REVIEWS

Subsynaptic Units as a Universal System-Forming and Regulating Factor of Brain Synapses

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Dense projections and postsynaptic density — specialized structures of the synaptic cytoskeleton — are regarded as a single functional system of subsynaptic units. From this viewpoint, interneuronal relations in health and after brain ischemia are analyzed. It is shown that hyperplasia and recombination of the system of subsynaptic units are the major mechanisms responsible for reorganization of functionally mature interneuronal junctions in the mammalian and human brain.

Key Words: *synapse; synaptic cytoskeleton; dense projections; postsynaptic density*

The relationship between form and content and between quality and quantity at the level of interneuronal relations are of particular importance, taking into account the role of synapses in neuronal plasticity and the molecular biochemistry, immunohistochemistry, and electron microscopy data [4,7,16,24,38,41, 47,65]. Basic structural components and biochemical composition of virtually all brain synapses have been identified. They include: 1) pre- and postsynaptic membranes with receptors and ionic channels and pumps, 2) synaptic cleft substance providing adhesion, transport, and deactivation of neurotransmitters, 3) intrasynaptic structures: simple (synaptic vesicles) and more complex vesicular (various vacuoles, rimmed vesicles, and multivesicular complexes) and filamentous-granular-vesicular (dendritic spikes), and 4) specific structures of presynaptic (dense projections) and postsynaptic (postsynaptic density) cytoskeleton [3,14,47,63,82]. Although large body of evidence has been collected, the problem of integrating structure(s) of the synapse unsolved [6].

The idea that paramembrane structures of the cytoskeleton is a single functional system of subsynaptic units (SSU) that integrates the activity of the entire synapse was put forward in the 1970s [1,5]. The search for the system-forming factors and their structural equivalents at the level of interneuronal junctions and new electron microscopic data on the structure of paramembrane zones in synapses [14,18, 22,30,44,70] led to the need of interpreting the contribution of dense projections (DP) and postsynaptic densities (PSD) to impulse transmission across synapses [1] in structural and functional terms. It was demonstrated that pre- and postsynaptic structures are structurally and functionally continuous and operate as a single complex [1,5]. Molecular mechanisms by which all synaptic structures are integrated in a system of discrete information transfer between neurons have been elucidated [3,24,27,34,47,63,71, 77,82].

New information regarding synaptic function and the need for morphological indicators of integrative-triggering activity of the brain motivated our interest into the idea of the SSU in the light of recent findings [7-11,13]. We have formulated a concept of the SSU [7,12] and applied it to the analysis of specific pathological and functional states of the brain [7-10,75]. According to this concept, a SSU that was created over a long period of natural selection, is an autonomous subsynaptic structural/functional system common to all brain synapses and

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designed for integrating the activity of the entire synapse and for realization of discrete unidirectional transsynaptic transfer of neurotransmitters in strictly proportioned quantities (quanta). This function of the SSU is based on its structural continuity and asymmetric structure and on the principle of reciprocal antagonistic relations between its pre- and postsynaptic parts. Such a function is accomplished through spatial reorganization of SSU resulting from successive local (the areas of Ca^{2+} concentration fluctuation) conformational changes in filamentous components of the DP and PSD and in general cohesive properties of pre- and postsynaptic zones united into a single cycle and regulated by Ca^{2+}. Quantitative characteristics (information content) of each synapse are determined by the size and shape of the SSU and by the location of the synapse in the neuron, while the type of synapse (inhibitory or excitatory) is defined by receptors on pre- and postsynaptic membranes [7,12].

1. Basic molecular and spatial organization of the SSU

Structurally, the components of SSU, which can be detected by special methods (staining with phosphotungstic acid), look like granular-fibrillary paramembrane entities of presynaptic, postsynaptic, or mixed origin in a functionally mature chemical synapse (Figs. 1 and 2). In the presynaptic zone, the SSU is represented by DP or protrusions that make up a presynaptic lattice [22,35,55,53,80]. We regard this lattice as an integral part of SSU but not as an independent structural/functional entity artificially singled out in the early studies of synapses; in some studies the sum of synaptic vesicles was referred to as synaptic complex [70] or active synaptic area [30]. Recent studies showed that presynaptic lattice cannot effectively function and even exist unless it is linked to the PSD [3,24,27,47, 63,82].

