Indicators of Neuritis Growth and Retraction in Tissue Culture and Histological Specimens

O. S. Sotnikov, G. I. Rybakova, L. I. Archakova, and V. G. Lukashin

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The dynamics of structural processes in cultured neurons was studied by time-lapse video recording and compared with the diversity of morphological structures in fixed preparations. Ellipsoid terminal structures indicate not the growth, but growth arrest of neurites and the start of their retraction (retraction bulbs). Growth cones are the only indicators of neuritis growth and development. Autotomy of individual terminals is a normal process and does not indicate neural pathology. The "neuroplasm protuberances" on fixed preparations do not indicate neuroplasm extrusion, but reflect an extreme state of retracting processes. The formation of varicosities results not from swelling, but from redistribution of the neuroplasm and often accompanies its contraction. Disruption of neural contacts and complete invagination of neurites into the cell body are not indicative of neuronal death. These cells can form new processes and make new interneuron contacts.

Key Words: *neuron culture; retraction of processes; growth cones; retraction bulbs; terminal autotomy*

The kinetic branch of histology was not properly developed because rapid structural rearrangements cannot be studied by histological examination of fixed preparation. Some problems can be solved indirectly using cell culture technique [10] and intravital microscopy of transparent invertebrates or transparent organs from vertebrates [4]. In neurohistology, these methods cannot completely replace routine morphology of fixed preparations, which can examine any organ and tissue.

Our aim was to search for unequivocal indicators of structural processes by examining fixed preparation needed for the study of structural dynamics in culture.

Laboratory of Neuronal Functional Morphology and Physiology, I. P. Pavlov Institute of Physiology, Russian Academy of Sciences, St. Petersburg. ***Address for correspondence:*** sotnikov@kolt.infran.ru. O. S. Sotnikov

MATERIALS AND METHODS

The study was carried out on cultured neurons from proteolytically dissociated ganglia of adult mollusks *Lymnaea stagnalis* and on total preparations of feline intestine. The neurons were cultured in serum-free RPMI-1640 medium [1]. Developmental processes in individual neurons and forming neural plexuses in the culture were studied by time-lapse video recording using computer-assisted phase-contrast microvideo setup. The observation period for one culture was 5-6 days. The marks on figures show the time from the start of video recording.

The structural dynamics of neural processes observed in culture was compared with a variety of morphological structures examined in individual neurons of intramural intestinal nervous plexuses, which in many respects resemble plexuses formed in culture. The total preparations of feline intestine were impregnated with silver nitrate after

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Bielschowsky—Gros, cleared, and processed routinely [5].

RESULTS

The growth cone was the obligatory terminal structure of the growing fibers revealed in culture at the very onset of regeneration of neuron processes (NP; Fig. 1, *a*). It looked like a small tri- or polygonal lamellar structure with several sharp projections and many filopodia resembling a goose-foot (*pes anserinus*). This structure unequivocally attests to the growth and regeneration of the neurite. By contrast, fibers with arrested growth demonstrate shortening of filopodia and rounding (balling) of terminals. During this process, the nerve fiber contracts and its growth cone turns into a retraction bulb (Fig. 1, *a*). During examination of the fixed preparations, both the growth cones and retraction bulbs are clear indicators of the two important and opposite events. In many neurological and neuropathological studies numerous retraction bulbs observed in fixed preparations of a damaged nerve were interpreted as the formation of efferent terminals [2] or as a sign of massive and intensive growth [6], while we considered these structures as an unequivocal indicator of growth arrest and start of NP shortening and contraction. The contraction feature of NP is a complex non-electrical function of the neuron [3,11].

