Chapter 6

Use of Cell Culture to Prove Syncytial Connection and Fusion of Neurons

O.S. Sotnikov

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/51214

1. Introduction

1.1. Discussion between neuronists and reticularists

We do not consider it possible to cast any doubts on the grounds of the Neuronal Doctrine; however, the interneuronal syncytial connection still does exist and the cytoplasmic fusion of neurons still is possible.

The reticular theory of the general syncytial cytoplasmic connection of neuronal processes as the principle of organization of the nervous system was already known to the German histologist Joseph von Gerlach [1]. It was supported by almost all neurologists of the XIX century [2] and was passionately defended by the famous Camillo Golgi.

The presence of the cytoplasmic syncytium[1] in the nervous system was defended by such known histologists as Nissl, Ranvier, Schwann, and others. The theory of nervous network had its attractive and convenient explanations [2]. By suggesting the general cytoplasmic connection of nerve fibers, it considered nervous network as anastomoses, roundabout pathways of blood vessels and allowed explaining comparatively easily the relatively fast recovery of functions in cerebral stroke. The reticular theory suggested not the discrete single, but the grouped functioning of neurons, which at present seems more realistic [3, 4]. However, this theory was not based on the most important — absolute scientific facts. The wonderful histological method invented by C. Golgi (*reazione nera*) and celebrated by S. Ramon y Cajal could not compensate low resolving power of the optic microscope. Not infrequently the superpositions of nerve processes one upon another were interpreted as their fusion and formation of network. The reticular theory had to be replaced by the Neuronal Doctrine.

[1] Here and further the term "syncytium" implies the true cytoplasmic connection of different neurons or different processes of one neuron rather than the quasisyncytium representing the interneuronal electric connection with aid of gap junction. The term "syncytium" is incorrectly borrowed by many electrophysiologists [15].

Lucky findings of mossy and climbing fibers by S. Ramon y Cajal proved convincingly the existence of nerve terminals and a possibility of individual existence of neurons. Then S. Ramon y Cajal and his supporters obtained numerous preparations in different parts of the nervous system, which convinced scientific community in the rightness of the Neuronal Doctrine of W. Waldeyer and S. Ramon y Cajal [5, 6]. Disputes of Cajal and Golgi represented one of the largest collisions of ideas in development of the scientific thought [7].

Discussion of neurohistologists was quite emotional. Santiago Ramon y Cajal thought that – hypothesis of the network is a terrible enemy, – contagion of reticularism, whereas Camillo Cajal opposed to him defiantly emotionally his views in his Nobel lecture [2].

It seemed that discovery of synapses with aid of electron microscope [8, 9] had become the absolute and last proof of victory of the Neuronal Doctrine. In most neurobiologists the erroneous opinion was formed about the absence in principle of the syncytial connection in the nervous system. However, the proof of the presence of synapses, strictly speaking, is not the proof of the absence of the syncytial interneuronal connection. This is the typical error (paralogism), a usual contrivance in discussions of sophists of the IV century B.C. [10].

All neurons have synapses (incomplete premise).
Synapse is the form of connection of neurons.
Hence, the form of connection of all neurons is synapse.

Both reticularists and neuronists assumed the exclusively one way of connection in the nervous system. Both the former and the latter in principle did not tolerate compatibility of the theories. This was characteristic both of S. Ramon y Cajal and of C. Golgi (*Neuronismo o Reticularismo*), either the neuronal or the reticular theory [11]. However, this classical approach in discussions "either – or" already at that time could have been replaced by the approach "both – and", as the third opinion already existed [12]. Some researchers adhering to the Neuronal Theory noted a possibility of the existence of the interneuronal syncytium in particular places and under some conditions [13, 14]. Even C. Golgi extended the theory of network only to organization of axonal branchings, whereas dendrites, in his opinion, terminated freely [2]. At present, in the literature there is the sufficient amount of evidences for accepting the concept and proofs of reticularists erroneous and nevertheless for claiming that the syncytial connection between neurons does exist.

2. Findings of syncytial connection in the nervous system

However, in the literature there already are the irrefutable facts of the presence in the nervous system of the true cytoplasmic syncytial interneuronal connection. After information of absolute facts of fusion of nerve processes in invertebrates [16], some authors were ready to recognize the giant neurons to be the non-nerve cells rather than to agree with facts of their syncytial connection [17]. It is impossible to ignore detection of the syncytial connection in molluscs, crustaceans, polychaetes, and other invertebrates [18-24]. All these works first of all offer absolute proofs of that the interneuronal syncytium in the nervous system in principle does exist.

Data about "fused neurons, that produce the giant axons...", are "example of a situation, that is against the strict letter of the neuron doctrine but can fit easily into the cell theory" [25]. It turns out that in nature the presence of chemical synapses in animals is quite compatible at solution of special tasks with the presence of the cytoplasmic syncytial connection. In nature the Neuronal Doctrine and structural elements of the reticular theory are compatible.

Of principal importance are the data that syncytial connections can also be formed between stumps of sectioned fiber at its regeneration by ingrowth of the central stump into the peripheral one [26-28]. By the example of formation of the earthworm giant axon, in some cases, the "Calenary theory" was even demonstrated to be true [29]; this theory was a variant of the syncytial fusion of neurons and suggested formation of nerve fibers by fusion of individual nerve cells into chains. This theory was held by one of pillars of neuronism van Gehuhten [2].

We were the first to reveal with aid of electron microscope the syncytial connection *in situ* in the piglet enteral nervous system [30]. Subsquently, this way of interneuronal connection was shown in molluscs [31, 32], in the crawfish peripheral nervous system [33], in cat autonomic ganglia [34], in rabbit hippocampus [35, 36], and in the human cerebral cortex [12, 37].

Morphology of fusion of living neurons at present has not yet been studied, and to do this, it is the most convenient with use of tissue culture.

3. Obtaining and cultivation of isolated mollusc neurons

By having obtained convincing data about the existence of interneuronal syncytial connection in invertebrates, we found it quite important to reveal such form of connection in living mollusc neurons in cell culture [38]. For this, the peripharyngeal ring of the mollusc *Lymnaea stagnalis* with all ganglia (Fig. 1) were performed in 0.4% pronase with temperature of 20°C (use of another protease is also possible). We used lyophilized pronase from *Streptomyces griseus* (Sigma). The mean molecular mass is 20000. The pronase solution was prepared on the basis of isotonic solution for molluscs.

Use of pronase for enzymatic treatment of ganglia was due to the following causes. Pronase represents a complex preparation composed of 11-14 proteases. Its hydrolytic activity is spread onto the large spectrum of proteins, peptides, amides, and amino acid esters [39]. Proteases are known to have properties of a neurotrophic factor. They stimulate growth and branching of neurites [40, 41]. M.A. Kostenko was the first to develop the method of proteolytic isolation of individual neurons with aid of pronase [42]. Subsequently this method of enzymatic dissociation has become widely used in electrophysiological and biophysical studies [43-46]. Other methods of cell disintegration also are known [47].

At treatment of ganglia with pronase, the earlier and greater damages are characteristic of elements of connective tissue and glia. This is due to that they are in the composition of sheaths and are the first to deal with acting factors. This also depends on that glial cells have very large surface of their lamellas [48] and accordingly the extremely high ratio of the membrane area to the cell volume (i.e., have very low thermodynamic and structural stability). They always are the first to respond to external actions. If time of the proteolytic treatment is decreased, some number of gliocytes can be preserved in culture.

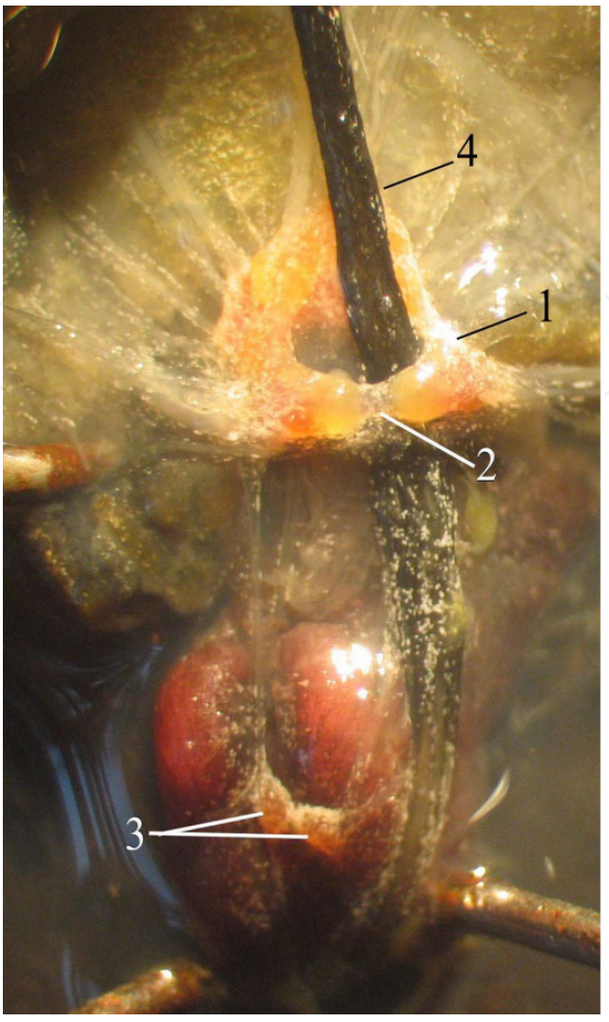

Figure 1. The peripharyngeal nerve ring of the mollusc *Lymnaea stagnalis* L *in vivo*.
1 – cerebral ganglion; 2 – intercerebral commissure; 3 – buccal ganglia; 4 – esophagus. MBS-2. Magnification – 20×.

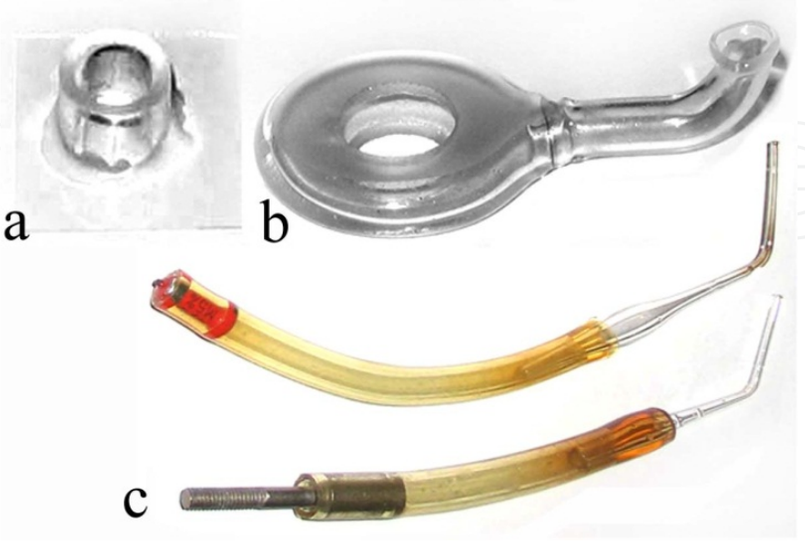

Figure 2. Microcameras (a, b) and Γ-shaped micropipettes (c), with whose aid isolated neurons were cultured and prepared.

After 40-60 min of incubation of ganglia in pronase, they were washed out from proteolytic enzymes in isotonic solution and got free from their connective tissue. To make sure in the viability of neurons, they were placed under a MSSO-IV42 microscope (LOMO, St. Petersburg) and by using a gold microelectrode with the tip diameter of about 20 μm in the glass isolation the extracellular leading of the spontaneous electric activity of individual cells was performed. They generated spikes with frequency of 1.5 ± 0.3 imp/s. The amplitude of spikes was equal to 22 ± 4 μV, while their duration – to 4 ms [38].

After cleaning from capsules, the procedure of multiple suction of ganglia (pipetting) into the specially designed Γ-shaped glass micropipettes with the tip diameter of 0.8 and 0.6 mm was performed (Fig. 2).

The experience shows that the molluscan neurons can be seeded onto the glass without special support. For cultivation of neurons we used microcameras of the following construction (Fig. 2, *a*, *b*).

1. The glass rings, 0.8 cm in height and 0.8 cm in diameter, attached with a mixture of paraffin and vaseline (1:1) to coverslips served the base of the camera.
2. The Carelli flask in our modification represents camera, 4 ml in volume, with two round holes on the upper and lower walls (Fig. 2, *b*), which for preservation of sterility were closed by coverslips and soldered with the paraffin—vaseline mixture. The cylindric camera branches designed for change of the nutrition medium were closed by a lid. The cells were seeded onto the lower coverslip, which allowed microscopy with aid of inverted microscope at high magnification (with objectives 40× and 60×). Besides, clearness and contrast of image were significantly improved. At filling of only the lower camera indentation (1.5-2 mm in depth) a meniscus of the medium was formed, and the surface tension produced aggregation of neurons near the camera center. Cells in this aggregate were not flattened, but could long survive by preserving the sphere-like shape and the granular cytoplasm. We managed to avoid aggregation of neurons at the complete filling of the camera with nutrition medium. The cells were attached to the surface of the lower coverslip, spread on it, and generated processes. Our proposed camera allows observing the details of growing neurons, invisible at low magnifications or resulted from light dispersion at its passage through the thick bottom of the glass or plastic Petri dish. Thus, for instance, it is clearly seen that the structures sometimes mistaken by researchers for cell processes are in reality dense cytoskeleton strands inside lamellas. For experimental studies, the serum-free nutrition medium of certain chemical composition was used. As the stock medium, we used the standard RPMI 1640 medium.

The thereby isolated cells were repeatedly washed with Ringer solution. The upfloating connective tissue and dying gliocytes were carefully removed with the same micropipette. As a result of micropipetting, we managed to obtain a significant amount of isolated glia-free neurons (Fig. 3). By varying pronase concentration, time of incubation, and activity of ganglion pipetting, it was possible to obtain either the isolated neuronal bodies or neurons with fragments of their processes. The more time of treatment with pronase and its concentration, the lower chances to isolate neuron with its processes.

