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CELL TECHNOLOGIES IN BIOLOGY AND MEDICINE

Binucleated and Multinucleated Neurons are Formed by Fusion

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In the era of molecular biology and atomic force microscopy, some important macroscopic issues such as simultaneous bidirectional axonal flow or neuronal multinucleosis remain unaddressed. However, these issues have to be addressed, because they distort the results of our current achievements. Using videorecording technique, we studied adhesive contacts between neurons and their processes and kinetics of anastomosis retraction between the cell bodies up to their complete fusion with introduction of neurites into the cell cytoplasm and formation of binuclear cells. Three proofs refuting the mechanism of binuclearity formation by amitosis are presented. Live trinuclear neurons without signs of amitotic division were identified. Electron microscopy showed that fusion of many living neurons into one simplest during centrifugation of isolated cells.

Key Words: *binucleated neuron; multinucleated neuron; neuronal fusion; neuronal symplast; fusion of nerve processes with the soma*

Binuclear neurons were discovered by Robert Remak in rabbits after Johannes Muller described dikaryons of other cells. Since then, 180 years have passed, but we still do not know how two nuclei appear in one neuron. Many people think that binuclear neurons are formed via amitosis (repeated mitosis). Later, these unusual cells were studied by about a hundred authors, including well-known neurogistologists [6,13,14,27]. Further studies have shown that such neurons can be detected in many pathological conditions, such as paralysis, schizophrenia, senile dementia, prolonged tremor, ganglioma, as well as under normal conditions [8,9,10,14,15]. This suggests that the process of binuclearity is non-specific [12]. Binuclear neurons can be found in mammals, fish, invertebrates, and humans [19,20]. Binuclearity precedes the formation of multinuclearity [24]. Neurons containing 7 and 9 nuclei were described in the sympathetic ganglia of patients with pulmonary tuberculosis. In general, dikaryons were found not only in the nervous system, but also in many other organs and tissues [18,23].

As this intriguing process is related to fundamental physiological problems of the nervous system *in vivo* and seems to be directly related to neural pathology, we studied the kinetics of binuclearity generation in closely contacting living neurons.

MATERIALS AND METHODS

In vivo studies were performed on ganglia of 35 *Lymnaea stagnalis* mollusks using a phase-contrast video microscope. Neurons were isolated from the ganglia by treatment with 0.4% pronase. We obtained 24 two-nuclear and 3 three-nuclear live neurons (0.165%) from 16,339 neurons studied. After 30-min centrifugation of the isolated neurons at 30 rpm, the cells

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were placed in a tissue culture. Among 6561 cells, 30 binuclear cells (0.459%) were found. These cells were fixed in a glutaraldehyde solution in a 0.1 M cacodylate buffer (pH 7.4) at 40°C for 1.5 h. After dehydration in ethanol solutions of ascending concentration, the material was embedded in an araldite mixture. Ultrathin sections prepared on an LKB-5 ultratome were stained with triple Reinhold contrast. The sections were examined and photographed under an LEO-10 electron microscope (Carl Zeiss). Neurons isolated from 55 *Lymnaea stagnalis* mollusks were cultured in RPMI-1640 medium for 5 days. The kinetics of neuronal fusion was studied under an MBI-13 phase-contrast video microscope (LOMO).

RESULTS

First, we studied spontaneous bi- and multinuclearity of live neurons of dissociated ganglia by phase-contrast microscopy (Fig. 1). As is seen from our observation, amitosis is hardly applicable to trinuclear cells, but they sometimes had two and three merged axons. These observations demonstrate that fusion of neuronal membranes is possible between both the bodies of neurons in warm-blooded animals and neuronal processes in invertebrates.

