, 14.2% MNA, 2% DMP). Ultrathin sections were prepared on ultratome ULTRACUT (Reichert-Jung, Austria), stained with uranyl acetate and lead citrate and investigated in electron microscope Morgagni 268D (FIE, Netherlands).

Statistical analysis. The findings of the fluorescence changes of stain Oregon Green BAPTA-1 AM reflecting the dynamics of $[Ca^{2+}]_i$ (frequency and duration of Ca^{2+}-oscillations) were represented as the mean (M) ± standard error of mean (m). The reliability of statistical differences of the samplings was determined by nonparametric Mann–Whitney test. The differences were considered statistically significant in $p<0.05$.

Results. Immunocytochemical study revealed the changes of mutual alignment and quantitative relation of neurons and glial cells in the process of culture development. On day 5 *in vitro* (DIV) in the depth of neuronal monolayer not numerous differentiated glial cells with simple processes

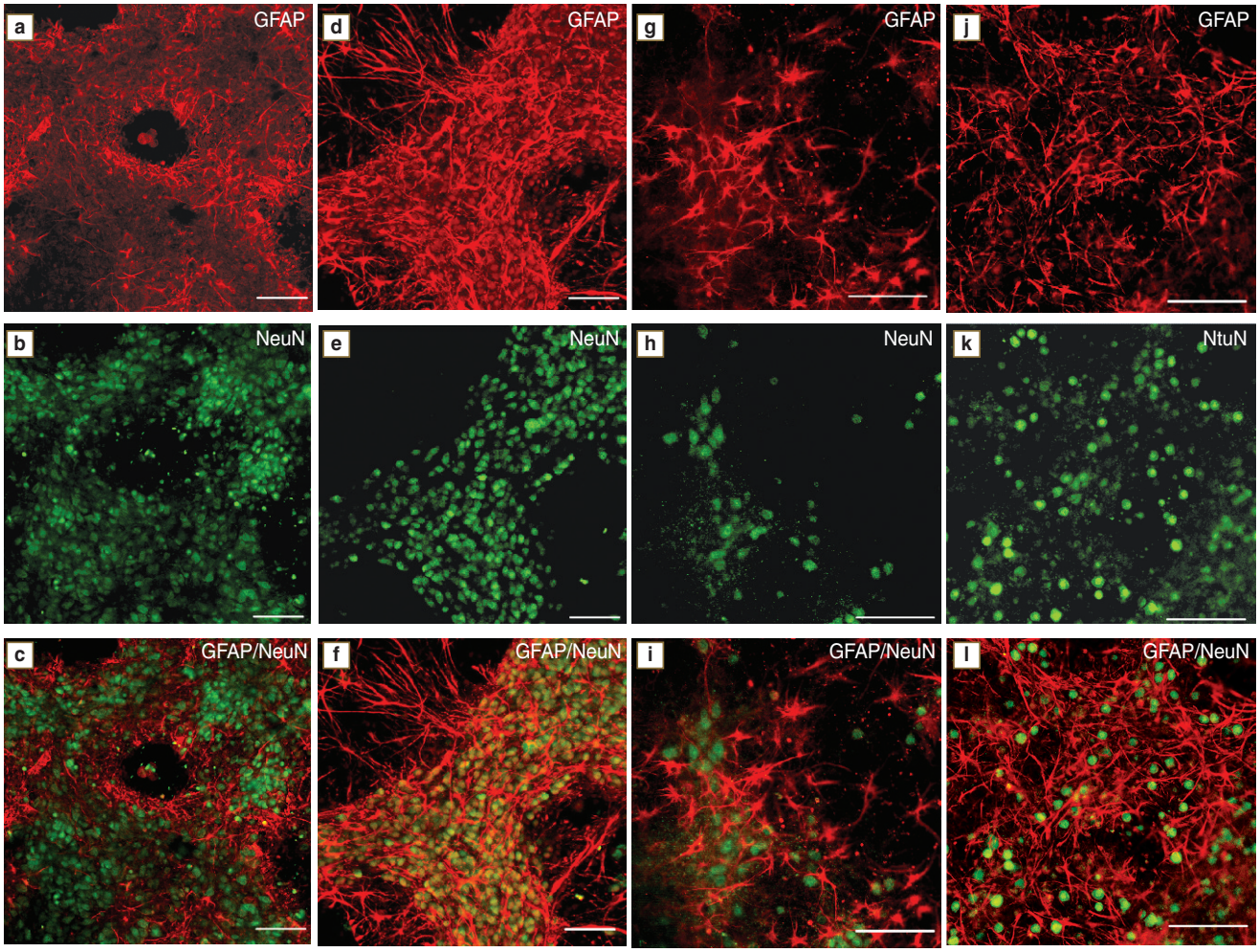

Fig. 2. Fluorescent images of dissociated hyppocampal structures on 5 (*a, b, c*), 10 (*d, e, f*), 14 (*g, h, i*) and 21 (*j, k, l*) day of development *in vitro*. Glial cells are marked by primary antibodies to GFAP (*a, d, g, j*), neuronal nuclei — antibodies to nuclear protein NeuN (*b, e, i, k*); scale — 50 μm