The medium directly for cultivation was prepared based on the stock single medium RPMI 1640 medium by its dilution with a special salt solution. The composition of the salt solution (mol): 75 NaCl, 5 KCl, 2.5 $CaCl_2$, 2.5 $MgCl_2$. To obtain the nutritive medium, 250 ml of the single RPMI 1640 medium were diluted in 1 l of the salt solution. Concentration of amino acids after dilution decreased 5 times. Such medium composition is optimal for successful cultivation of dissociated neurons of *Lymnaea stagnalis*.

pH of the prepared medium raised to 7.6 with aid of Tris-HCl, and for sterilization filtrated immediately the medium by passing it through Millipore membranes with diameter of pores of 0.22 μm by using a Peristaltic miniflon pump type 304 (Poland). In the process of filtration, pH of medium reached the value of 7.8 optimal for cultivation. For control of pH, there was used a pH-meter-millivoltmeter of the "pH 150" type ("Izmeritel", Gomel).

The light microscopy was performed by using a MBI-13 inverted phase-contrast microscope (LOMO, St. Petersburg) with a thermostated camera and water-heat filter.

Observations were performed only on living neurons. The neuron viability signs are the clear external cell contour (the intact membrane), the granular cytoplasm that has the clear light dispersion and fills completely the body contour; the nucleus contours are not seen. Considered damaged were the neurons, in which the granular cytoplasm was separated at small sites from the outer cell body contour with a layer of homogenous flooded cytoplasm. Not infrequently the nucleus contours are well expressed. The hopelessly traumatized cells usually had the aggregated cytoplasm with large flooded submembranous spaces and the Brownian movement in them of granules or the visible disturbance of the membrane integrity. The sign of insufficient treatment with pronase served preservation of fragments of the glial membrane connected with the cell body. The neuron was as if in the villous membrane with uneven contours, while its own "membrane" was not seen.

Observation on behavior of regenerating processes can begin at the very first day; however, at long illumination under microscope, especially in the absence of water filter, neurites stop growing and branching.

Thus, the exposed detailed procedure allows obtaining the viable single neurons that regenerate nerve processes (Fig. 3, *d*) and form interneuronal contacts and extensive nerve plexuses. It is important to emphasize that we are dealing here with culturing of the glia-deprived brain neurons of the adult animals and that the culture medium is strongly identified and does not contain uncertain growth factors. This makes it possible to take into account sufficiently exactly the effects of various outer agents on living neurons, their processes, and interneuronal contact.

To reveal the principal possibility of formation of syncytium in the nervous system, it is necessary, first, to reproduce on living cells the process of fusion of outer cellular neuronal membranes and, second, to prove combination of the neuroplasm of two different cells. The most convenient for this is use of living neurons in the tissue culture.

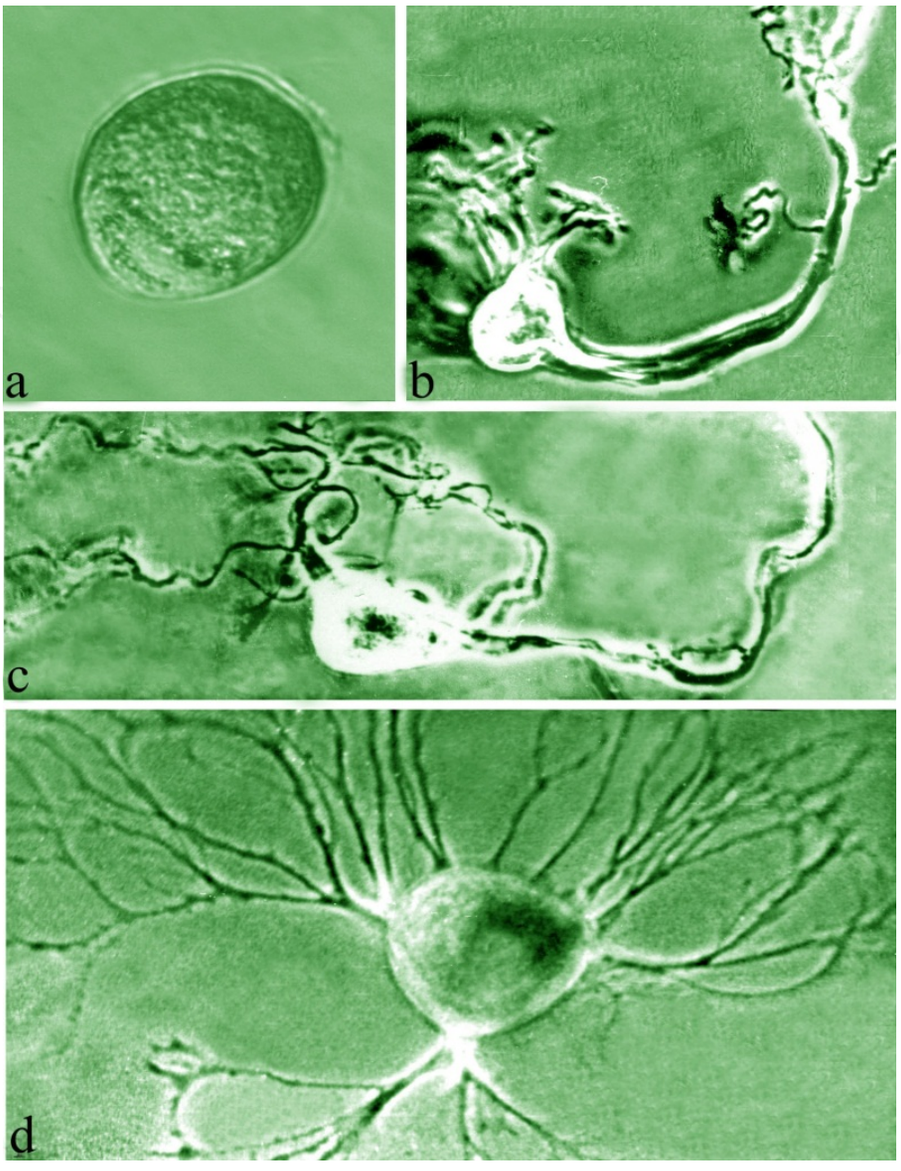

Figure 3. Isolated molluscan neurons after treatment with pronase.
a – neuron isolated without processes; b, c – variants of neurons isolated with processes; d – isolated neuron at the 4th day of cultivation in the RPMI 1640 medium. Supravital microscopy. Obj. 20Ph, eyep. 10.

4. Structural kinetics of neurons during formation of syncytium

The primary neuronal cultures were studied in phase contrast, which increased essentially the degree of detection of structural details with aid of a light microscope. However, the main virtue of the method of supravital studies was a possibility of studying the structural kinetics of the culture, the ability of the method to reveal structural transformation of neuron in time. This advantage of the method also allowed developing peculiar absolute criteria making it possible to prove formation of syncytial connections in living neurons at the light microscopy level [32, 49]. The studies were carried out by using the many-day (3-6 days) automatic time-lapse microvideo shooting and computer analysis. To prove formation of the neuronal cytoplasmic connection, we used criteria that allow with aid of videostudy

of kinetics of living neurons differentiating fusion of processes from their contact. For this purpose, as the theoretical substantiation (criterion of syncytial connection) there was used inverted statement of the law of the Wallerian degeneration [50]. Astonishing as it is, detection of syncytium was helped by the concept of the Wallerian degeneration that at its time was one of important proofs of the absence of syncytial connection in the nervous system.

Since after separation of the nerve process from the neuronal body (the trophic center) it should necessarily degenerate, so if such process does not degenerate after separation of the body of its neuron, this means that it is in the cytoplasmic connection with body of the other neuron.

We were the first to propose the novel way of revealing syncytial connection between neurons by using the light microscopy observations of dynamics of structural processes in the tissue culture.

Figure 4 shows the initial establishment of "end-to-end" contacts between filopodia (3) of the lamellar processes of cells A and B. Then, after 15 min, contacts start to form between lamellae 1, 2 neighboring cells A, B and the boundary between them becomes unclear. It can be suggested that these lamellae have a syncytial coupling, as with time they form a single intracytoplasmic cytoskeletal filament (Fig. 4, d-f, 4) which, being continuous, runs from one lamella to other lamellae. Cell A is connected with cell B via lamella 2 of cell B (Fig. 4, d, 4). However, this remains insufficient as evidence of a syncytial coupling between these lamellar processes, though, as often occurs in primary cultures, cell B dies (Fig. 4, e, 5) and its lamellar processes 2 and cytoskeletal filament 4 remain intact. Lacking its body (the trophic center), process 2 of cell B does not undergo Wallerian degeneration, as occurs in all other cases when neuron processes are detached from their bodies. In the present case, they persist for 4 h, i.e., until the end of the observation period. Furthermore, by contracting, cells A and B are brought closer together (the distance between them decreases by 9.2% and the anastomosis connecting them straightens, Fig. 4, d, f). This is possible only in one case, if the process preserving viability got time to acquire a new trophic center, i.e., established the direct cytoplasmic connection with the other cell. This process is schematically presented in three stages (Fig. 5).

Exchange of neuroplasm can be observed after fusion of processes. Thus, the plexus of tubular nerve processes presented in Fig. 6, a, b shows that growing neurite 1 establishes contact with process 2 of the other cell. Further video frames show displacement of the cytoplasm of varicosity 3 of process 1 into process 2 of cell 4 (Fig. 6, b-f), which provides further support for the formation of syncytial connections between different cells by fusion of their processes. The cytoplasmic varicosity 3 of process 1 slowly, over 6 h 14 min, moves to the site of contact between processes 1 and 2 (Fig. 6, c) and, passing it (Fig. 6, d), moves from process 1 into process 2. The varicosity then approaches cell 4 (Fig. 6, e) and fuses with it (Fig. 6, f). It is suggested that displacement of the cytoplasmic varicosity from the process of one neuron to the process of the other one can only occur when there is a syncytial connection between the neuroplasm of their processes.

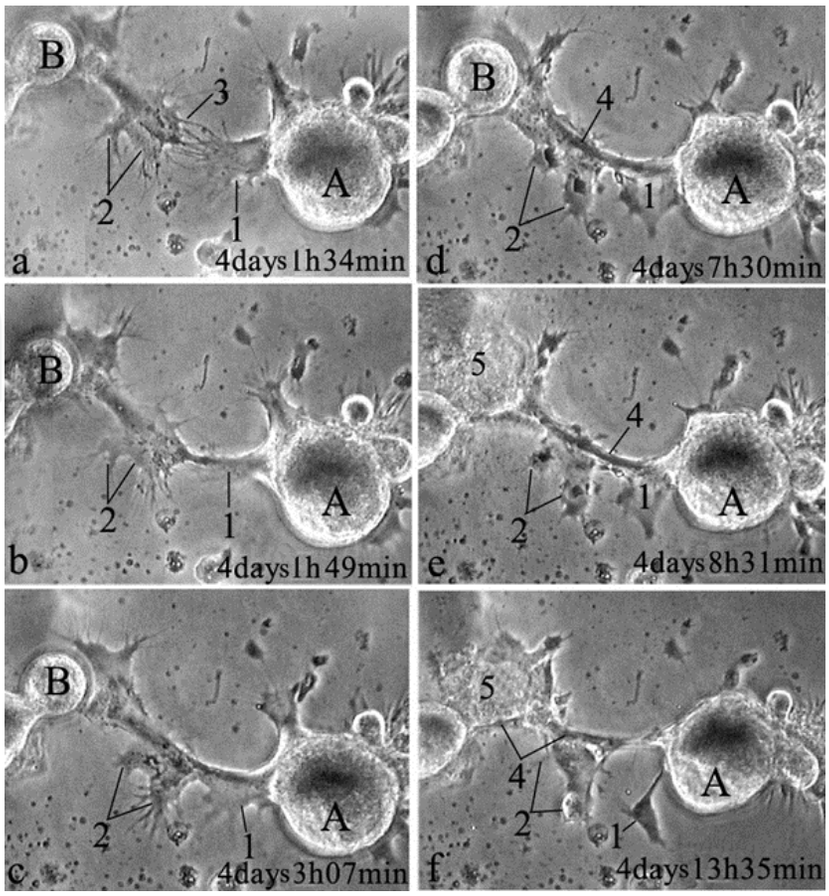

Figure 4. Dynamics of formation of syncytial connection between processes of neurons of the mollusc *Lymnaea stagnalis* in tissue culture.

a-f – stages of formation of syncytium (time – from beginning of culturing); A, B – neurons forming syncytium; 1 – process of neuron A; 2 – lamellar process of neuron B; 3 – combined filopodia of growth cones of cells A and B prior to fusion of their lamellar processes; 4 – the formed single cytoskeletal structure inserted into lamellopodia of both cells; 5 – dead neuron. Tissue culture, time-lapse computer videoshooting. Obj. 20Ph, eyep. 10.

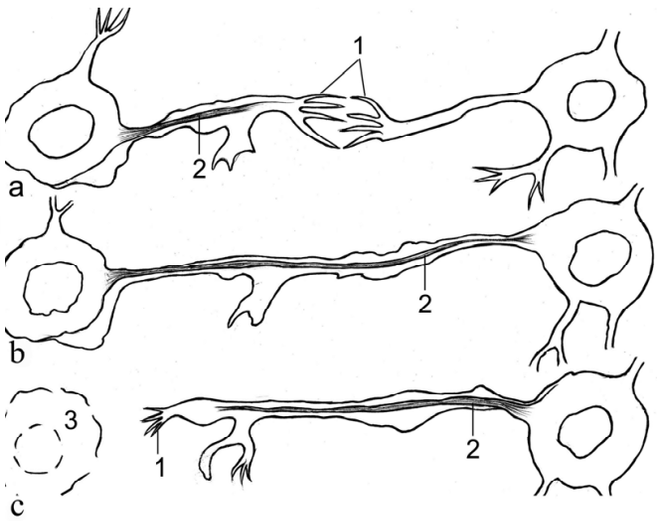

Figure 5. Schematic presentation of formation of syncytial collection between processes of two neurons.

a-c – stages of process; 1 – filopodia of growth cones; 2 – formation of the cytoskeletal strand common to two neurons; 3 – the dead neuron.