Isolated live mononuclear cells begin to fuse in ~10 min after making contact. The contact surfaces of these cells form a single flat surface, and the contours of the cell bodies form an 8-shaped structure (Fig. 2, *a*). The gap between the merged neurons is clearly seen

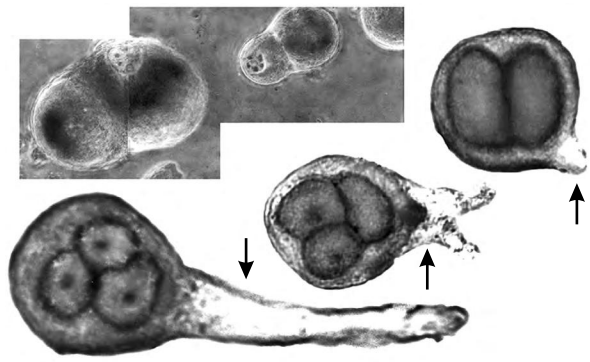

Fig. 1. Tri- and binuclear fused live neurons of the brain of *Lymnaea stagnalis* mollusk. Live-cell imaging. Phase contrast. Ob. 40Ph, eyep. 10. Arrows show fusion of two and three processes in bi- and trinuclear cells.

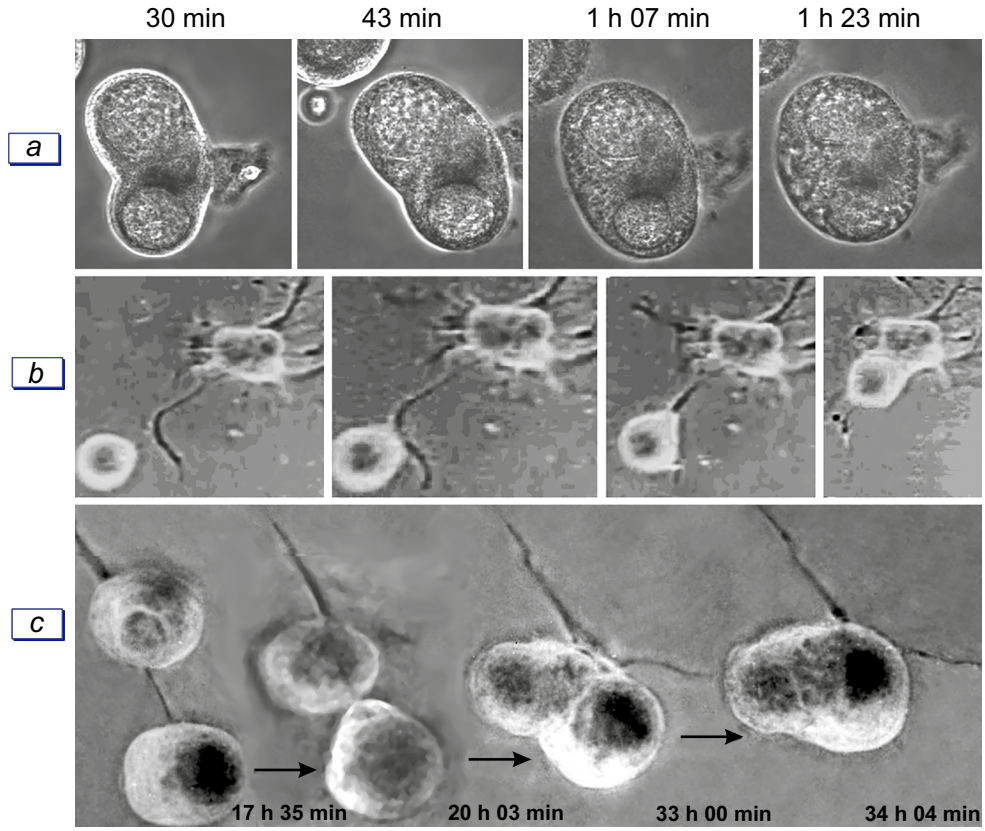

Fig. 2. Kinetics of fusion of single living neurons in tissue culture. Vital phase-contrast microscopy, ob. 40Ph, eyep. 10. *a*) Formation of a living spherical cell from an 8-shaped merging cell structure; *b*) fusion of a nerve process with the body of a neighboring cell, retraction of the process, and contact of two bodies before fusion; *c*) retraction of the process, contact of two neuronal bodies, and their fusion in the tissue culture.

under an electron microscope. During fusion, it breaks up into many vacuole-like fragments arranged in a single row (Fig. 3, *b*). Cytoplasmic bridges connect the neuroplasm of two cells (Fig. 3, *a*) and a dikaryon is formed. Thus, we can experimentally obtain a simplast from many closely contacting neurons (Fig. 4, *b*). An alliance of two nuclei and fused neuroplasm is clearly seen in ultramicroscopy, but there are no signs of mitosis in the nuclei (Fig. 4, *a*). This is the second proof of fusion, but not division of nerve cells.