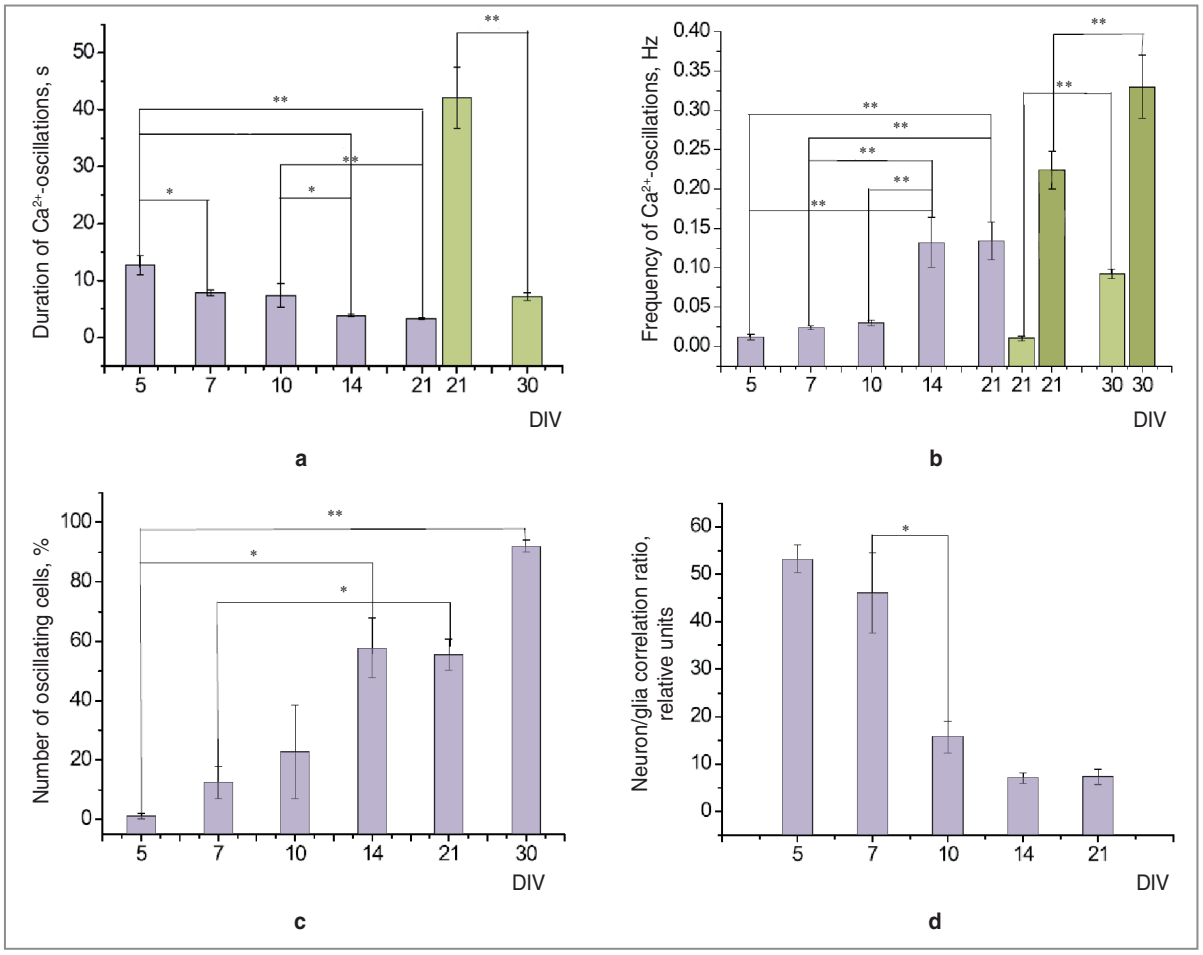

Fig. 3. Dependence diagram of duration (*a*) and frequency (*b*) at different stages of development of hippocampal cultures *in vitro*: lilac — mean value for oscillations, light green — for superoscillations, dark green — for oscillations inside "superoscillations"; *c* — the number of working cells, and *d* — a neuron/glia correlation ratio at different time intervals of development of dense hippocampal cultures *in vitro*; * — $p<0.05$; ** — $p<0.001$

(Fig. 2, *a–c*) were identified. By day 10 the neurons did not already form a continuous monolayer, but spread among many glial elements with processes of more irregular form (Fig. 2, *d–f*). On day 14 and 21 in the cultures glial cells were prevailing, and single neurons were looked as a sparse thin monolayer (Fig. 2, *g–l*).

First Ca^{2+}-oscillations reflecting the changes $[Ca^{2+}]_i$ were recorded on 5 DIV, though only in 1% of neurons (Fig. 3, *c*), and were characterized by low frequency (0.012±0.003 Hz) and longer duration (12.0±1.64 s) (Fig. 3, *a, b*). On day 7–10 the oscillation frequency slightly increased (up to 0.030±0.007 Hz), and their duration decreased up to 8.0±0.55 s. The 14 DIV was characterized by significant falling of oscillation duration (up to 3.7±0.2 s) and the increase of their frequency up to 0.14±0.03 Hz. The number of neurons with spontaneous oscillations increased up to 54% (Fig. 3, *a, b, c*). On the 21 DIV Ca^{2+}-oscillations did not differ statistically in duration and frequency from the 14 