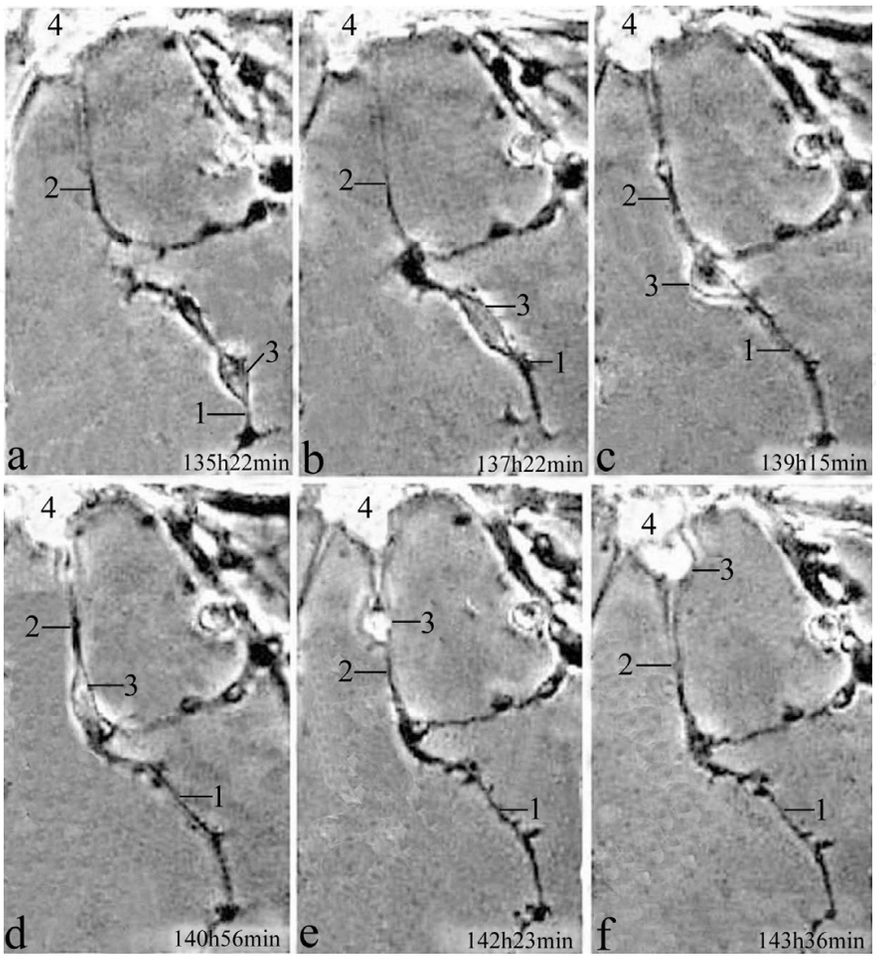

Figure 6. Formation of syncytial connection between processes of two cells and translocation of the cytoplasmic varicosity from one process into the other.

a-f – stages of formation of syncytium; 1 – process of the lower cell; 2 – process of the upper cell; 3 – the varicosity that flows over through the place of fusion of two processes; 4 – body of the upper cell. Tissue culture, time-lapse computer videoshooting. Time from the beginning of culturing is shown. Obj. 20Ph, eyep. 10.

Such behavior of cytoplasmic varicosities, in our opinion, can serve another criterion of formation of the cytoplasmic syncytial connection of neurons.

In the dissociated culture, in isolated neurons that have no contacts with other neurons there are formed multiple contacts between their processes (autapses) (Fig. 7, *a*). Some of these processes seem to form syncytial connection, as cytoplasmic varicosities from some processes are freely translocated onto neighbor processes. Processes *1* and *2* (Fig. 7, *b*) approach the process *3*, while their cytoplasmic varicosities overcome sites of their connections (Fig. 7, *d-f*). This phenomenon occurs for 5 h between all processes of the isolates neuron

Such are the facts of fusion of neurons with formation of the syncytial interneuronal connection in tissue culture. However, by the example of cells of other types it is shown that their syncytial connection is easily transformed into the cell fusion. Dynamics of fusion of neuronal bodies has not yet been studied at present.

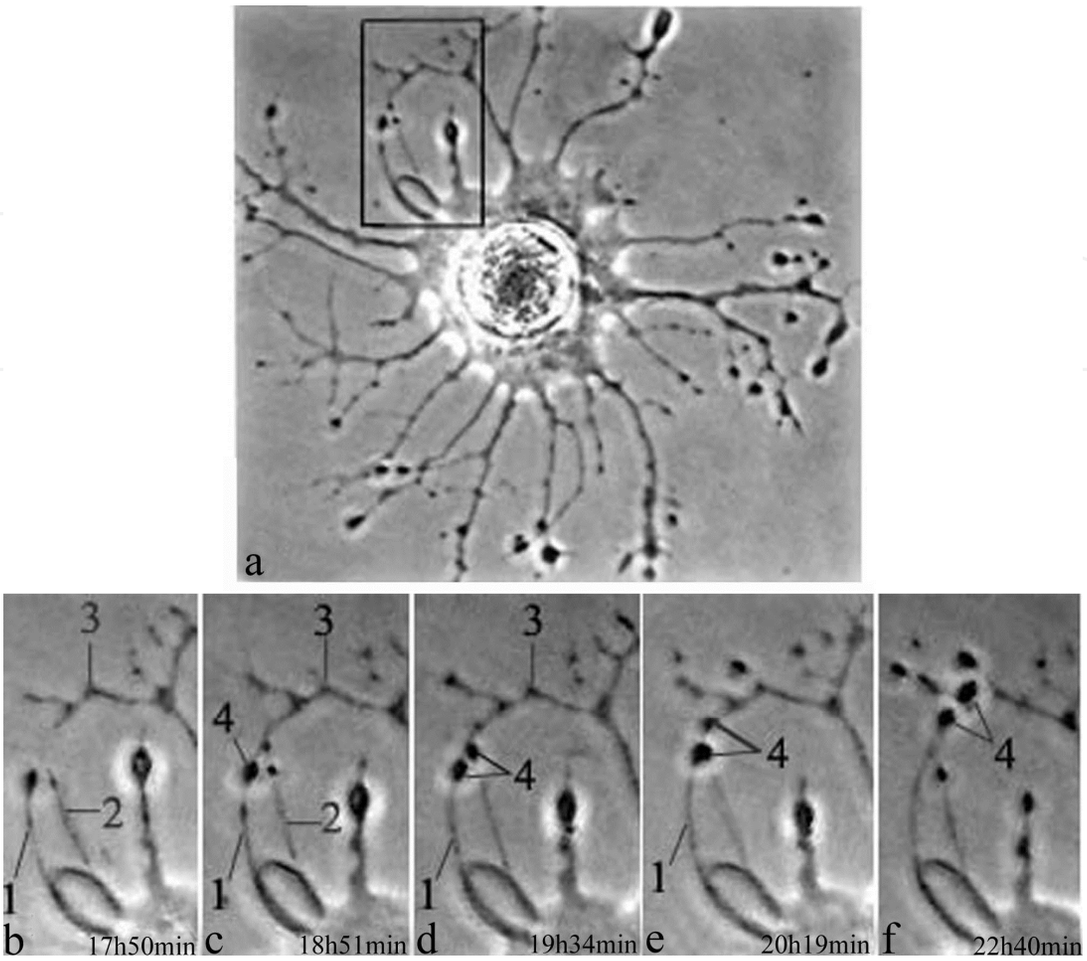

Figure 7. Translocation of cytoplasmic varicose thickenings along the syncytially connected processes of the same neuron (a).

b-f – stages of overflowing of cytoplasmic thickenings, 1, 2 – nerve processes of the lower neuron, 3 – process of the upper neuron, 4 – cytoplasmic varicosities translocating from the lower to the upper processes. Tissue culture, time-lapse computer videoshooting. Time – from the beginning of culturing. Obj. 20Ph, eyep. 10.

5. Spontaneous syncytial fusion of bodies of molluscan living neurons

It seemed unachievable to wish obtaining in the supravital experiment the fusion of living neuron bodies with formation of binucleated cells. However, numerous experiments on proteolytic dissociation of ganglia with obtaining isolated neurons got success several times. Preparations of living, just isolated mollusc cells turned out to contain binucleated neurons. The fact that indeed these were individual binucleated, rather than attached paired cells is indicated, first, by the treatment itself of cells with pronase that regularly, in 100% of cases, dissociates molluscan ganglia into single neurons by eliminating all gliocytes and fibroblasts. Second, these cells sometimes had common fused nerve processes (Fig. 8). Lastly, the angle between the fused cells (the angle of waist) exceeded 125°, which has been proven electrophysiologically [51] to be the convincing evidence for fusion of neurons.

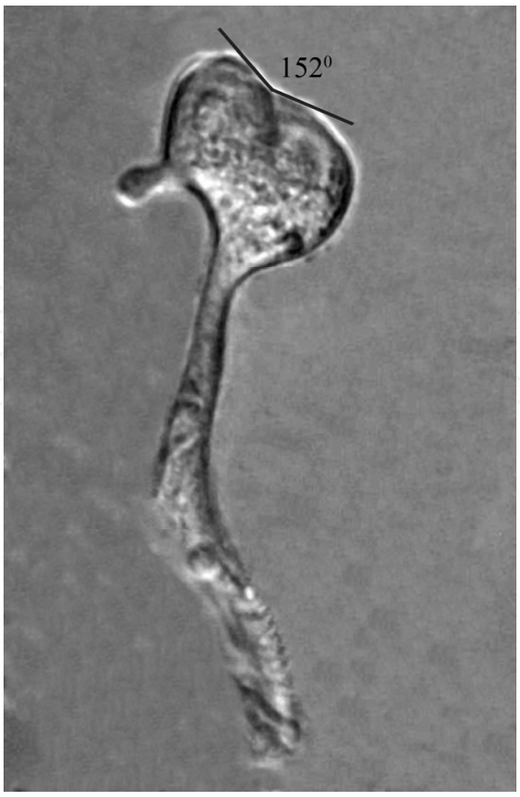

Figure 8. Spontaneous fusion of two neurons isolated from the molluscan perinuclear ring. Obj. 40Ph, eyep. 10.

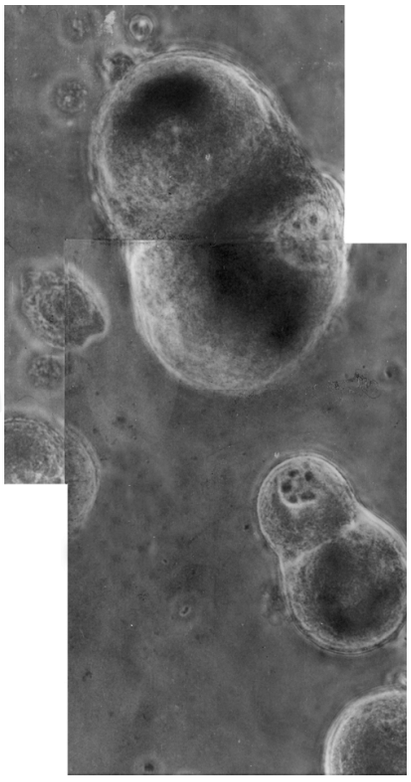

Figure 9. Binucleated neurons of *Lymnaea stagnalis* after two days of incubation in the cultural RPMI 1640 medium. Phase contrast. Obj. 20Ph, eyep. 10.

Unfortunately, the revealed fused neurons and the very process of the cell fusion directly after isolation are observed quite seldom. However, after careful washing out from pronase, after two days of incubation of neurons in the culturing medium there is noted a significant number of the fused neurons whose fusion angles are much higher than 125º (Fig. 9). Such cells also have other absolute features of fusion.

This has allowed us to suggest that neurons, by using their own potentials for fusion, some time after isolation, without help of draining agents, are able to form intersomatic syncytial cytoplasmic connections and to fuse. But since in the experiment there was involved a complex of proteolytic enzymes (0.4% pronase), a possibility existed that the cell fusion had been provoked by proteolytc enzymes. To check this, we performed a special series of experiments on electron microscopy study of effect of pronase on molluscan neurons.

Nowhere in this experiments could we detect formation of typical specialized membranous contacts and syncytial perforations. It can be concluded that the treatment itself of neurons with pronase does not induce their fusion. It only removes glial interlayers between the nerve cell bodies by promoting the fusion.

6. Development of method of artificial syncytial cytoplasmatic fusion of neurons *in vitro*

Our application of several procedures used at fusion of non-nervous cells [52, 53] had no success. Attempts at using polyethyleneglycol as a draining agent turned out to be unsuccessful, as these neurons do not endure the temperature necessary for keeping polyethyleneglycol in melted state. For fusion of neurons with aid of latex balls of polysterol there was needed the culture medium deprived of Ca and Mg ions causing aggregation of balls. Such medium is also poorly endured by molluscan neurons. As a result, these methods turned out to be poorly effective for the studied neurons.

At developing the new way [54], two groups of experiments were performed. In the first group, isolated neurons were studied in usual culture for 2-5 days, while in the second group, the structural processes were analyzed in the cell aggregate after two days.

Since earlier nobody has performed the experimental fusion of non-infected neurons, we developed a special method for fusion of the molluscan cells that have the satellite gliocytes.