We believe that the final convincing proof of the mechanism and kinetics of neuronal fusion can be obtained from the video showing isolated neurons growing in tissue culture (Fig. 2). The contact of a single nerve process with the body of a nerve cell is visible. Adhesion of membranes of the body and the process causes rapid retraction and straightening of the free end and entire process, which leads to contact and beginning of fusion of cell bodies connected by anastomosis (or commissure) diverging after division of daughter cells. In fact, in living neurons, they are retracting anastomoses of neurons that form a binuclear neuron (Fig. 2, *c*). According to Gibbs' law of minimal surface energy, fused dikaryon automatically changes its 8-shaped configuration to O-shaped one and turns into a typical spherical cell resembling a mononuclear neuron. Figure 2, *b* shows the stages of adhesion and process retraction during fusion of living cells. The contact between the cells is accompanied by flattening of the contact surface and fusion of neurons. Therefore, we believe that the reduction of living neurons, visualization and electron microscopy demonstrated a new physiological process of neurolemma contact and neuronal fusion. We think that these data provide three absolute proofs of the mechanism underlying the formation of binuclear and multinuclear neurons by their fusion. This also refutes the current idea of amitosis as a secondary (repeated) wave of mitosis.

The fact that binuclear cells can be formed by the fusion of neurons and glial cells should be considered as further evidence that binuclearity is a cell-wide property, the result of fusion, also inherent in neurons. Nuclei in cell hybrids cannot be duplicated due to mitosis, they can only occur as a result of fusion [5,21,22].

Thus, *in vivo* video studies revealed live trinuclear cells under normal conditions of their formation, which excludes the possibility of amitosis, because mitosis leads to the formation of only two daughter cells. Second, we demonstrated that individual live neurons extracted from the brain of a mollusk can merge and form dikaryons and multinucleated symplasts. This is

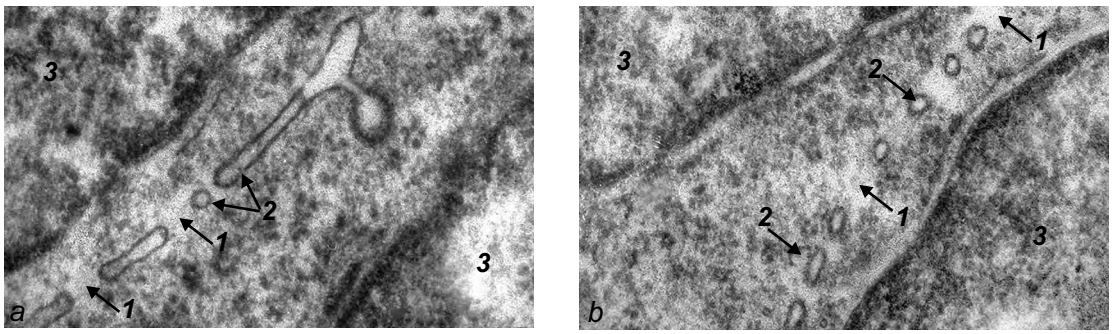

Fig. 3. Electron microscopy image of two binuclear cells upon fusion of two mononuclear neurons, ×80,000. *1*) Cytoplasmic bridges; *2*) residual vacuole-like structures in the intercellular gap; *3*) dikaryon nuclei.