DIV (Fig. 3, *a, b*), however, their pattern changed: among spontaneously occurring single changes $[Ca^{2+}]_i$ with frequency of 0.17±0.02 Hz and duration of 3.40±0.17 s, there appeared "superoscillations" with duration of 42.0±5.36 s and frequency — 0.010±0.003 Hz consisting of a large number of single oscillations with frequency of 0.20±0.02 Hz.

By day 30 only "superoscillations" were observed and they differed in its pattern from those observed on day 21 DIV: their duration decreased up to 7.0±0.65 s, and frequency increased up to 0.09±0.006 Hz along with the frequency increase of single Ca^{2+}-signals within "superoscillations" up to 0.32±0.04 Hz. Moreover, the total number of active neurons on 30 DIV increased up to 92 %.

Ultrastructural analysis of 5 DIV cultures revealed in neuropil vacant postsynaptic densities and a large amount of immature (desmosome-like, gap-junctional and symmetric) non-vesicular contacts forming atypical for mature brain, dendro-dendritic (Fig. 4, *a, b*), somato-somatic (Fig. 4, *c*) and somato-dendritic (Fig. 4, *d*) contacts which able to transmit electrical signals in this period of synapse development. On 7 DIV desmosomes localized along with symmetric or asymmetric junctions, formed mixed contacts (Fig. 5) representing an intermediate stage of development of a chemical synapse. In this period of culture development, the first mature synapses were both asymmetric axo-dendritic (Fig. 5, *b*), and symmetric including axo-spinous (Fig. 5, *d*), in which presynaptic terminals contained