Ganglion neurons of the mollusc *Limnaea stagnalis* first were freed from the ganglion connective tissue and satellite gliocytes with aid of proteolytic treatment. Neurons were carefully washed out from pronase. Then they were studied in the Eagle MEM cultural medium (Sigma, England). A part of cells were aggregated by centrifugation (3000 *g*, 15 min) and preserved in the cultural nutritive medium for 2 days by allowing the neurons to restore their natural capabilities for adhesion and fusion. Then, with aid of a light phase-contrast microscope, semithin sections were analyzed, while with aid of the standard transmissional electron microscope, ultrastructure of borders of contacting neurons was studied.

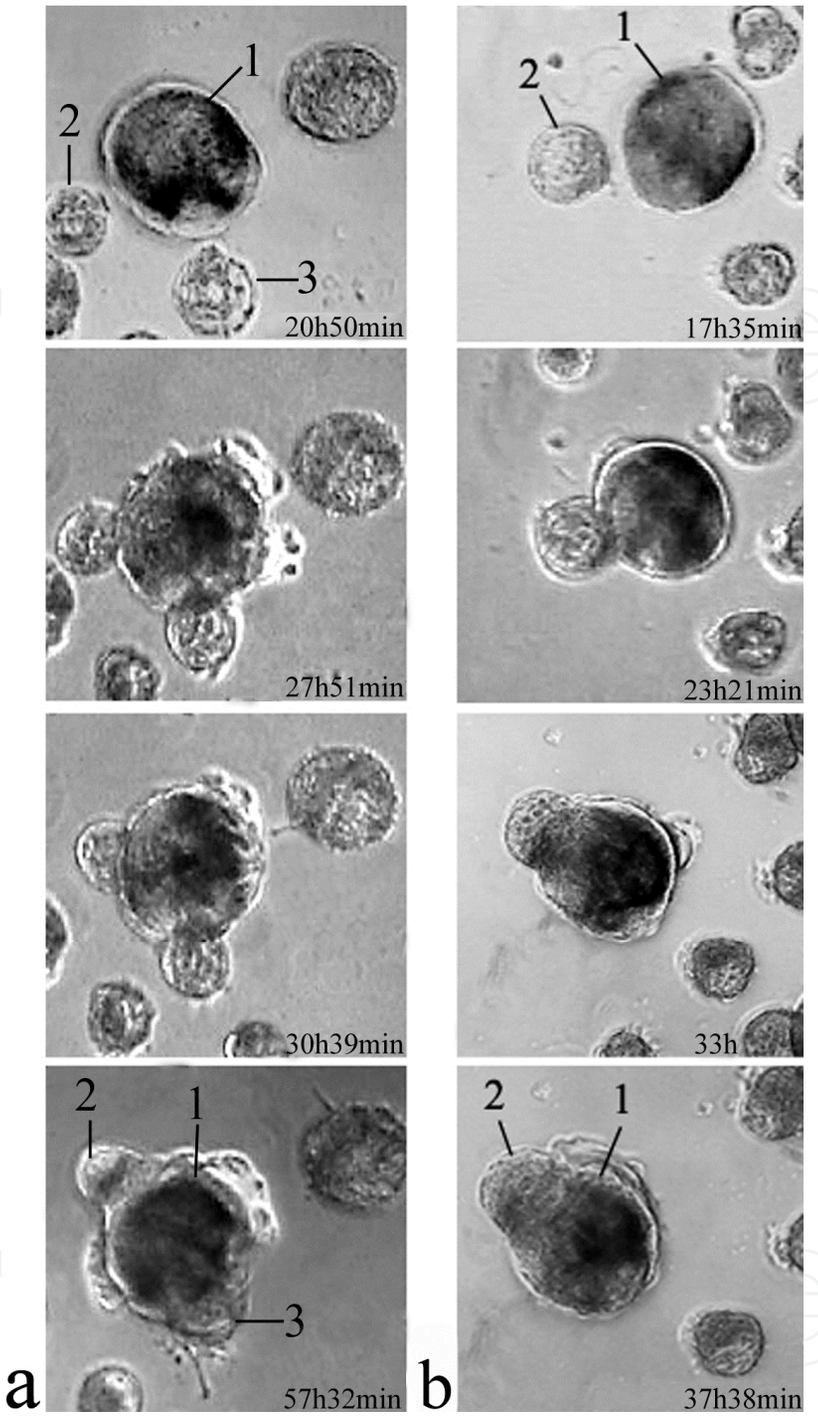

Figure 10. Dynamics of fusion of large and small neurons in tissue culture.
a – fusion of two neurons for 57 h; b – fusion of three neurons for 37 h; 1, 2, 3 – fusing neurons. Time-lapse videoshooting. Obj. 20Ph, eyep. 10.

Experimental aggregates of neurons were carefully washed out from pronase with cultural medium and were kept under the "normal" conditions natural for cultivated neurons. Therefore, we consider the processes revealed in the aggregates as natural, depending only on potentials of the cells themselves. It is suggested that treatment with pronase promoted only the removal of glial membranes.

Under conditions of the performed experiment the single living neurons often form paired or multicellular aggregates and fuse between each other. Fig. 10 presents dynamics of fusion of neurons in tissue culture. Initially, neurons are approached to each other by using the dotted contact. Later, the area of contact is enlarged and acquires shape of the large flatness.The angle of waist, of the node between the fusing cells increases. At fusion of small neurons this phenomenon is expressed worse due to large curvature of small spheres. Lastly, the smaller neuron is invaginated almost completely into the larger cell.

At the second day of culturing, the neuronal processes start growing and provide contacts of neurons; by contracting, the processes make the neuronal bodies adjacent to each other (Fig. 11). The contacting neuronal bodies form the 8-like structures that are separated by vacuole-like structures (Fig. 11, c, d). These vacuole-like structures at the borders of cells are clearly seen with aid of computer image treatment.

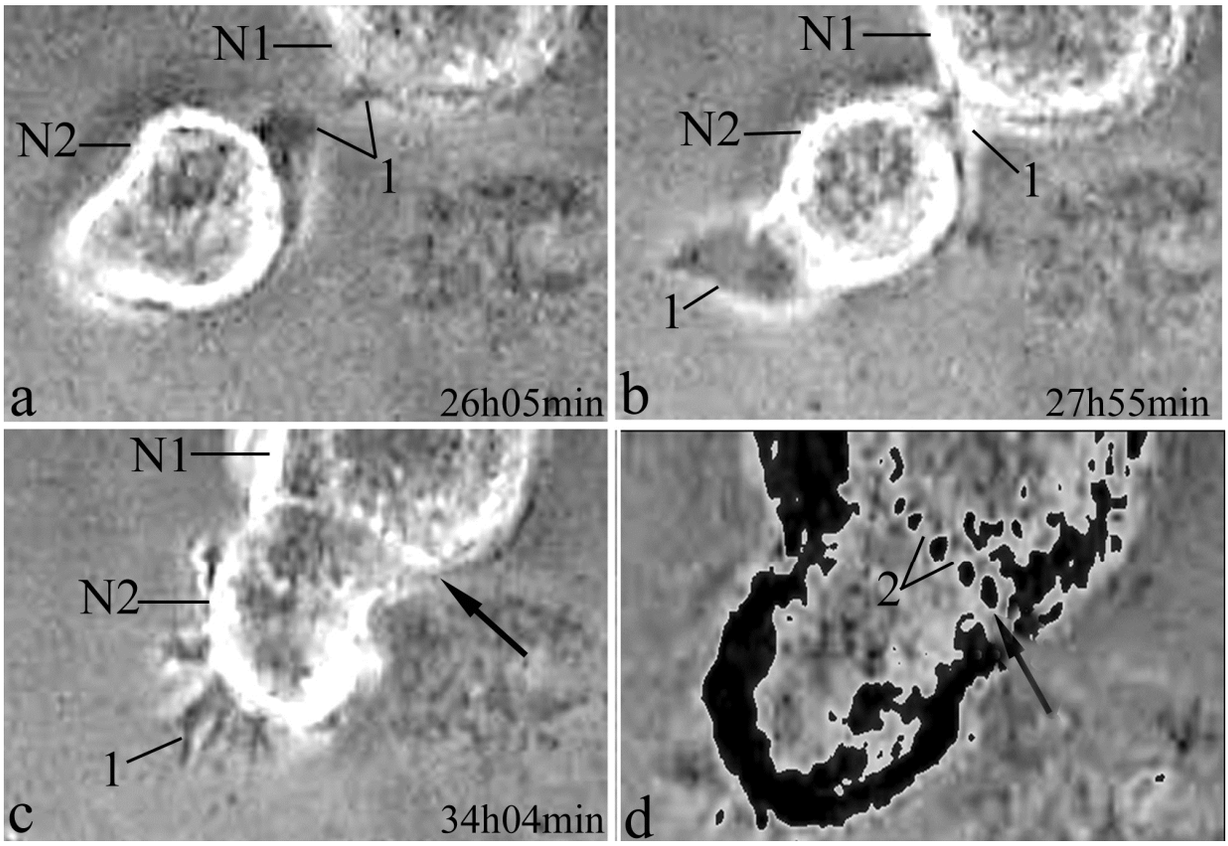

Figure 11. Formation of syncytial connection of two isolated neurons tissue culture.
a-c – approaching and fusion of neurons revealed in phase contrast; d – vacuole-like structures revealed with aid of computer Solarise effect (the same neurons as those in Fig. 1, c); 1 – nerve processes; N1, N2 – neurons; *arrows* – vacuole-like structures. Time – from the beginning of shooting. Obj. 40Ph, eyep. 10.

In semithin sections, we have managed to reveal along the contacting neuron edges the multiple outgrowths (the cytoplasmic feet) that are firmly adjacent to the "feet" of the neighbor cell. The paired feet are separated from each other by large vacuole-like "empty" structures that represent the local sharply enlarged fragments of the intercellular space (Fig. 12). The alternating bridges and the vacuole-like structures are clearly arranged along the cell borders and can serve a reliable orienteer of these borders under light microscope, especially at using the computer software ACDSee. It is these bridges that represent the cytoplasmic connections uniting the cytoplasm of adjacent cells. Here it is worth noting that the described vacuole-like local fusions are completely identical to such structures found at fusion of filopodia of growth cones [55, 56]. We have managed to detect the formed trinucleated neurons forming, in fact, the multinucleated symplast (Fig. 13).

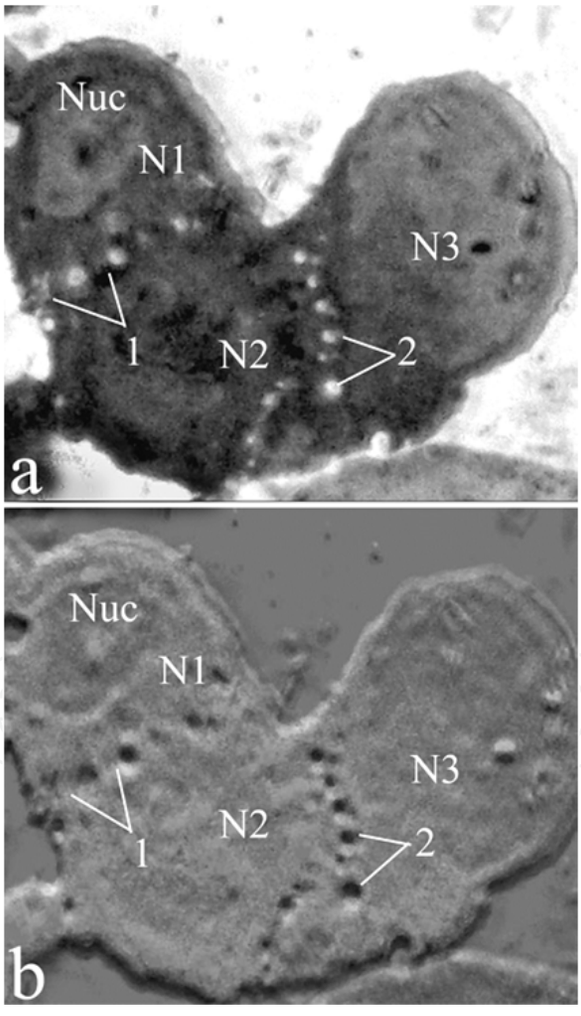

Figure 12. Light-optical signs of syncytial fusion of neurons in semithin sections.
a – additional staining with toluidine blue; b – vacuole-like structures revealed with aid of computer effect Emboss; 1 – cytoplasmic fusion bridges; 2 – vacuole-like enlargements of intercellular cleft; N1, N2 – adjacent neurons; Nuc – nuclei. Obj. 40Ph, eyep. 10.

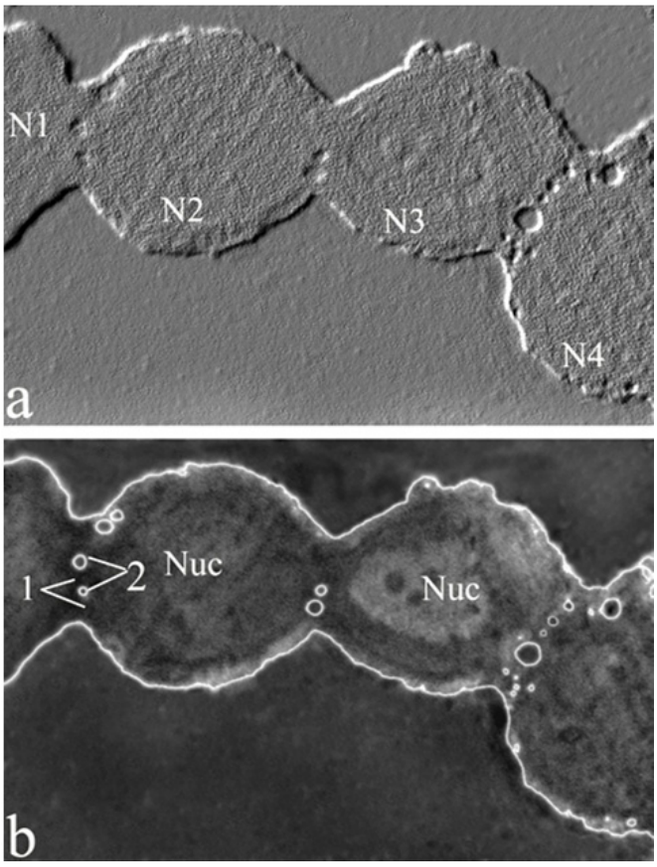

Figure 13. Chain of syncytially connected (draining) neurons.
a – computer effect of Emboss; b – computer effect Solarise; 1 – cytoplasmic bridges of fusion; 2 – vacuole-like enlargements of intercellular cleft; N1-N4 – fused neurons; Nuc – nucleus. Obj. 40Ph, eyep. 10.

