Heterokaryon Formation as a Method for Neuron Regeneration in Postischemic Injury to Cerebral Cortex in Rats

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Rat prefrontal cortex was examined by light and electron microscopy after stroke induced by photothrombosis. An appreciable number of binuclear neurons with morphologically similar and different nuclei was detected in the perifocal zone and adjacent intact tissue. The satellite oligodendrocyte nucleus was frequently the second nucleus in binuclear neuron. Control specimens also had binuclear neurons, but their number was much lower. It is hypothesized that neuron fusion normally and after injury is a manifestation of physiological and reparative regeneration of these cells.

Key Words: *cell fusion; neuron; heterokaryon; nervous system regeneration*

Neuronal stem cells were revealed in the bone marrow on the verge of the millennium [3,5,7]; these cells are initially present there or form by transdifferentiation; this discovery led to revision of the concept assumed by that time, according to which mammalian neurons stop proliferate soon after birth and regenerate only intracellularly. All revising studies were carried out by the transgender transplantation method. If a transgene (most often green fluorescent protein, GFP) is found several months after transplantation in a brain cell expressing neuronal markers, this cell is considered as a neuron formed *de novo* from a stem cell by direct differentiation or due to plasticity. Similar findings were documented in the analysis of material from dead women receiving male bone marrow transplants, the Y-chromosome serving as the transgene [6,12]. Later (in 2002-2003) several publications noted that the "classical" evidence of plasticity (presence of a stem cell transgene in a differentiated cell) proved nothing, because another explanation of the phenomenon was possible: the stem cell did not transform into a definitive cell and created differentiation markers by itself, but just fused with a differentiated cell and acquired ready markers without passing the appropriate developmental pathway and developing these markers by itself [2,4,8-10, 13,14].

Attempts at solving the problem at a new level consisted in creation of more sophisticated modifications of the same transgender method. The transgene was introduced not only in donor, but in recipient cells as well; another variant was to introduce into the recipient genome a construct, reaction with which was the only way for the donor transgene to manifest. No *de novo* formed neurons were found in these experiments, only fusion of stem cells with neurons [2,13].

The results of all the above experiments were analyzed by fluorescent or confocal microscopy. We failed to find reports on the use of electron microscopy for the analysis of a neuron formed *de novo* or by fusion, a binuclear heterokaryon. The value of studies on intact brain is that they showed

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a possible way of physiological regeneration of neurons: fusion with a cell, which the authors called a stem cell. However, even considering the fluorescent and confocal microscopic images presented in these reports completely reliable, we have to admit that they only prove that cell fusion really can take place under certain conditions, impossible outside so complex an experiment. It is impossible to study the mechanisms of fusion under natural conditions by the transgender models, because these models are very artificial and far from actual health and disease conditions. Lethal irradiation, used in these experiments, massive doses of immunosuppressants, transplantation of great volumes of foreign cells into the blood (but not in their natural niche), despite the immunological pressing, surely distort the common regeneration schemes and can modify the cells participating in the process. All this is true for clinical observations. In addition, the study is impossible, if a female recipient was transplanted female bone marrow.

This study was undertaken to clear out whether cells do fuse in reparative and physiological regeneration in cases, when all the above-listed distortions of common health and disease processes are ruled out.

MATERIALS AND METHODS

Experiment was carried out on 9 outbred male rats (200-230 g). Ischemic stroke was created in 5 animals by a method described previously [11]. The animals were injected with a fluorescent stain (Bengal Rose), the periosteum above the right prefrontal zone was exposed under narcosis, and a site 3 mm in diameter was irradiated with a light beam (λ=560 nm) for 20 min. Under these conditions, bengal rose damages the endothelium at the site of irradiation, aggregates platelets, and they occlude the vessel. Damage to the prefrontal cortex by the method of photothrombosis leads to the formation of a local ischemic infarction involving the entire thickness of the cortex, which is clearly separated from the adjacent intact tissue by a well-discernible borderline [1]. Controls were subjected to the same operation without the dye.