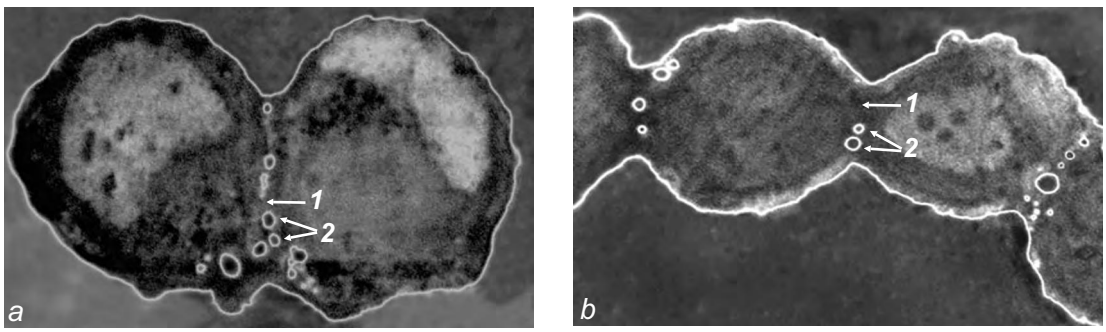

Fig. 4. Experimental fusion of neurons after treatment with 0.4% pronase (10 min). Electron microscopy, ×10,000. *a*) Fusion of two neurons forming a dikaryon visualized by computer embossing; *b*) symplast formation visualized by computer solarization. *1*) Cytoplasmic bridges of syncytial cell fusion; *2*) chains of vesicles as residual structures of the intercellular gap.

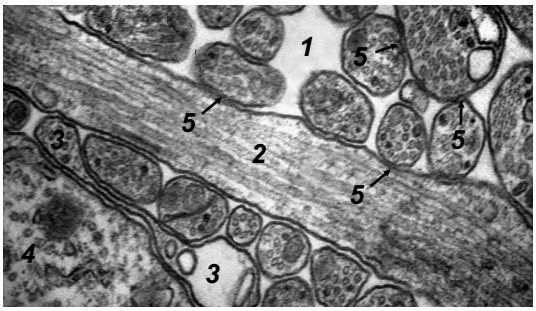

Fig. 5. Glia-free nerve fibers of the ganglion of the mollusk after treatment with 0.4% pronasa. Electron microscopy. ×10,000. *1*) Empty spaces earlier filled with processes of glial cell processes; *2*) microtubules; *3*) contracted stump of a glial cell process after its amputation; *4*) neuron; *5*) gap junction.

an absolute proof of the phenomenon of binuclearity as a result of fusion of differentiated neurons. The kinetics of cell contact and formation of dikaryons by cell fusion has been experimentally demonstrated on cultured neurons.

Fused cells with binuclear heterokaryons are used to treat patients with multiple sclerosis and other diseases [11,16,17]. The binuclearity of hybrid neurons also confirms membrane adhesion and fusion of neurons. The phenomenon of binuclearity was used to introduce healthy nuclei or functional genes into damaged cells [9,16]. In light of recent progress in the field of stem cell transplantation, fusion of neurons with bone marrow mesenchymal cells [7,26] and cortical neurons with microglial cells [4,25] is widely used. However, for a serious analysis of the physiological and pathological role of the described phenomenon, additional studies are needed.

Binuclear and multinuclear neurons are described in almost all types of pathologies, therefore, there is no doubt that this is a non-specific effect of hundreds of pathological processes. It is also known proteolytic enzymes and their modulators participate in all types of cellular pathology do not participate. As the neuronal bodies and neurites are normally protected by layers of glial processes, the neuropathological processes, apparently, should begin from the neuroglia. Our experiments on the effect of pronase (complex 14 proteases) on the nervous system of rats, frogs, leeches, and mollusks revealed primary damage (retraction and degradation of gliocytes) (Fig. 5). In denudated contacting neurons [1,2,24], connexin gap junctions and syncytial perforations appear. The neurolemma is exposed and possibly traumatized. In face of the problem of pathology, the cell "finds" a natural way out — contact with the membrane of the neighboring neurite. Membrane adhesion easily connects their shells, while opening of the gap junctions and syncytial perforations form neuroplasmic bridges that unite the neuroplasm of both cells. According to the Gibbs law of minimum surface energy, the 8-shaped structure of merging cells transforms into a ball with minimum surface at the same volume, that is, the thermodynamically most stable binuclear neuron, which has a reduced surface of possible damage and an increased number of mitochondria — energy sources of cell neuroplasm. Naturally, this process can well be considered therapeutically positive spontaneous cure. In live trinuclear neurons, fusion of some processes is clearly seen (Fig. 1). A unique nonanuclear neuron in the sympathetic ganglion was previously described [3]; this neuron had a complex of 8 normal and one regenerating process. This suggests that the cells described in the article live and regenerate. Electrophysiological studies of neurons treated with pronase, purified from gliocytes, and already having electrical fusion, demonstrate reverberation activity, that is, even ability to cognitive functions [1]. All of this implies the formation mechanism of neuronal dikaryons, multinational and new ways of research in medicine.

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