With aid of electron microscope, indeed, it can be showing that bridges serve loci of fusion of two cells (Fig. 14). Although in some electron pictures the bridges of two contacting neurons can be separated with their external membranes (Fig. 14, b), the majority of membranes separating the cytoplasm of neighbor cells in the foot area turn out to be destroyed (Fig. 14, c, d). Instead of the external cell membranes separating the neuron cytoplasm, there are revealed only their short residual fragments that locally preserve the intercellular cleft, about 20 nm in width. In other places the neuroplasms of adjacent cells pass directly into each other (Fig. 15). Actually the cells turn out to be fused.

Thus, in these experiments, we were the first to manage modeling the syncytial connection between neurons *in vitro*, to prove their fusion, and thereby to confirm the principal similarity of neurons with other, non-nervous cells in the subject of intercellular interactions. Besides, results of these experiments, in our opinion, answer the chief question of the discussion about the principal possibility or impossibility of the syncytial connection of neurons. Not the initial small membrane pores and perforations have been demonstrated, but almost complete destruction of membranes of paired neurons and fusion of their cytoplasm have been shown. Fusion of neurons has been shown to occur not simultaneous on the entire contacting surface.

Initially, there are formed the fusion bridges, the local sites where membranes of contacts are destroyed and intercellular clefts between them are sharply enlarged in places and remind vacuoles (Fig. 16). This is the first absolute parameter of fusion.

The second absolute parameter of fusion of neurons is proven by McCarthy et al. [51]. It consists in that the adjacent cells first have the shape of the figure eight with the acute angle of waist. Then such structure gradually loses the shape of zero, i.e., strives for the shape of ball. The authors showed experimentally, with aid of microelectrode technique, that if the waist angle between paired contacting neurons exceeded 125°, these neurons had already fused (Fig. 16, *c, d*).

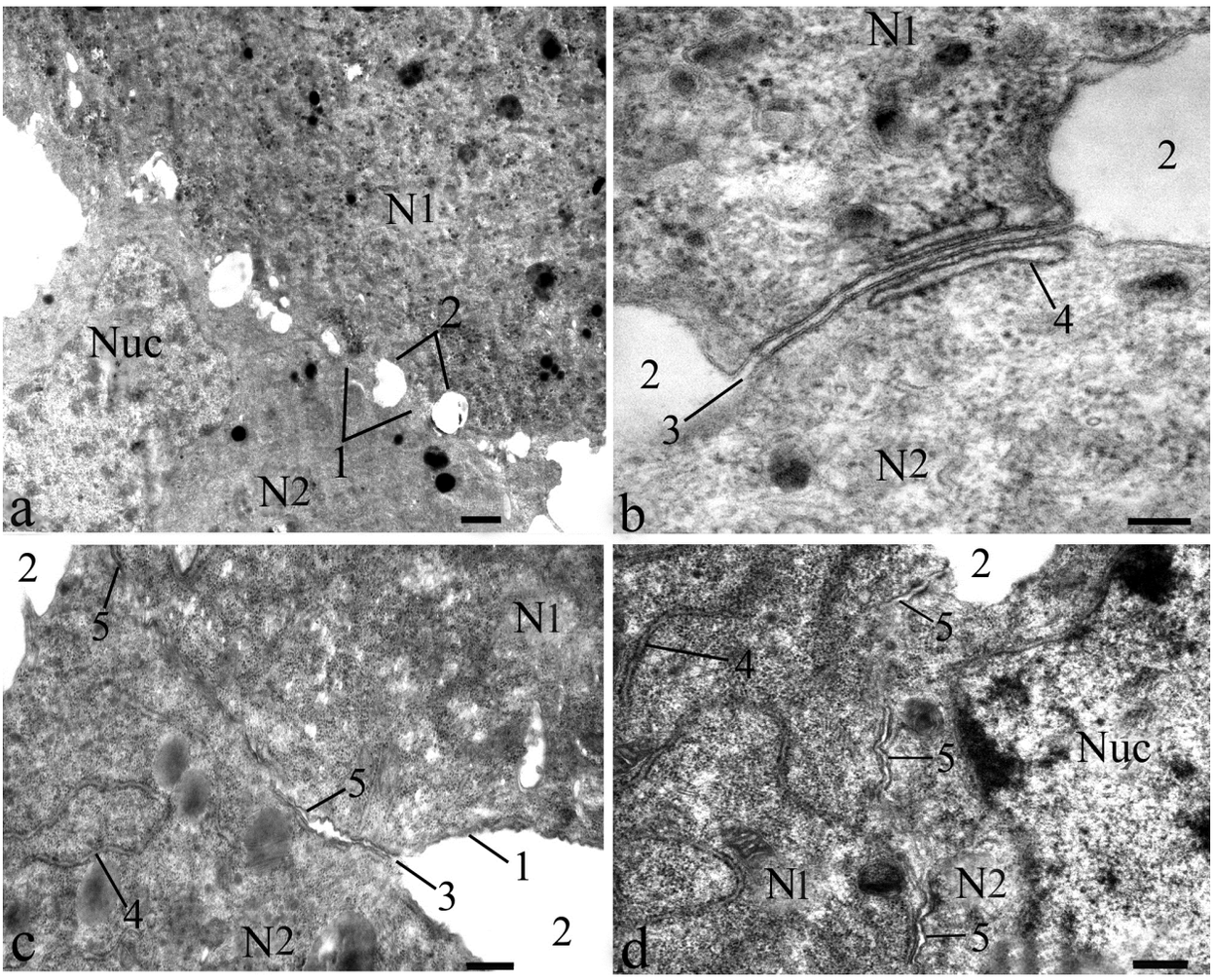

Figure 14. Borders of fused neurons.
a – multiple formations of cytoplasmic bridges of fusion and vacuole-like enlargements of intercellular cleft between two contacting neurons; b – preserved external cell membranes on the border of cytoplasmic bridges of two neurons; c, d – variants of destroyed borders between two fused neurons in the area of cytoplasmic bridge; 1 – cytoplasmic bridges of contacting neurons; 2 – vacuole-like enlargements of intercellular cleft; 3 – intercellular cleft; 4 – cistern of endoplasmic network; 5 – residual fragments of destroyed membranes on the border of two neurons; N1, N2 – adjacent neurons; Nuc – nucleus. Bar: a – 0.05 nm; b-d – 0.1 nm.

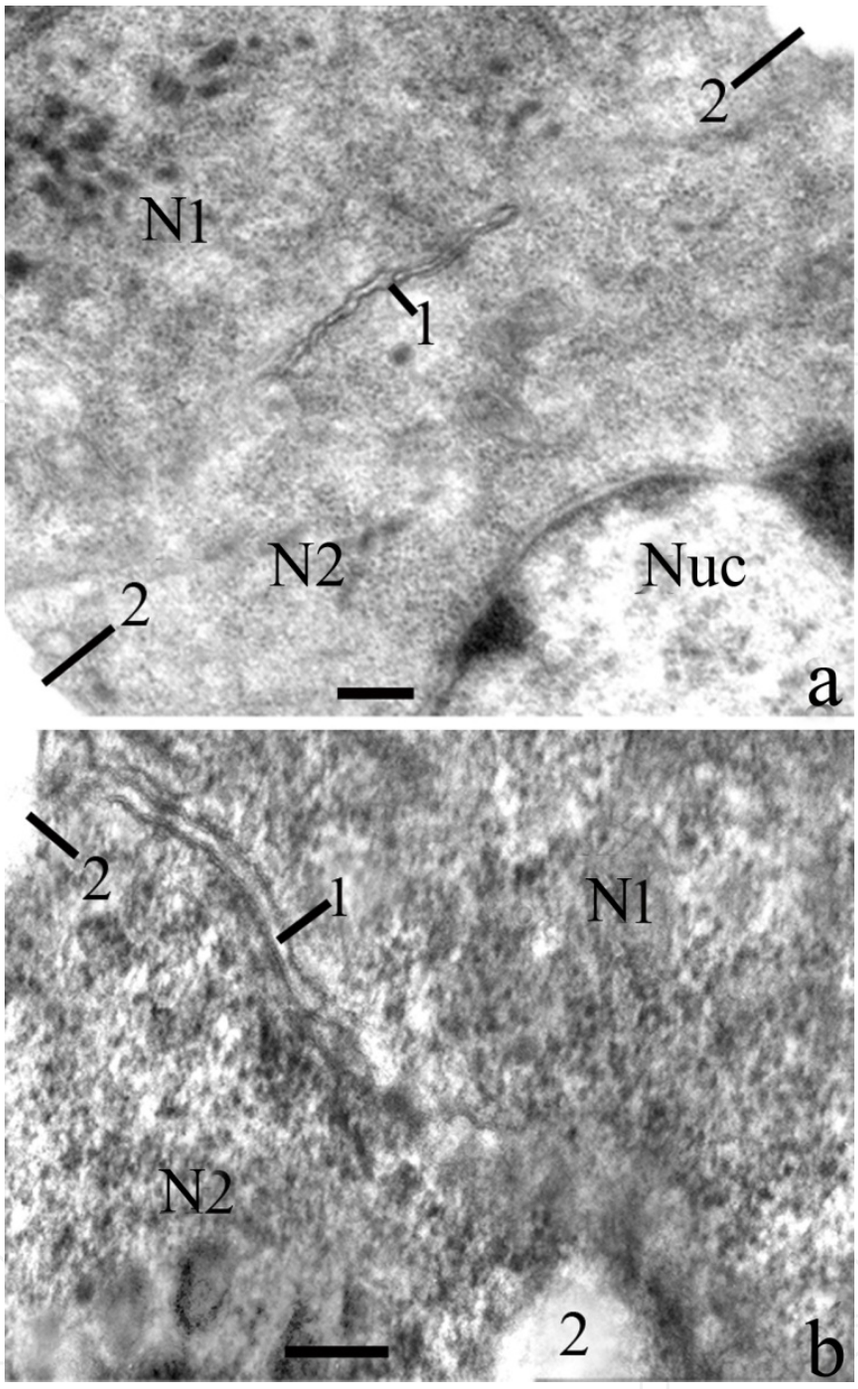

Figure 15. Residual fragments of destroyed external membranes in the area of bridges of adjacent neurons. a, b – variants of fragments; 1 – remnants of adjacent membranes with rounded ends; 2 – vacuole-like enlargements at the site of intercellular cleft. N1, N2 – adjacent neurons; Nuc – nucleus. Bar – 0.1 nm.

The data obtained in model experiments in tissue culture about the syncytial fusion of neuronal bodies are leading us to another, seemingly independent problem of mechanism of formation of binucleated and polynucleated cells [12].

The doubtless proves of formation of neurosyncytia have also revealed in tissue culture with aid of the phase-contrast microscopy of the structural neuron kinetics.

1. Syncytial connection between nerve processes is found in the case that nerve processes of some neurons, which contact with branches of another neuron do not die if the body of this neuron dies or is amputated. By the law of Wallerian degeneration the process separated from the cell body is to die necessarily. But if it does not degenerate, it means that it is connected cytoplasmically with the other neuron body.
2. The cytoplasmic syncytial connection of neurons can also be considered in the case that cytoplasmic varicosities are translocated from branch of one neuron to the branch of the other, contacting neuron.