Seven days after irradiation, the animals were narcotized and the brain was perfused with 2.5% glutaraldehyde. This was followed by standard fixation and embedding in epoxy resin for electron microscopy. Semithin sections were stained with toluidine blue, ultrathin sections were contrasted with uranyl acetate and lead citrate. The preparations were examined under an Axiostar plus microscope (Carl Zeiss) and LEO 912 AB OMEGA electron microscope. Fused neurons in the examined section were counted under a light microscope.

RESULTS

Many neurons and glial cells at the site of injury were necrotic and degenerative, which was seen from deformation and loss of cytoplasmic structures. Binuclear neurons with nuclei of similar structure (dikaryons) and of different structure (heterokaryons) were seen in the semithin sections from the ischemic and perifocal zones. The incidence of binuclear neurons in this zone was one per 18,900± 1100 μ^2. Binuclear neurons were more rare in the left cortex: one per 91,500±3450 μ^2 (4.8 times more rarely than in the damaged cortex) and still more rare in the control.

Electron microscopy of the same specimens showed that it was impossible to differentiate with certainty fused cells from cells closely adhering to each other, but separated by the plasma membranes, by analyzing semithin sections in a clear field. Sometimes two neurons were so closely pressed to each other, that the interface between them could not be discerned on a semithin section, and they seemed to be a dikaryon, while electron microscopy showed the difference (Fig. 1, *a*, *b*).

A stem cell is called the second fusion partner in transgender experiments, though it is in fact not proven. The only thing known about this cell is that it carries a donor transgene. The results of our pilot studies, carried out (in contrast to transgender experiments) only on cells of one individual, showed that at least in some cases the neuron fused with a satellite oligodendrocyte (Fig. 2). The motive forces of fusion could be so potent, that the resultant cells were not binuclear, but multinuclear neurons (Fig. 3).

The increase in the number of binuclear neurons in the zone of brain injury was comparable to the control and suggested that fusion was a way of reparative regeneration of neurons. During the formation of heterokaryon the second nucleus is reprogrammed by the neuron cytoplasm for the neuron-specific function, this increasing 2-fold the number of genes maintaining this function. Our observations support the idea put forward by D. S. Sarkisov suggesting that CNS neurons regenerate not by cell multiplication, but by amplification of the number of cell structures. On the other hand, fusion of neurons is a heretofore unknown way of intracellular regeneration, developing the regeneration theory and the cellular theory. One more mode of cell-cell interactions was detected: a cell exhausted by functional loading or injury can receive a potent material (structural) support from the

neighboring cells: the second genome. This way of regeneration seems to be biologically the most rational for neurons. Really, if a neuron separates from a previously existing one and appears in the cortex, then, in order to become a properly func-