Retraction is an important function of individual neurons and neural plexuses under normal and pathological conditions. This function is realized via actin-myosin AII interaction [7,14]. The retraction rate of NP varies from 0.36 to 3.36 μ/min, which does not depend on the cell size and corresponds to the regeneration rate of the nerve fibers. Retraction can be easily visualized during damage to neurites (Fig. 2, *a*). This process is involved into the formation of diastasis in cut nerves, which is frequently observed during neurosurgery. For prevention of dramatic development of diastasis, retraction should be decelerated. There are numerous papers devoted to activation of neurite retraction or termination their growth [8], while inhibition of NP contraction is little studied except our work [12]. NP is a labile structure. In the same growing neuron, the retraction bulbs periodically emerge together with growth cones. Under normal conditions, the growth cones are easily transformed into retraction bulbs and vice versa (Fig. 1, *b*). In histological preparations of intramural intestinal nervous plexus of healthy adult cats, the growth cones and retraction bulbs are persistent terminal structures (Fig. 1, *c*). For example, they were revealed in dendrites of type II Dogiel cells (17% cases). There is an unstable equilibrium between the appearance of these structures, but growth cones prevail at the initial stages of culturing, while the number of the retraction bulbs increases dramatically in response to homeostasis disturbances (elevated temperature, medium exhaustion *etc*.), which reflects intensification of NP contraction.

An important consequence of retraction of live substrate-attached NP is the process of self-amputation, in which the terminals are detached from the initial processes (Fig. 3, *a*). This amputation occurs only with retraction bulbs and only during activation of NP contraction. It should be emphasized that self-amputation of terminals is normally observed in fixed preparations of intramural intestinal nervous plexus of healthy adult cats (Fig. 3, *b*). In fixed histological preparations, individual ball-shaped amputated cytosomes can serve as the markers of active *in vivo* retraction process instead of pathological one. However, generalization of this process is observed during pathological alterations of neuronal environment. In culture, repeated autotomy of the same neurites is observed, where ruptures appear not only in retraction bulb vicinity, but also proximally in segments between the varicosities located distantly from the terminal parts of the processes. When fused, the varicosities form degenerative spheroids. In this way, the tissue events transform into Wallerian degeneration (Fig. 2, *b*). In this context, so-called neuroplasm protuberances should be noted (Fig. 2, *d*). This structures were observed in neurohistological preparations and interpreted as extrusion process with cellulofugal flow of the neuroplasm. The comparison of these structures with changes in cultured neurons (Fig. 2, *c*) shows that these features should also be considered as manifestations of NP retraction, which is a cellulopetal process. Despite cell deformation clearly indicating cell pathology, these neurons were found in the intestinal plexus of healthy adult cats. Probably, the pathological processes in individual nervous elements do not necessarily mean pathology in the entire organism or organ. It is also true for the extreme retraction of the damaged fibers. Although contraction of these fibers frequently terminates in complete invagination into the neuron body (Fig. 2, *a*), it does not necessarily mean the death of neurons. By contracting its process, the neuron decreases its surface (*S*) under the same volume (*V*) thereby diminishing the thermodynamic index of structural instability *S*/*V*. The resulting spheroid shape is the most stable structure, which helps neuron to survive. After the spheroidization period, the cultured neurons usually regenerate new NP.