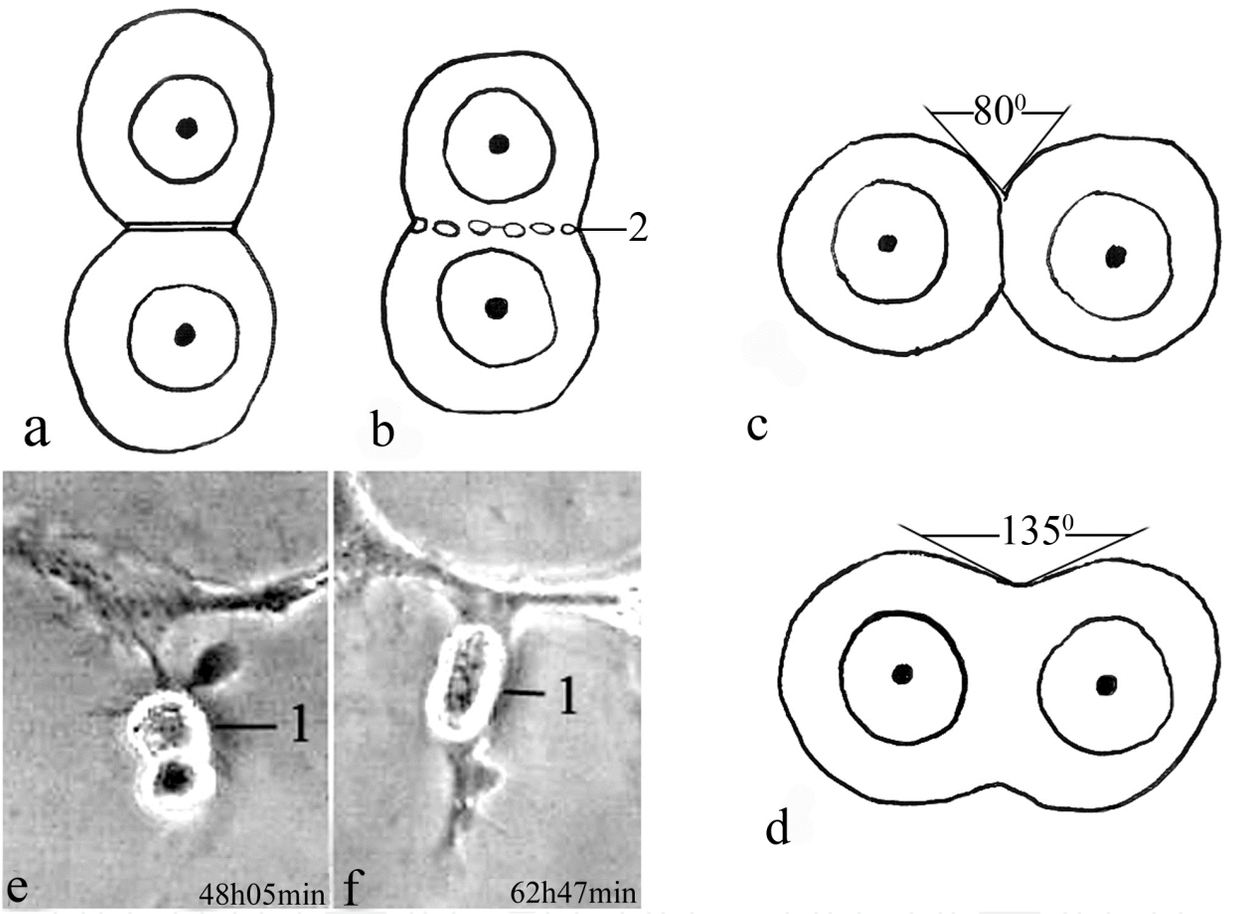

Figure 16. Light optical morphological parameters of syncytial fusion of neurons.
a, b – formation and visualization of fusion bridges and of bordering vacuole-like structures, rounding of the total structure of two fused cells; c, d – an increase of the waist angle of two fused cells higher than 135°; e, f – rounding of fused neurons; 1 – neuronal body; 2 – boundary vesicle-like structures; e, f – phase contrast. Obj. 20Ph, eyep. 10.

3. In semithin sections and under electron microscope, parameters of fused neurons serve the contacting "feet" of adjacent cells forming the fusion bridges, in which the residual fragments of destroyed boundary membranes of adjacent neurons are detected. The same criteria of syncytial connection serve the vacuole-like structures, significant local enlargements of intercellular clefts located between the fusion bridges. The local vacuole-like enlargements are also seen at phase-contrast microscopy on living fused neurons.

4. As seen with aid of a microelectrode leading and of recording of membrane permeability to stains with different molecular masses, an increase of the waist angle of attached neurons higher than 125° corresponds to conversion of gap junction pores into syncytial perforation and to fusion of neurons [51].
5. Since we have proven the binucleated neurons to be formed by the cytoplasmic syncytial fusion, their presence also is the absolute parameter of the existence of the syncytial connection in the nervous system.

7. History of study of binucleated neurons

The binucleated nerve cells were first revealed by I. Remak [57]. He was a pupil of the famous physiologist I. Müller who was the fist to describe the binucleated non-nerve cells. Since then the binucleated neurons were studied hundred times by numerous well-known histologists, such as Ranvier, Schtër, Dogiel, Schpielmayer, Bielschowski, Këliker, Alzheimer, and others [58, 59]. Their description had been included into previous textbooks and manuals past [60-62].

This problem has also been studied at present [63-65]. The binucleated and polynucleated neurons have been revealed in various central and peripheral parts of the nervous system [66, 67]: in large hemisphere cortex, hippocampus, globus pallidus, brainstem, cerebellum, pituitary, spinal cord, spinal ganglia, boundary sympathetic trunk, prevertebrate ganglia. The binucleated neurons were studied in human, monkey, horse, pig, sheep, rabbit, cat, dog, rat, mouse, guinea pig, frog, cod. In investigation of our Laboratory, they have been detected in intramural nerve plexuses of the normal cat gut (Fig. 17) and in dog [68] in toxicosis on the background of the portal vein (Fig. 18). More than a half of the authors studied the normal material.

In pathology there has also been described a great number of bi- and polynucleated neurons [69-74].

In the monograph of N.E. Yarygin and V.N. Yarygin [75] as well as in works of Ehlers [76], Shabadash and Zelikina [77] there are presented counts of binuclear neurons in norm. Their number in peripheral ganglia varies in different animals from 13 to 89%.

The issue of binucleated cells involves the problem of syncytial connection in the nervous system and the problem of non-divisibility of highly differentiated neurons. The most important is the issue of mechanism of formation of phenomenon of neuron binuclearity. The point is that from the very beginning of the studies and till now the appearance of binucleated neurons has been explained by division of mononucleated nerve cells. But since mitosis in differentiated neurons of adult animals is impossible, the hypothesis of the so-called amitosis was proposed, when the neuron nucleus is divided by direct perecording, without mitotic division figures. Since nobody has seen cytokinesis (the process of division of the cell body proper), there was formulated the additional hypothesis of the possibility of the incomplete amitosis restricted only by division of the nucleus. But division of the neuronal nucleus also was seen by nobody. All suggestions are based on voluntary selection of static fixed histological preparations.

This construction looks schematically like in Fig. 19, *a*. But the point is that in static histological preparations it is impossible to determine direction of vector of the process [78]. It is selected arbitrarily, based on the beforehand suggested hypothesis. Based on the same preparations it is also possible to model the process of the opposite direction: two neighbor cells are touched, form membrane contact, establish syncytial connection, and then are fused into one binucleated cell (Fig. 19, *b*).

However, for strange reason, almost all authors chose hypothesis of amitosis as mechanism of binuclearity and even do not discuss the possibility of mechanism of syncytial cytoplasmic fusion of neurons.

True, H. Apolant [79] mentions such possibility, but thinks it to be incompatible with physiological data. Indeed, the neuronal doctrine negates completely the possibility of interneuronal syncytium and, hence, does not accept the possibility of syncytial fusion. But in the culture of isolated neurons we have shown the binucleated neurons to be formed by syncytial fusion of usual mononucleated cells.

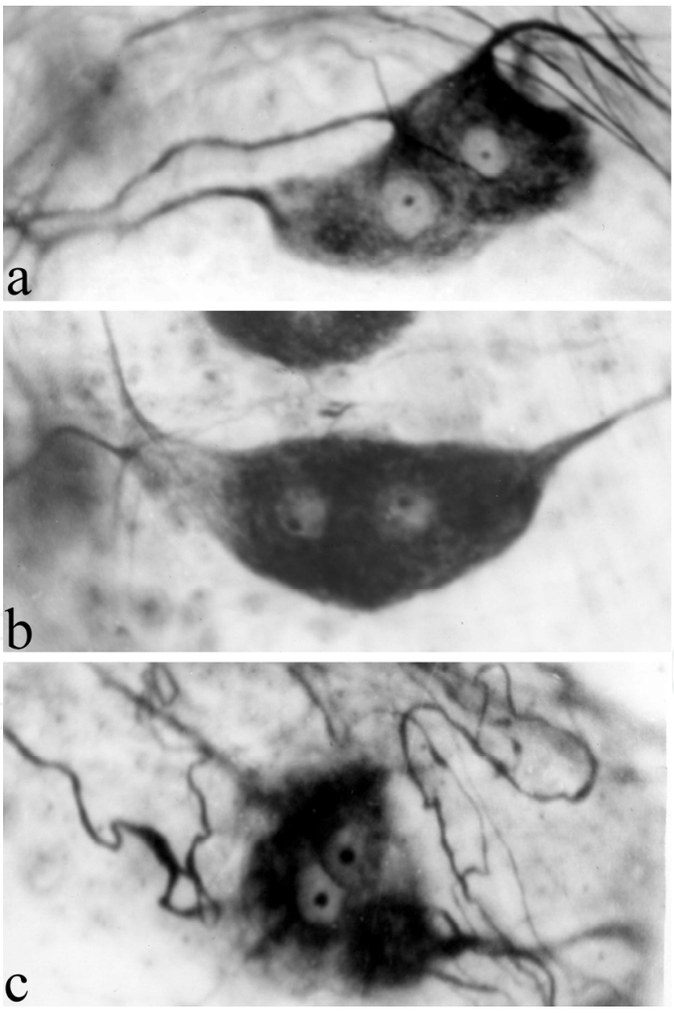

Figure 17. Binucleated neurons from intramural plexus of normal gut.
a – binucleated neuron with independent neurocytes; b – binucleated bipolar neuron with the generalized processes; c – a variant of binucleated neuron. Impregnation by Bielshowski-Gros. Obj. 40, eyep. 10.

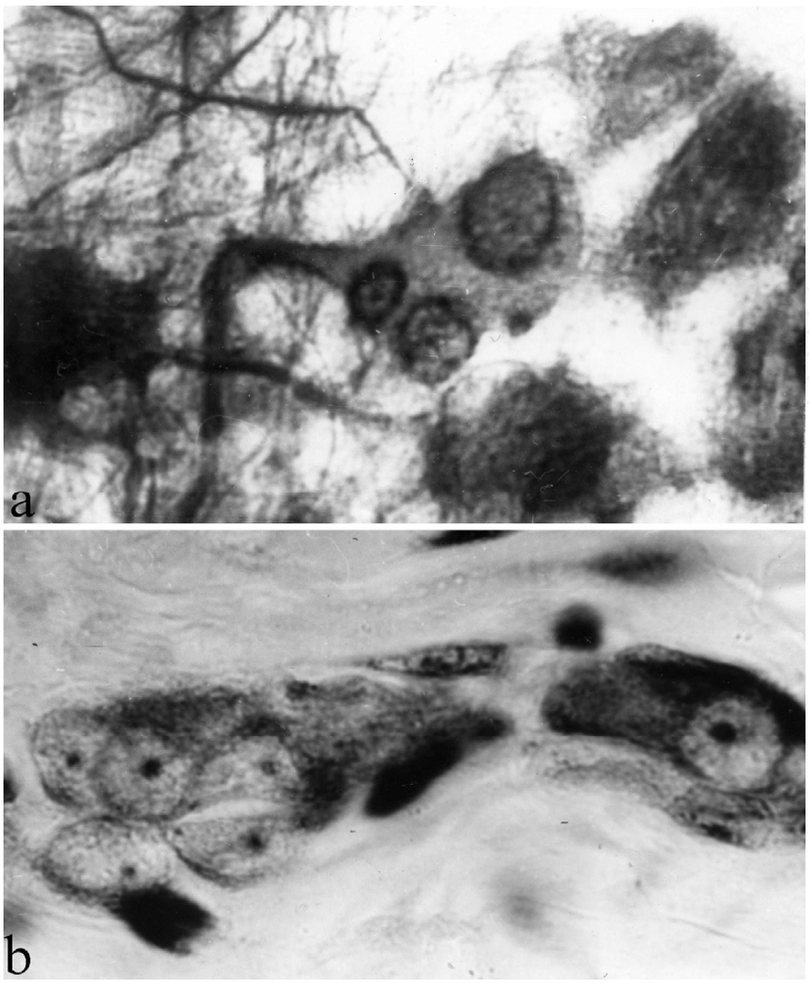

Figure 18. Polynucleated neurocytes in the node of submucosal plexus of the dog jejunum. The 15th day after the operation of the portal vein stenosing (by Chepur [68]). Procedures of Gomori in modification of Chilingaryan (a) and Einarson (b). Obj. 40, eyep. 10.

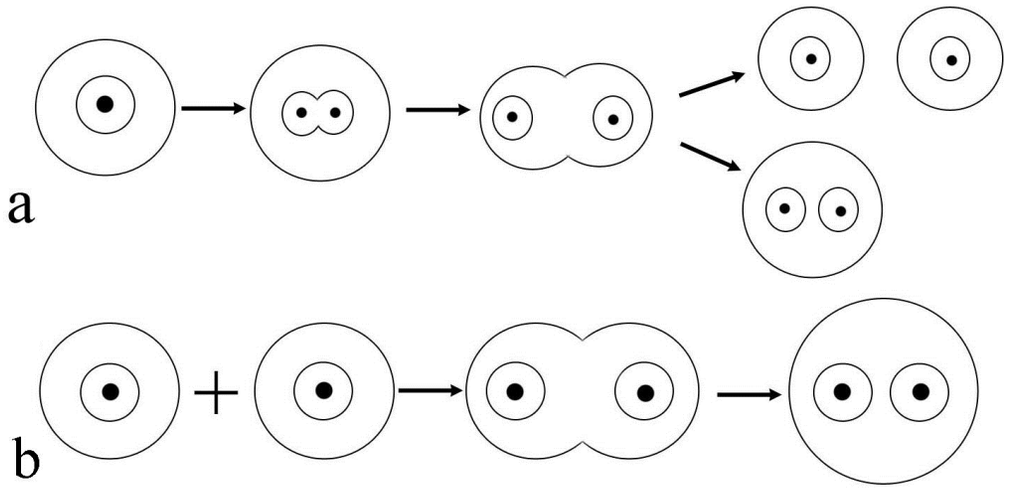

Figure 19. Schematic presentation of suggested mechanisms of formation of binucleated cells.
a – formation of binuclearity of neurons by amitotic cell division; b – formation of binuclearity by syncytial fusion of neurons.

8. Physiological proofs of interneuronal syncytial connection and of formation of binuceated neurons

The first electrophysiological study of neurons after their syncytial fusion with the neuroblasoma C 1300 neuroid cells seems to have been performed by A. Chalazonitis and coauthors [80]. The cells of the obtained hybrid line reminded the initial sympathetic neurons, were electroexcitable, sensitive to acetylcholine, and able to generate action potential. It is not necessary to prove that the hybrids are obtained by the cytoplasmic syncytial fusion. There also exist other examples that the presence of syncytial connection of neurons follows indirectly from physiological experiments.