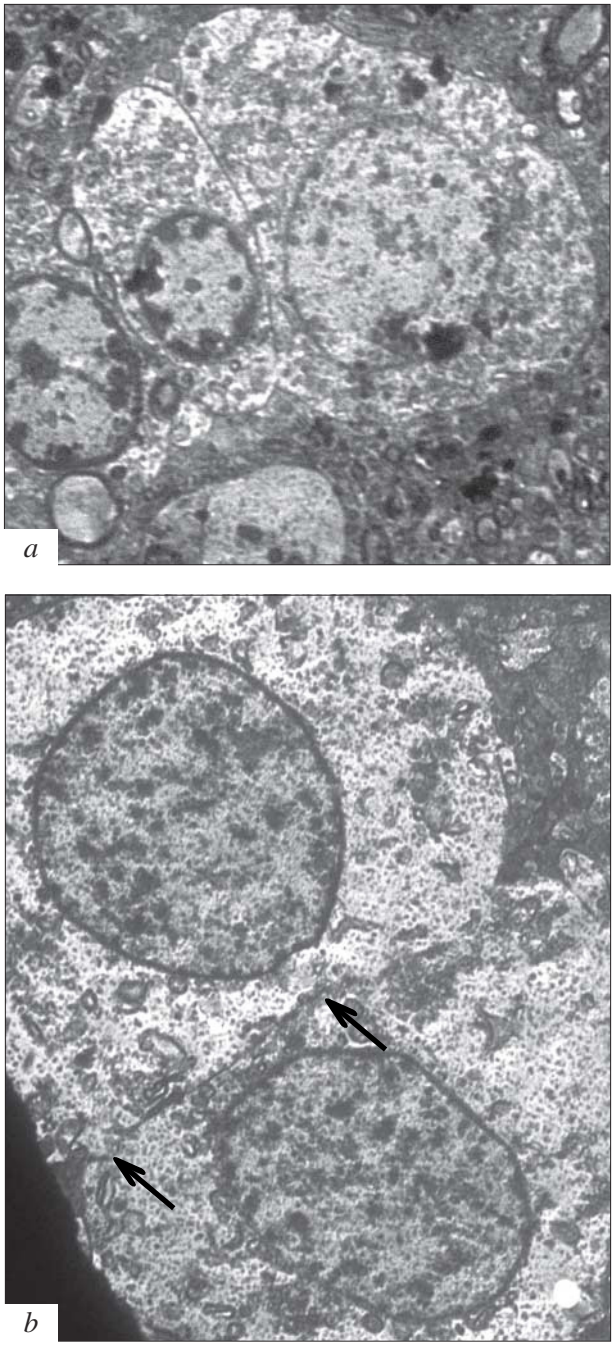

Fig. 1. Electron microscopic analysis of closely lying neurons, seen in an optic microscope as a dikaryon (×10,000). *a*) electronogram shows membrane between the cells, indiscernible in an optic microscope and indicating that there are two mononuclear neurons; *b*) electronogram shows fusion of an appreciable part of cytoplasm of two neurons, with the membrane between the cells retained in a just limited area (shown by arrows); in other words, this image shows a dikaryon.