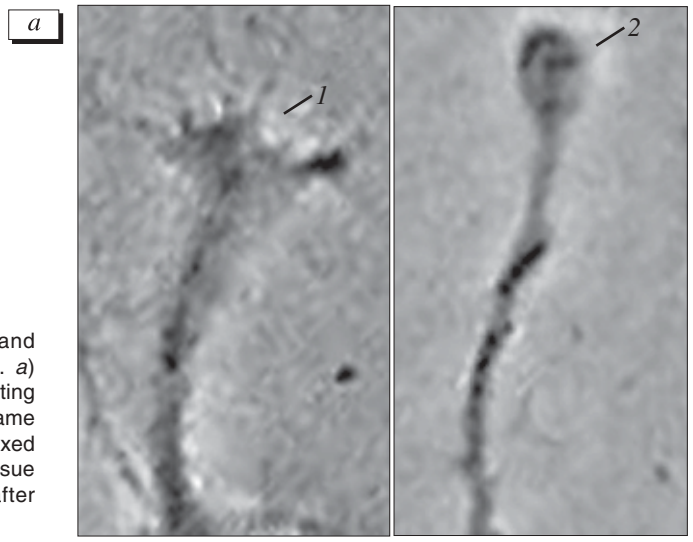
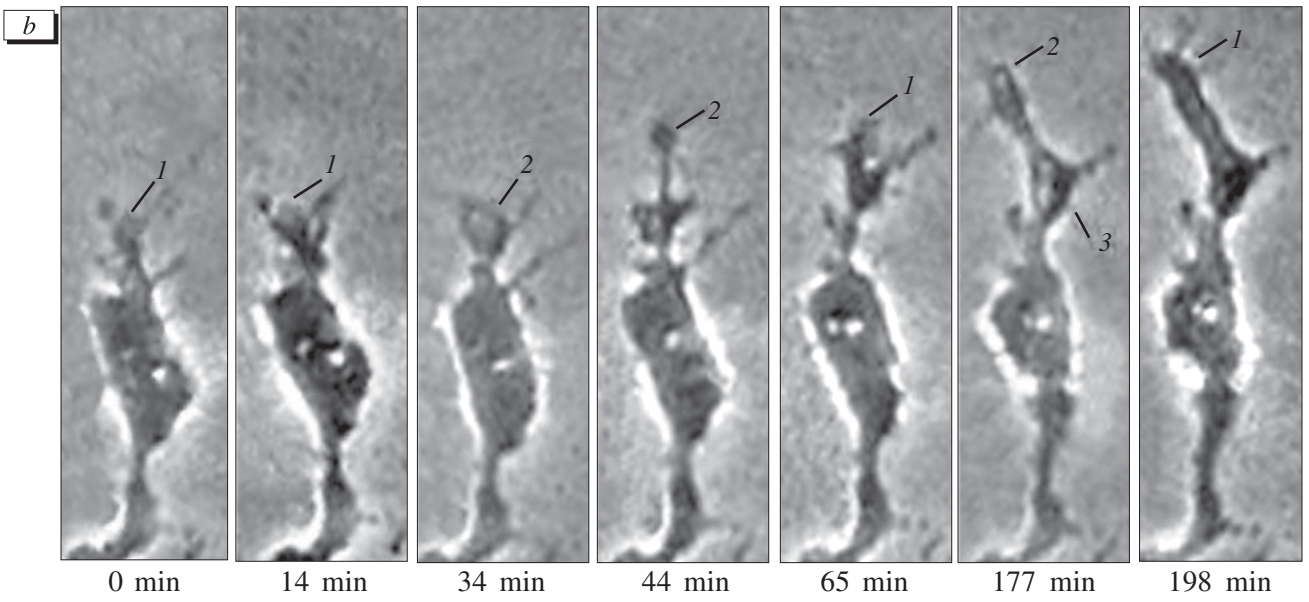
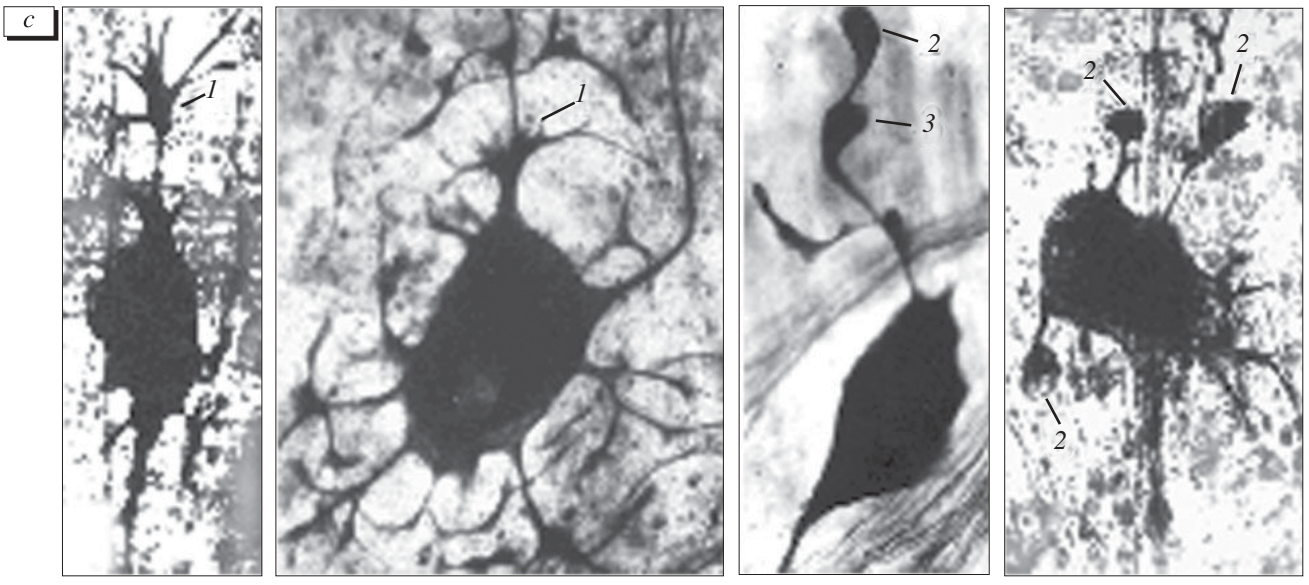