In our work there remains one more uncovered point: whether there are physiological proofs of the existence of the syncytial interneuronal connection and what is the physiological effect of formation of syncytium in tissue culture.

In several experiments it was shown that such stains as Fura-2 and Calcium Green that are too large to overcome gap junction pores sometimes do penetrate across the boundary membranes. After injection into one cell they turned out to appear in the adjacent cell [81] as if the cleft contact would be converted into the syncytium. When studying the neuronal coupling only by the electrophysiological method, it is always difficult to distinguish the cleft contact from the electrical syncytial interconnection of neurons [82].

For the last few years there was intensively studied the role of interneuronal cleft contacts in hippocampus and cerebral cortex [83-85]. The electropermeable contacts were found to be the cause of formation of the high-frequency synchronization of the spike activity [63, 86]. It was shown that the “connexin-specific” blockators of cleft contacts: octanol, halotan, and carbenoxolen [87] cannot prevent completely effects of the presence of intercellular electrical connection. Suggestion of electrical connection of the “non-connexin type” appeared [88, 89]. This form of connection can be syncytium, as it is known that it is the syncytial perforations by their nature, which cannot be blocked by chemical agents.

It is also proven that the main protein providing intercellular connections in hippocampal cleft contact is connexin-36. It also serves the 100% marker of these contacts in vertebrates. However, in special studies of knockout animals deprived of connexin-36 and hence of cleft contacts [90, 91], the capability of hippocampus for synchronization of spikes as well as other effects are partly preserved [81, 92, 93]. Hence, apart from the cleft contact pores, hippocampus contains some intercellular connections between neurons, i.e., possibly the cytoplasmic syncytium.

Further. As long ago as in the 1990s a mysterious phenomenon was detected: the pseudorabies virus in the tissue culture was able to penetrate from neuron to neuron and to other cells by passing the intercellular medium [94-96]. This allowed suggesting formation of membranous perforations between cells. This virus was shown to be able to fuse neurons with the surrounding glia by forming binucleated cells and multinucleated hybrid symplasts [97].

Lastly, a work was published [51] whose authors infected culture of isolated neurons of rat sympathetic ganglion with the low pathogenic pseudorabies virus (PRV-151) and produced a massive formation of cleft contacts between adjacent neurons. The authors were able to demonstrate the ability of the dye with low molecular weight, Lucifer yellow (457 MW) to penetrate across the cleft contact membranous pores 9-12 h after infection of the culture. The dyes with the higher molecular weight could not be translocated from neuron to neuron. However, 24-26 h later, the dextran Texas red, the dye with the higher molecular weight (3000-40000 MW) began to penetrate from one neuron into the other one. This means that the cleft contact pores were transformed into syncytial cytoplasmic perforations; in other words, the neurons fused and acquired the dumbbell-like shape. The waist angle between fused neurons became more than 125° and several contacting neurons actually formed binucleated cells or the multinucleated symplast. Thus, with aid of the electrophysiological procedure there were shown the morphological equivalent and the physiological manifestation of the syncytial fusion of neurons.

It is important to emphasize that 18-20 h after infection of culture the incomplete electrical coupling (interneuronal electrical connection) was still observed, although a weak ability of the Lucifer yellow to penetrate from one cell into the other already was obvious. There were noted the incomplete coincidence of rhythms of adjacent cells, deletion of spikes or their conversion into spikelets (the noise-like spikes). But after 24-26 h the paired neurons became completely connected. They reproduced synchronously both spikes and spikelets, i.e., worked as the single binucleated cell.

Thus, these authors have managed to: first to obtain a model of the massive goal-seeking formation of gap junctions; second, with aid of microelectrode electrophysiological procedures to demonstrate conversion of small membranous pores of these contacts into syncytial perforations, i.e., to fuse neurons in the same way as we managed to do this in tissue culture without using virus.

In our work, by morphological methods it was also shown that syncytial perforations appeared in the place of gap junctions as a result of their perforation [25]. Hence, apart from the existing static concepts of structure of gap junctions there should be formulated a novel concept of transformation of cleft contacts from perforation of contacting membranes up to the complete fusion of neurons.

Some consequences of fusion of neuronal bodies can be revealed with aid of mathematical modeling [98, 99]. Due to development of the process of the neuronal body fusion and a rise of fusion pores between these neurons, the degree of synchronization of impulses of this pair of neurons increased. In the end of the fusion process the impulse activity of these neurons became practically synchronous. Besides, after fusion of two identical spherical neurons and at their transition to the shape of one sphere with preservation of the total cytoplasm volume the dikaryon surface will be reduced by approximately 20% as compared with that prior to fusion. Also reduced was the capacity of the cell body membrane. The newly fused nerve cell should be more sensitive to controlling signals of synapses.

Also attention is to be paid to data of study of neocortex of the 14-day rat embryos [12]. The syncytial cytoplasmic connections in these embryos were detected at the time when

the developed chemical synapses still were absent. Besides, it is known that in pathology, when chemical synapses are damaged for the first time, a clear increase of the number and size of syncytial perforations is noted [35]. Possibly, in these cases, when the fine procession of information in chemical synapses is absent or attenuated, brain uses rough, but simple and stable form of impulse transduction between neurons with aid of syncytial pores and perforations. This primitive way is resistant to chemical actions and pessimal impulse activation and hence can have certain compensatory significance. Besides, the presence of the syncytial cytoplasmic connection between neurons allows the direct exchange with energetically important substances and proteins between the relatively preserved and the damaged neurons. This also can be considered as compensatory phenomenon.

In our neurohistological studies, on many objects there were recorded syncytial cytoplasmic interneuronal connections [100]; however, studies on living neurons in the tissue culture were necessary to demonstrate kinetics of their formation and transformation into fusion of cells and multinuclearity; it was necessary to develop the way of massive artificial obtaining in the tissue culture of interneuronal syncytial connections in experiment. Such way was developed [54]. We have managed to obtain fusion of two or several neurons, i.e., to model formation of bi- and multinucleated neurons and to confirm fusion of cells with aid of electron microscope.

The fact that syncytial perforations are formed on the basis of cleft contacts, while individual perforations are transformed with time into the total fusion of neurons with formation of binucleated cells allows formulating notion about the single process of readjustment of paired membranes. The process begins with formation of dotted and expanded membranous contacts, their fusion, formation of small pores and large perforations. Enlargement of perforations leads to fusion of neuronal bodies and formation of binucleated cells.

Acceptance of cytoplasmic syncytial connections in the nervous system as the morphological and physiological reality removes contradiction of the neuronal theory and cellular theory. Similarity of the main properties of all cells including their ability to form the cytoplasmic syncytium and fusion has been confirmed.

Author details

O.S. Sotnikov
Pavlov Institute of Physiology of the Russian Academy of Sciences, St. Petersburg, Russia

Acknowledgement

The work is supported by the Russian Foundation for Basic Research. The author is grateful to associates of the Laboratory A.A. Laktionova, N.M. Paramonova, T.V. Krasnova, E.A. Gendina and L.Z. Pevzner for assistance in creation of this Chapter.

9. References

[1] Gerlach J (1872) Von den Rückenmarke. In: Handbuch der Lehre von den Geweben des Menschen und der Thiere. 2; 30: In Tech. pp. 665-693.

[2] Ramon y Cajal S (1985) Recollection of My Life. Moscow. Meditsina: InTech. 283 pp. In Rus.

[3] Bullock TH, Bennett MVL, Johnston D (2005) The Neuron Doctrine, Redux. Science. 310: 791-793.

[4] Mazzarello P (2007) Net Without Nodes and Vice Versa, the Paradoxical Golgi-Cajal Story: a Reconciliation? Brain Res. Bull. 71: 344-346.

[5] Piccolino M (1988) Cajal and the Retina: a 100-year Retrospective. Trends Neurosci. 11: 521-525.

[6] Peters A (2007) Golgi, Cajal, and Fine Structure of the Nervous System. Brain Res. Rev. 55: 256-263.

[7] Torres-Fernández O (2006) The Golgi Silver Impregnation Method: Commemorating the Centennial of the Nobel Prize in Medicine (1906) Shared by Camillo Golgi and Santiago Ramón y Cajal. Biomedica. 26: 498-508.

[8] Robertis ED, Bennett E (1955) Electron Microscope Observations on Synaptic Vesicles in Synapses of the Retinal Rods and Cones. J. Biophys. Biochem. Cytol. 1: 47-58.

[9] Palay SL (1958) The Morphology of Synapses in the Central Nervous System. Exp. Cell Res. 5: 275-293.

[10] Makovetskii AN (1940-1941) Sophists. Baku. In Tech. 1-2: 48 pp. In Rus.

[11] Ramon y Cajal S (1954) Neuron Theory or Reticular Theory? Objective Evidence of the Anatomical Unity of Nerve Cells. Madrid. Instituto "Ramon y Cajal": In Tech. 8: 144 p.

[12] Sotnikov OS, Frumkina LE, Novakovskaya SA, Bogolepov NN (2011) Fusion of Brain Neurons in Rat Embryos. Morfology. 139: 18-21. In Rus.

[13] Dogiel AS (1893) To the Question of Relation of Nerve Cells to Each Other. In: Histological Studies. I, Tomsk, P.I. Mokushin's Typo-Lithography, 23 pp. In Rus.

[14] Jabonero V. (1956) Studien über die Synapsen des Periferen Vegetativen Nervensystems. III. Das Distale Nervosa Synzytium und die Plexiforme Synapse auf Distanz. Z. Mikr.-anat. Forsch. 62: 407-451.

[15] Amzica F (2003) Physiology of Sleep and Wakefulness as it Relates to the Physiology of Epilepsy. J. Clin. Neurophysiol. 19: 488-503.

[16] Young JZ (1939) Fused Neurones Synaptic Contacts in the Giant Nerve Fibres of Cephalopods. Philosoph. Transact. Roy. Soc. London: Ser B, Biol. Sci. 229: 465-503.

[17] Maximov AA, Bloom W (1938) A Text–Book of History, W.B. Saunders Company, Philadelphia, 380 pp.

[18] Sotnikov OS, Kamardin NN, Rybakova GI, Solovieva IA (2009). Cytoplasmic Syncytial Interneuronal Connection in Molluscs. J. Evol. Biochem. Physiol. 45: 223-232. In Rus.

[19] Günter J (1975) Neuronal Syncytia in the Giant Fibres of Earthworms. J. Neurocytol. 4: 55-62.

[20] Hagiwara S, Morita H, Naka K (1964) Transmission Through Distributed Synapses Between the Giant Axons of a Sabellid Worm. Comp. Biochem. Physiol. 13: 453-460.

[21] Nicol JAC (1948) Giant Axons of *Eudistylia vancouveri* (Kinberg) Transact. Roy. Soc. Canada, XIII, (III): 107-124.
[22] Nicol JAC, Young JZ (1946) Giant Nerve Fibre of *Myxicola infundibulum* (Grube). Nature. 158: 167-168.
[23] Young, JZ (1936) Structure of Nerve Fibres and Synapses in Some Invertebrates. Cold Spring Harbor. Symp. Quant. Biol. 4: 1-6.
[24] Young JZ (1938) The Functioning of the Giant Nerve Fibres of the Squid. J. Exper. Biol. 85: 170-185.
[25] Guillery RW (2007) Relating the Neuron Doctrine to the Cell Theory. Should Contemporary Knowledge Change Our View of the Neuron Doctrine? Brain Res. Rev. 55: 411-421.
[26] Birse SC, Bittner GD (1976) Regeneration of Giant Axons in Earthworms. Brain Res. 113: 575-581.
[27] Derimer SA, Elliot EJ, Macagno ER, Muller KJ (1983) Morphological Evidence That Regeneration Axons Can Fuse with Axon Segment. Brain Res. 272: 157-161.
[28] Hoy RR, Bittner GD, Kennedy D (1977) Regeneration in Crustatian Motoneurons Evidence for Axon Fusion. Science. 156: 251-252.
[29] Sotnikov OS, Frumkina LE, Maiorov VN, Paramonova NM, Laktionova AA, Bogolepov NN (2012) Rehabilitation of Interneuronal Syncytial Connection in the Nervous System. Pacific Med. J. 2: 75-83. In Rus.
[30] Sotnikov OS, Lagutenko YuP, Malashko VV, Gusova BA, Podolskaya LA (1994) Kinetics of Avesicular Interneuronal Membranous Contacts. III Congr. Anatom. Histolog. Embryol. Ross. Federat. Minzdrav, Tyumen, 189. In. Rus.
[31] Sotnikov OS, Kamardin NN, Rybakova GI (2009) Cytoplasmic Syncytial Interneuronal Connection in Molluscs. J. Evol. Biochem. Physiol. 45: 223-232. In Rus.
[32] Sotnikov OS, Malashko VV, Rybakova GI (2006) Fusion of Nerve Fibers. Proceed. Biol. Sci. 410: 361-363. In Rus.
[33] Sotnikov OS, Fomichev NI, Laktionova AA, Archakova LI (2010) Glio-neuronal and Glio-glial Syncytial Cytoplasmic Connections in Peripheral Nerve Trunks of the Crawfish *Astacus leptodactylus*. J. Evol. Biochem. Physiol. 46: 429-434. In Rus.
[34] Archakova LI, Sotnikov OS, Novakovskaya SA (2010) Syncytial Cytoplasmic Ganglion Cells in Adult Cats. Neurosci. Behav. Physiol. 40: 447-450.
[35] Paramonova NM, Sotnikov OS, Krasnova TV (2010) Interneuronal Membrane Contacts and Syncytial Perforations in CA2 Hippocampal Area After Brain Trauma. Bull Exp. Biol. Med. 150: 100-103.
[36] Sotnikov OS,, Paramonova NM (2010) Cytoplasmic Syncytial Connection – One of Three Forms of Interneuronal Connection. Usp. Physiol. Science 41(1): 45-57. In Rus.
[37] Sotnikov OS, Novakovskaya SA, Solovieva IA (2011) Syncytial Perforations of Human Embryo Neuronal Membranes. Russ. J. Devel. Biol. 42(1): 48-52.
[38] Kostenko MA, Sotnikov OS, Chistyakova IA (1999) Methods and Methodological Approaches to Studies of Isolated Neurons of Brain from Adult Animals (*Lymnaea stagnalis*) in Tissue Culture. Neurosci. Behav. Physiol. 29: 455-459.