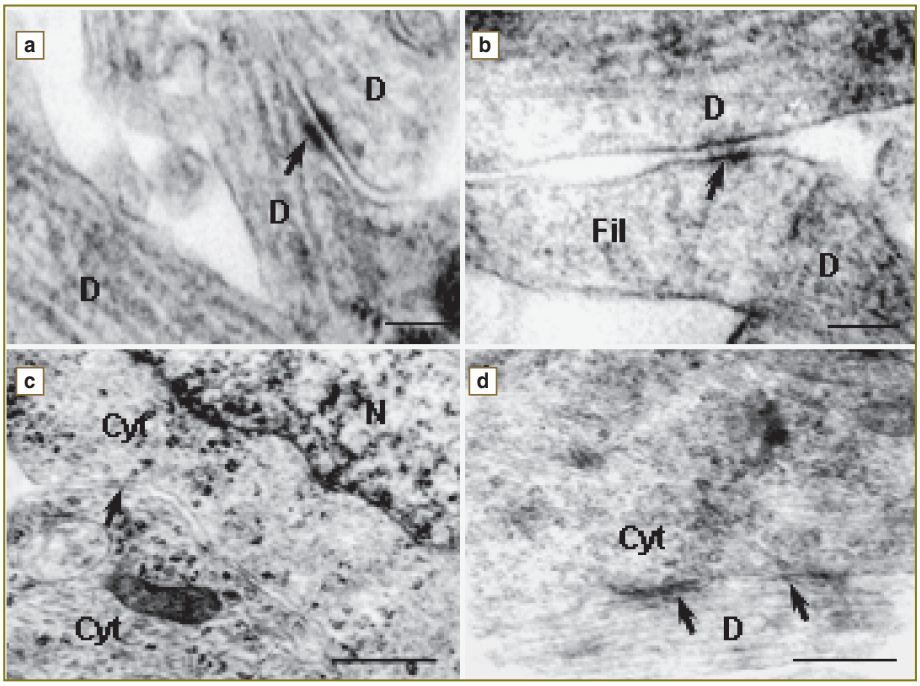

Fig. 4. Ultrastructure of hippocampal cell culture, 5 DIV. Desmosome-like dendrodendritic (*a, b*), somatosomatic (*c*) and somatodendritic (*d*) avesicular contacts (arrows). Ax — axon; D — dendrite; Fil — filopodium; Cyt — cytoplasm; N — nucleus; SpA — spinous apparatus; Sp — spine; sv — synaptic vesicles; Scale 1 μm

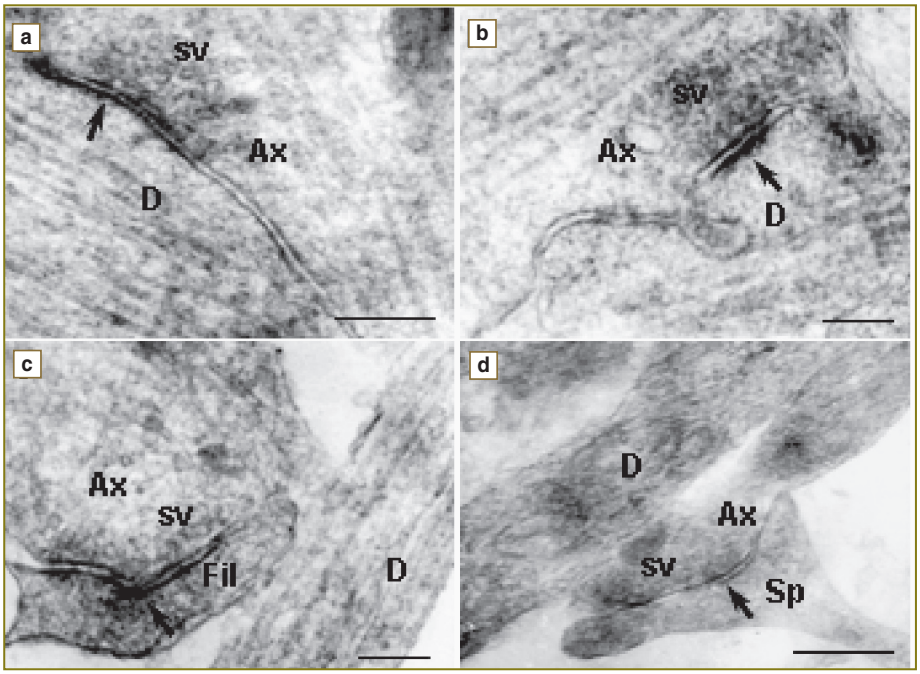

Fig. 5. Ultrastructure of of hippocampal cell culture, 7 DIV. Mixed axodendritic (*a, c*), symmetric axodendritic (*b*) and symmetric axospinous (*d*) vesicular synaptic contacts (arrows). Conventional signs: See Fig. 4. Scale 1 μm