Fig. 1. Growth and retraction of neurites in tissue culture and markers of these processes observed in fixed preparations. *a*) growth cone (*1*) and retraction bulb (*2*) in tissue culture; *b*) remitting dynamics of growth cones (*1*) and retraction bulbs (*2*) in the same NP in culture; *c*) growth cones (*1*) and retraction bulbs (*2*) of fixed neurons from intestinal nervous plexus; *3*) varicosity. *a*, *b*) tissue culture, phase-contrast microscopy, ×200; *c*) impregnation after Bielschowsky—Gros, ×400.





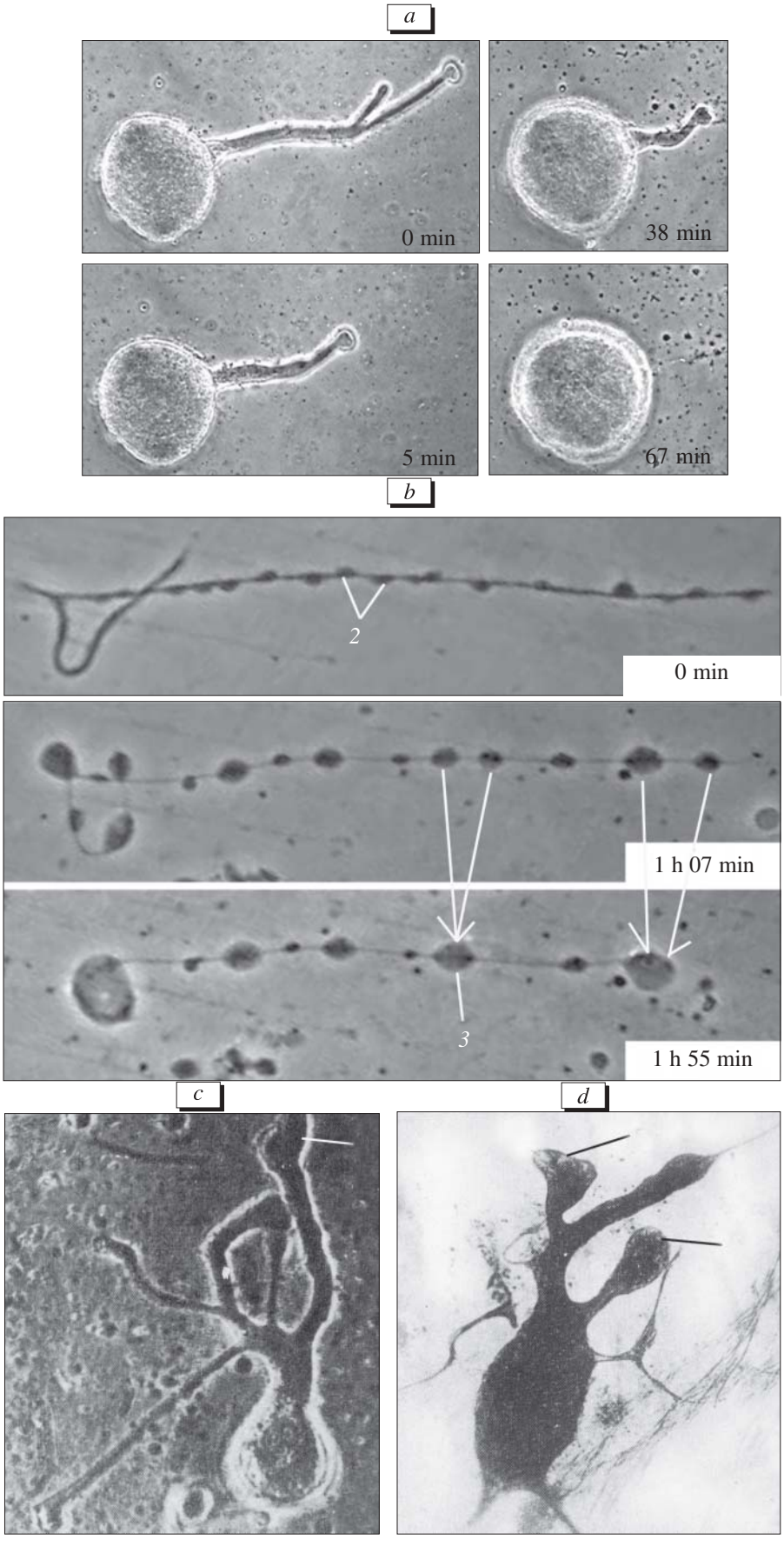


Fig. 2. Morphological phenomena of extreme retraction of NP. *a*) contraction stages of traumatized NP; *b*) transformation of varicosities (*2*) into degenerative ovoids (*3*) via fusion of varicosities (arrows); *c*) neuroplasm protuberances (*1*) in live neuron; *d*) neuroplasm protuberances in fixed neuron from intestinal plexus. *a*, *b*, *d*) intravital phase-contrast microscopy, ×200; *c*) impregnation after Bielschowsky—Gros, ×400.