[39] Petrova IO (1976) Proteolytic Enzymes of Actinomycetes. Moscow. Nauka: In Tech. 26 pp. In Rus.
[40] Leprince P, Register B, Delree P (1991) Proteases et Inhibiteurs de Proteases: Implications Multiples Dans le Development et le Vieillissement Cerebral. Rev. ONO. 12-13: 30-38.
[41] Scher M, Aidoo R, Chung P (1991) Plasminogen Activator is Regulated in Peripheral Nerve After Injury and During Regeneration. J. Cell Biol. 115(2): 117.
[42] Kostenko MA (1972) Isolation of Single Nerve Cells from Brain of the Mollusc *Lymnaea stagnalis* for Their Further Culturing *in vitro*. Cytologia. 14(10): 1274-1279. In Rus.
[43] Kostyuk PG., Kryshtal OA (1981) Mechanisms of Electrical Excitability of the Nerve Cell. Moscow. Nauka: In Tech. 108 pp.
[44] Akaike N, Nishi K, Oyama Y (1983) Characteristics of Manganese Current and Its Comparison With Current Carried by Other Divalent Cations in Snail Some Membrane. J. Membr. Biol. 76: 289-297.
[45] Zhang W, Lin GJ, Takeuchi H (1996) Effect of L-Glutamic Acid and Its Agonists on Snail Neurons. Gen. Pharmacol. 27(3): 487-497.
[46] Sotnikov OS, Chalisova NI (1988) Supravital Methods of Morphological Investigations. In: Manual of Culturing of Nervous Tissue. Moscow. Nauka: In Tech. pp. 79-84. In Rus.
[47] Fikhte BA, Gurevich GA (1988) Deintegrators of Cells. Moscow. Nauka: In Tech. 185 pp. In Rus.
[48] Sotnikov OS (1985) Dynamics of Structure of Living Neuron. Leningrad. Nauka: In Tech. 159 pp. In Rus.
[49] Sotnikov OS, Malashko VV, Rybakova GI (2008) Syncytial Coupling of Neurons in Tissue Culture and Early Ontogenesis. Neurosci. Behav. Physiol. 38: 223-331.
[50] Waller A (1850) Experiments on the Section of the Glossopharyngeal and Hypoglossal Nerves of the Frog and Observations of the Alterations Produced Thereby in the Structure of Their Primitive Fibres. Philos. Trans. 140: 423-429.
[51] McCarthy KM, Tank DW, Ehquist LW (2009) Pseudorabies Virus Infection Alters Neuronal Activity and Connectivity *in vitro*. PLoS Pathol. 5(10): 1-20.
[52] Efrussi B. (1976) Hybridization of Somatic Cells. Moscow. Mir: In Tech. 189 pp. In Rus.
[53] Lentz BR (2007) PEG as a tool to gain insight into memrane fusion. Eur. Biophys. J. 36(4-5):315-326.
[54] Sotnikov OS, Laktionova AA, Paramonova NM (2011) Way of Modeling of Syncytial Connections Between Cells *in vitro*. Patent RU 2010 114371. Decision of Granting the Patent 2011.05.30. In Rus.
[55] Luduena MA, Wessels NK (1973) Cell Locomotion, Nerve Elongation and Microfilaments. Dev. Biol. 30: 427-440.
[56] Spooner BS, Luduena MA, Wessells NK (1974) Membrane Fusion in the Growth Cone-Microspike Region of Embryonic Nerve Cells Undergoing Axon Elongation in Cell Culture. Tissue Cell. 6(3): 399-409.
[57] Remak R (1837) Weitere Mikroscopishe Untersuchungen über die Premitivfasern der Nervensystems der Wirbelthiere. Notizen aus dem Bau der Natur und Helkinde. 3(47): 36-38.

[58] Das GD (1977) Binucleated Neurons in the Central Nervous System of the Laboratory Animals. Experientia. 33(9): 1179-1180.
[59] Mair H, Budka H, Lassman H (1989) Vacuolar Myelopathy with Multinucleated Giant Cells in the Acquired Immune Deficiency Syndrome (AIDS). Light and Electron Microscopic Distribution of Human Immunodeficiency Virus (HIV). Acta Neuropathol. 78(5): 497-503.
[60] Spielmeyer W (1922) Histopathologie des Nervensystems. Berlin. Verlag von J. Springer: In Tech. 498 pp.
[61] Botar J (1966) The Autonomic Nervous System. Budapest. Akademiai.: In Tech. 357 pp.
[62] Ermokhin PN (1969) Histology of the Central Nervous System. Moscow. Meditsina: In Tech. 243 pp. in Rus.
[63] Maier N, Nimmrich V, Draguhn A (2003) Cellular and Network Mechanisms Underlying Spontaneous Sharp Wave – Ripple Complexes in Mouse Hippocampal Slices. J. Physiol. 550(3): 873-887.
[64] Kawataki T, Sato E, Sato T (2010) Anaplastic Ganglioglioma With Malignant Features In Both Neuronal And Glial Components – Case Report. Neurol. Med. Chir. 50(3): 228-231.
[65] DiLorenzo DJ, Jankovic J, Simpson RK (2010) Long-Term Deep Brain Stimulation for Essential Tremor: 12-year Clinicopathologic Follow-up. Mov. Disord. 25(2): 232-238.
[66] Tambuyzer BR, Nouwen EJ (2005) Inhibition of Microglia Multinucleated Giant Cell Formation and Induction of Differentiation by GM-CSF Using a Porcine *in vitro* Model. Cytokine. 31(4): 270-279.
[67] Mizuguchi M (2007) Light Microscopic Analysis of Cellular Networks in the Pineal Gland of the Golden Hamster as Revealed by Methylene Blue Labeling. Congenit. Anom. 47(1): 2-8.
[68] Chepur SV (2002) Morphofunctional Characteristics of the Nervous System Structures in Norm and Regularities of Their Changes in Hepatic Encephalopathy. Doctorate Dissert. Pavlov Institute of Physiology: St. Petersburg. 390 pp. In Rus.
[69] Yamanouchi H, Jay V, Rutka JT (1997) Evidence of Abnormal Differentiation in Giant Cells of Tuberous Sclerosis. Pediatr. Neurol. 17(1): 49-53.
[70] Zhu X, Siedlak SL, Wang Y (2008) Neuronal Binucleation in Alzheimer Disease Chihppocampus. Neuropathol. Appl. Neurobiol. 34(4): 457-465.
[71] Paltsyn AA, Kolokolchikova EG, Kostantinova NB (2008) Formation of Heterokaryons as the Way of Regeneration of Neurons at Postischemical Damage of Rat Brain Cortex. Bull. Exper. Biol. Med. 146(10): 467-470. In Rus.
[72] Hirohata S (2008) Histopathology of Central Nervous System Lesions in Behçet's Disease. J. Neurol. Sci. 267(1-2): 41-47.
[73] Konstantinova NB (2010) Role of Cell Fusion in Reparative Regeneration of the Brain Cortex. Candidate Dissertation, Research Inst. Gener. Pathol. Pathophysiol. Ross. Acad. Med. Sci. Moscow: 24 pp. In Rus.
[74] Samosudova NV, Reutov VG, Larionova NP (2011) Fusion of Cells-Granules of Frog Cerebellum at the Toxic Effect of Glutamate and a NO-Generating Compound, Morphologia. 140(4): 13-17. In Rus.

[75] Yarygin NE, Yarygin VN (1973) Pathological Adaptive Neuron Changes. Moscow. Meditsina: In Tech. 175 pp. In Rus.
[76] Ehlers P (1951) Über Altersveränderungen an Grenzstrang-Ganglien vom Meerschweinchen. Anat. Anz.. Bd. 98, H. ½. S. 24-34.
[77] Shabadash AL, Zelikina TI (1968) Cytochemistry of Experimental Karyotomy in Nevrocytes of Mammalian Autonomic Ganglia. Proc. Acad. Sci. USSR. 183(4): 944-947. In Rus.
[78] Wang XJ, Li QP (2007) The Roles of Mesenchymal Stem Cells (MSCs) Therapy in Ischemic Heart Diseases. Biochem. Biophys. Res. Commun. 359(2): 189-193.
[79] Apolant H (1896) Über die Sympathischen Ganglienzellen der Nager. Arch. Mikroskop. Anat. Entwick.. Bd. 47: S. 461-471.
[80] Chalazonitis A, Greene LA, Shain W (1975) Excitability and Chemosensitivity Properties of a Somatic Cell Hybrid Between Mouse Neurol Laltoma and Sympathetic Ganglion Cells. Exp. Cell Res. 96: 225-238.
[81] Buzsáki G (2001) Electrical Wiring of the Oscillating Brain. Neuron. 31(3): 342-344.
[82] Thompson RJ, Zhou N, MacVicar BA (2006) Ischemia Opens Neuronal Gap Junction Hemichannels. Science. 312(5775): 924-927.
[83] Venance L, Rozov A, Blatow M (2000) Connexin Expression in Electrically Coupled Postnatal Rat Brain Neurons., Proc. Natl. Acad. Sci. USA. 97(18): 10260-10265.
[84] Bruzzone R, Hormuzdi SG, Barbe MT (2003) Pannexins, a Family of Gap Junction Proteins Expressed in Brain. Proc. Natl. Acad. Sci. 100(23): 13644-13649.
[85] Hamzei-Sichani F, Kamasawa N, Janssen WG (2007) Gap Junctions on Hippocampal Mossy Fiber Axons Demonstrated by Thin-Section Electron Microscopy and Freeze Fracture Replica Immunogold Labeling. Proc. Natl. Acad. Sci. 104(30): 12548-12553.
[86] Schmitz D, Schuchmann S, Fisahn A (2001) Axo-Axonal Coupling a Novel Mechanism for Ultrafast Neuronal Communication. Neuron. 31(5): 831-840.
[87] Draguhn A, Traub RD, Bibbig A (2000) Ripple (Approximately 200-Hz) Oscillations in Temporal Structures. J. Clin. Neurophysiol. 17(4): 361-376.
[88] Ylinen A, Bragin A, Nádasdy Z (1995) Sharp Wave-Associated High-Frequency Oscillation (200 Hz) in the Intact Hippocampus: Network and Intracellular Mechanisms. J. Neurosci. 15(1): 30-46.
[89] Stebbings LA, Todman MG, Phillips R (2002) Gap Junctions in Drosophila: Developmental Expression of the Entire Innexin Gene Family. Mech. Dev. 113(2): 197-205.
[90] Kistler WM, De Jeu MT, Elgersma Y (2002) Analysis of Cx36 Knock-out Does not Support Tenet that Olivary Gap Junctions are Required for Complex Spike Synchronization and Normal Motor Performance. Ann. NY Acad. Sci. 978: 391-404.
[91] Buhl DL, Harris KD, Hormuzdi SG (2003) Selective Impairment of Hippocampal Gamma Oscillations in Connexin-36 Knock-out Mouse *in vivo*. J. Neurosci. 23(3): 1013-1018.
[92] Deans MR, Gibson JR, Selitto C (2001) Synchronous Activity of Inhibitory Networks in Neocortex Requires Electrical Synapses Containing. Connexin 36. Neuron. 31: 477-485.

[93] Traub RD, Pais J, Bibbig A (2002) Containing Roles of Axonal (Pyramidal Cell) and Dendritic (Interneuron) Electrical Coupling in the Generation of Neuronal Network Oscillations. Proc. Natl. Acad. Sci. USA. 100(3): 1370-1374.

[94] Card JP, Rinaman L, Schwaber JS (1990) Neurotrophic Properties of Preudorabies Virus: Uptake and Transneuronal Passage in the Rat Central Nervous System. J. Neurosci. 10(6): 1974-1994.

[95] Ch'ng TH, Enquist LW (2005) Neuron-to-Cell Spread of Pseudorabies Virus in a Compartmented Neuronal Culture System. J. Virol. 79(17): 10875-10889.

[96] Ch'ng TH, Spear PG, Struyf F (2007) Glycoprotein D-Independent Spread of Pseudorabies Virus Infection in Cultured Peripheral Nervous System Neurons in a Compartmented System. J. Virol. 81(19): 10742-10757.

[97] Reichelt M, Zerboni L, Arvin AM (2008) Mechanisms of Varicella-Zoster Virus Neuropathogenesis in Human Dorsal Root Ganglia. J. Virol. 82(8): 3971-3983.

[98] Pokrovskii AN, Sotnikov O.S. (2011) Mathematical Modeling of Consequences of Fusion of Neuronal Bodies. Proc. Intern. Conf. Voronezh. Izdatelsko-Poligraficheskii Tsentr VGU: In Tech. 233-234. In Rus.

[99] Pokrovsky AN, Sotnikov O.S. (2008) Difficulties in Mathematical Modelling of Control Processes in One-Type Neuron Populations. II International Conference on Inductive Modelling. Kyiv. NAS of Ukraine: In Tech. 112-115.

[100] Sotnikov OS (2008) Statics and Structural Kinetics of Living Asynaptical Dendrites. St. Petersburg. Nauka: In Tech. 397 pp. In Rus.