numerous synaptic vesicles. After 2 weeks *in vitro* mixed contacts were still present in neuropil (Fig. 6, *a*), though typical symmetric (inhibitory) axo-somatic (Fig. 6, *c*) and numerous asymmetric (excitatory) mature synapses were appeared, the main population of them consisting of axo-spinous contacts (Fig. 6, *d*). Moreover, some perforated synapses were formed (Fig. 6, *b, d*), to improve the efficiency of neurotransmission. From 21 to 30 DIV, some complex perforated, divergent and convergent contacts (Fig. 7, *c, d*), as well asthe elements of spine apparatus in spines (Fig. 7, *a, b, d*) were found.

It should be emphasized that for the first three weeks *in vitro* the number of interneuronal contacts in neuropil containing formed postsynaptic density was eventually increased indicating the intensification of the processes of morphofunctional synapse differentiation.

Discussion. The characteristic feature of the vertebrate central nervous system development is the initial formation of excess amount of neurons, a part of which (over 50%) dies in the ontogenesis [10]. The same pattern was observed in the development of hippocampal embryonal cells *in vitro* (See Fig. 2). In the first days many neurons formed non-synaptic contacts providing morphofunctional stabilization of neuronal network at early stages of its

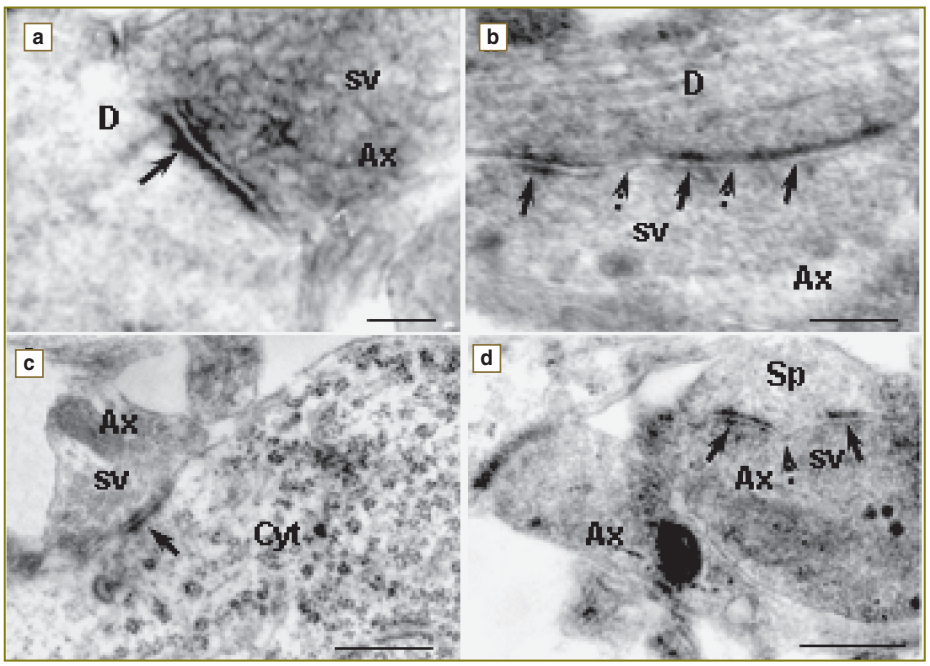

Fig. 6. Ultrastructure of of hippocampal cell culture, 14 DIV. Mixed axodendritic (*a*) and axosomatic (*c*), perforated axodendritic (*b*) and axospinous (*d*) synapses (solid arrows). Dotted arrows are perforations. Conventional signs: See Fig. 4. Scale 1 μm