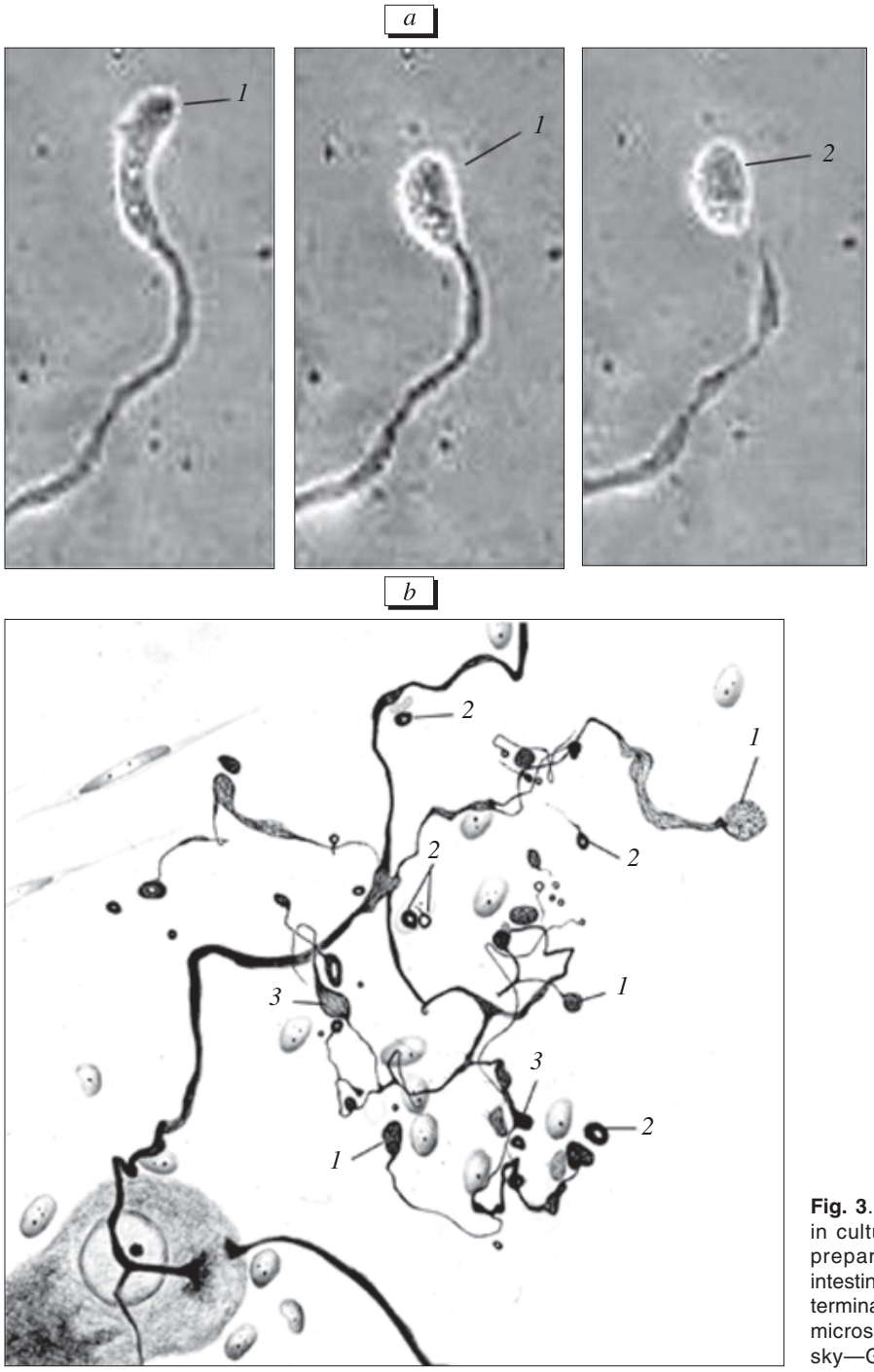


Fig. 3. Stages of self-amputation of a nerve terminal in culture (*a*) and markers of this process in fixed preparation of type II Dogiel cells isolated from intestinal plexus (*b*). *1*) retraction bulbs; *2*) amputated terminals; *3*) varicosities. *a*) intravital phase-contrast microscopy, ×200; *b*) impregnation after Bielschowsky—Gros, ×400.

Of particular importance are bead-like ellipsoidal varicosities in NP. It was demonstrated for the first time in live cultured neurons that these varicosities initially appear on the terminal parts of nerve branches [5]. These varicosities appear because of thermodynamic instability of fine nerve branches, which are characterized by a large relative surface *(S/V)*. This ratio grows in an inverse proportion to fiber radius and indicates its structural instability. The varicosities on fixed preparations can also indicate easily reversible processes of morphological, thermodynamic, and physiological changes in nerve fibers (Fig. 3, *b*). However, the varicosities are routinely seen under normal conditions in very fine preterminal fibers, where they are indicative of structural instability. During pathology, massive formation of varicosities is frequently accompanied by changes of their shape from oval to spheroid (Fig. 2, *b*) and rupture of the fiber between the beads. This phenomenon is a clear marker of

transition from reversible transformation of NP to degeneration of neurons. It should be remembered that the formation of varicosities on CNS dendrites is always accompanied by disappearance of spines [13]. Now it can be concluded that this phenomenon is related to general contraction of entire dendrite structure [9].