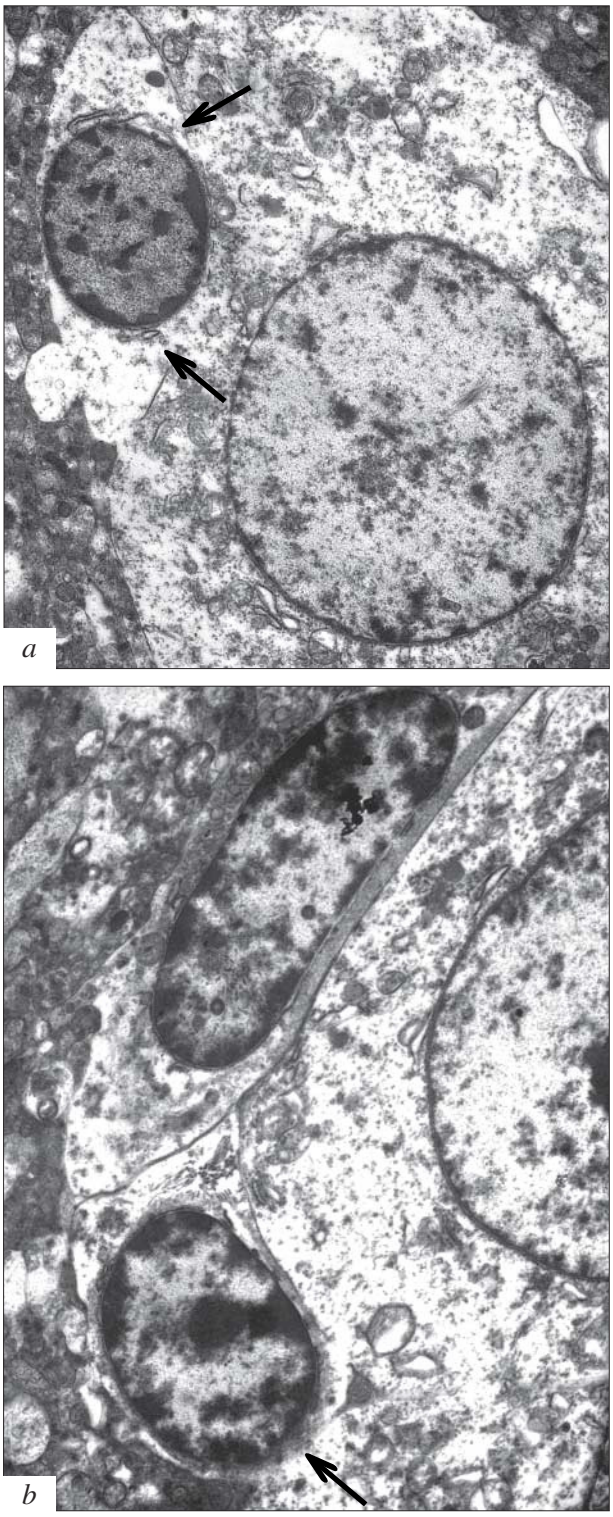

Fig. 2. Neuron fusion with a satellite oligodendrocyte. *a*) beginning of fusion: oligodendrocyte nucleus is still surrounded by its own cytoplasm, but the interface between the cells has already disappeared at the site shown with arrows (×15,000); *b*) one of the two neuron satellites is an isolated cell, the nucleus of the other (shown by arrow) retains the marginal position, but the cytoplasms of oligodendrocyte and neuron have fused (×17,000).

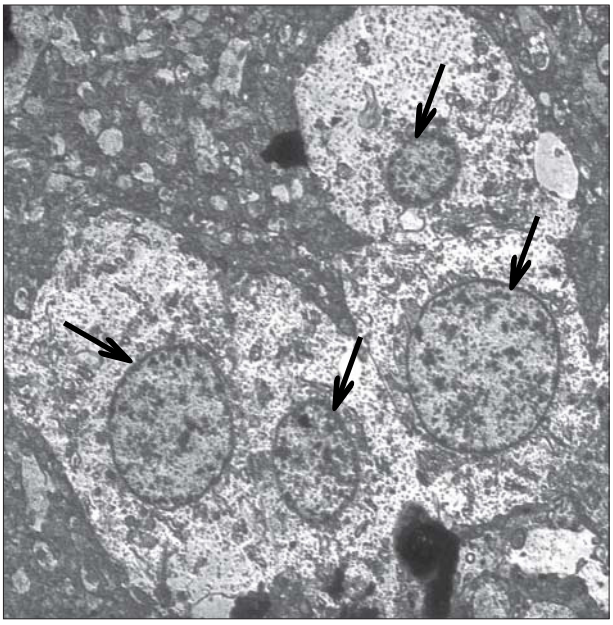

Fig. 3. Fusion with formation of a tetranuclear neuron (×7000).

tioning neuron, it has to establish thousands of contacts with other neurons. This seems impossible in an adult nervous system, which is already formed, with its intricate dense network of neuronal and glyocyte axons; at least, this cannot happen very soon. Fusion is presumably what happens to a damaged, but still viable neuron (otherwise it is impossible). The function of a cell can be restored and even enhanced directly after re-programming of the second nucleus, if the cell retains not all of its relations, but an appreciable number of them and gets such a support as the second genome.

The presence of binuclear neurons in the control suggests that physiological regeneration is realized by the same mechanisms which more markedly (quantitatively) manifest in disease. The fact that physiological regeneration of the cortex is realized by cell fusion gives us hope that when we clear out the mechanisms of fusion and the way to regulate them, it will be possible to delay age-specific changes in the nervous activity.

Since the method for detection of heterokaryons used in our study involves no intervention of any kind (as in transgender transplantations) and is based on analysis of specimens (biopsy or autopsy), it can be used without modifications of any kind for the study of regeneration of the nervous system in humans.

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