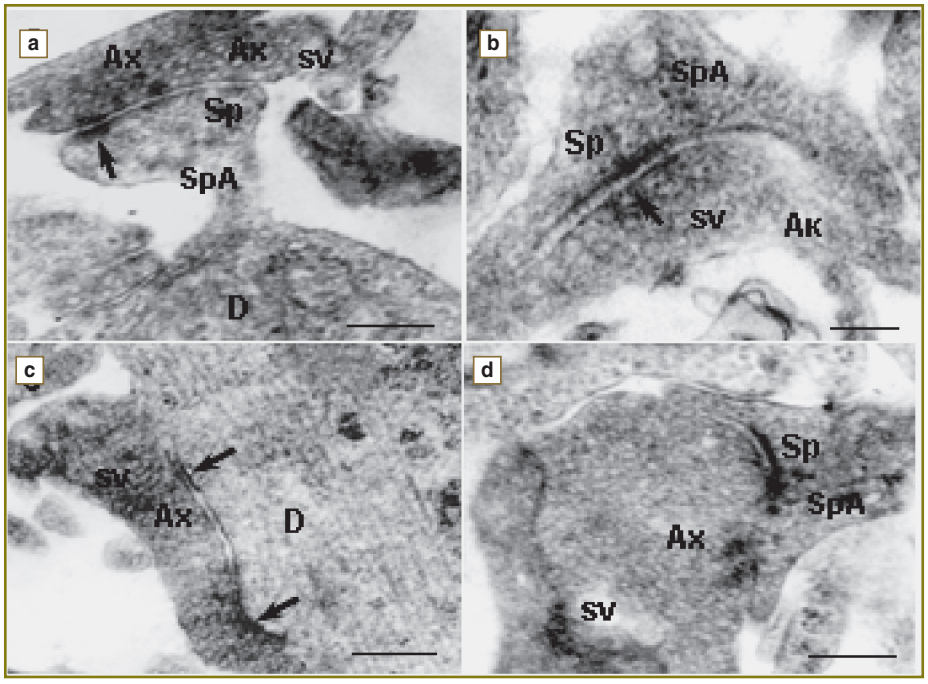

Fig. 7. Ultrastructure of hippocampal cell culture, 21 (*a*), 25 (*c, d*) and 30 (*b*) DIV. Axospinous (*a, b, d*) and perforated axodendritic (*c*) synapses (arrows). Fragment *d* shows elaborated divergent synapse. Conventional signs: See Fig. 4. Scale 1 μm

development. The presence of numerous free postsynaptic density confirms the idea of the authors [11] about the availability of postsynaptic site of neuronal membrane to form a mature contact, though on the 5th DIV the network activity is almost absent, since Ca^{2+}-oscillations are recorded only in 1% of neurons. The increase of Ca^{2+}-activity on the 7th DIV appears to be associated with the formation of first chemical synapses, the appearance of which has been also mentioned by other authors [5]. It is just the period when the first burst activity in the form of sporadic spikes and synchronized spike bursts were recorded [12, 13]. On the 10th DIV immunocytochemical analysis revealed the formation of morphofunctional clusters, in which glial cells formed necessary conditions for neurons functioning that was reflected by the increase of the number of active cells up to 20% (See Fig. 3, *c*). Since the second week *in vitro* the decrease of the duration of Ca^{2+}-oscillations and the increase of their frequency coincided in time with further complication of ultrastructure of chemical synapses, among which mature axo-spinous contacts were predominated, that appears to improve the efficiency of synaptic transmission [14]. High activity of synaptic transmission is confirmed by filling of axon terminals by synaptic vesicles. 21 DIV was characterized by the appearance of calcium

"superoscillations" accompanied by the complication of bioelectrical activity of neuronal network in the form of superbursts of spikes [8, 15, 16]. In addition, at ultrastructural level practically total disappearance of immature functional contacts was observed that was in agreement with the data on synaptogenesis *in vivo* [4, 17, 18].

Thus, our morphofunctional study concerning the dynamics of neuronal network development *in vitro* correspond as a whole to general understanding of the main neurogenetic stages and indicate that hippocampal cultures can be an adequate biological model of the developing brain neuronal networks. It should be noted that the second week in vitro is characterized by stable synchronic activity, and the main synaptic population consists of mature axo-dendritic and axo-spinous contacts.

Conclusion. Dissociated hippocampal culture can be considered as an adequate biological model of brain neuronal networks. Maturation of cultured hippocampal neuronal network occurs in the period from 14 to 21 days, when the main population of excitatory synapses consists of mature axo-dendritic and axo-spinous asymmetric contacts. Functionally, the cultures at this developmental period are also characterized by stable synchronous burst activity and the ratio of neurons and glia corresponds to one in intact brain.

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