F. Nissle was the first who tried to determine the state of a neuron by certain indices on fixed preparations. He termed certain pathohistological pictures as "equivalents" of the reversible or irreversible pathology in contrast to the markers of specific intravital processes [11]. The "dark contracted" cells revealed by Nissle staining do not attest to constriction of real live cells. Our special video recording studies showed that only those cells became contracted after fixation, which intravitally demonstrated detachment of submembrane watered layer of the neuroplasm or its swelling. This study revealed no intravital contraction of the neurons and formation of varicosities. From the time of Golgi, this phenomenon is viewed as an equivalent to the reversible pathology in the nervous system, although some researchers, when trying to reveal the development of varicosities based on static histological preparations, consider it as a varicose swelling or varicose atrophy [13]. From our point of view, both concepts are erroneous. The intravital video studies carried out on isolated fibers [5] or on tissue culture showed that the development of varicosities is not swelling or atrophy, but redistribution of liquid fraction of the cytoplasm from one part of the NP (which becomes thinner during this process) into the swelling neighbor parts.

Thus, our study of intravital tissue culture and comparison of our results with the data obtained on fixed preparations not only revealed the signs indicating some changes in the nervous system, but also allowed us to use these morphological markers for identification of processes and their underlying mechanisms. Such approach enhances the diagnostic potency of the pathohistological inferences and helps to correct some theoretical errors of neuromorphologists.

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