Dense projections are specific cytoskeletal structures of the terminal having stable physical links with the presynaptic membrane and unstable links with the surrounding filamentous network and synaptic vesicles [27,63]. The fibrillary network (cytoskeleton) of the terminal consists of filaments 3-12 nm in diameter and 35-180 nm in length; the organelles of the synaptic terminal are confined to this network, and their mobility is determined by the conformational state of filamentous proteins and local Ca^{2+} concentration [22,28,52,53,56,68,69,73]. Presumably, DP have a similar, but more dense structure. The filaments of DP and unorganized cytoskeleton are linked to synaptic vesicles by the protein synapsin I, while the contact of synaptic vesicles with presynaptic membrane during exocytosis occurs through sequential incorporation into the proteins synaptogamin, synaptobrevin, syntaxin, and synaptoporin; these links are also regulated by Ca^{2+} [2,3,19,27,28, 50,52,67].

Owing to conformational changes in these proteins caused by calcium-dependent phosphorylation and dephosphorylation, the filamentous network and DP of the terminal can act as a contractile substrate that modulates the motility of organelles and synaptic vesicles and is regulated by Ca^{2+} and ATP [71,72].

The cytoskeleton and DP of the terminal contain actin-, myosin- and tubulin-like proteins [2,3,15,28, 33,37,71,72,78]. In cross sections of synapses prepared after selective contrasting of the brain with alcoholic solution of phosphotungstic acid [7,11,17, 22,35,47,54,63,80], DP look like triangular (pyramidal), oval, and round or polyhedral (globular) structures [7,11,17,35,47,55,63]. In some studies, heterogeneous staining of DP was noted, their center being less electron-dense than the periphery [57, 59,63], suggesting that different portions of DP occur in different conformational states during synapse functioning or that DP consist of distinct subunits [7,12,47].

In a homogeneous synaptic population, functionally mature DP vary from 20 to 80 nm in height and 35 to 100 nm in the width of their base, and the distance between the centers of adjacent DP is about 81 nm [7,47]. This implies that the shape and size of DP can be altered only to certain limits during their functioning in normal or pathological states [7,11,17,18,35,47,57,63,79,80].

In the SSU, DP are arranged in a hexagonal or trigonal sequence providing up to 3 binding sites for synaptic vesicles per DP. Small synapses have up to 7 DP, medium synapses up to 19, and large synapses up to 37 [80]. The hexagonal arrangement of DP corresponds to the arrangement of intramembrane "anchor" particles demonstrated by the freeze-fracture technique in the presynaptic membrane at the DP level. These particles and filamentous proteins from the spectrin family ensure an orderly attachment of DP to the neuronal plasma membrane and to other components of SSU [2,3,15,16,77].

In the postsynaptic zone, the SSU is represented by a PSD or postsynaptic complex [14,24,27,34,64]. Similar to PSD, presynaptic lattice performs its function exclusively within the SSU. According to the degree of development, synapses can be divided into two groups: with well-defined (type I) and poorly-defined PSD (type II) by Gray [44]. Component *a* of PSD, which has the highest electron density, is located under the postsynaptic membrane, and it is followed by component *b* of medium electron den-

sity. A third possible component of the PSD may be the round subsynaptic bodies whose electron density is high in the center and low at the periphery. Subsynaptic bodies are linked to component *b*, occur at equal distances from the postsynaptic membrane, and form the postsynaptic lattice. The most common synapses are those with prominent *a* and *b* components [47]. The mean thickness of PSD varies from 20 to 60 nm in different synapses. The thinnest PSD are found in axospike synapses of the cerebellum and the thickest, in those of the cerebral cortex [7,10,43,47]. The thickness of synaptic PSD is not critical for defining the type of synapse; chemical composition of the PSD and the receptor abundance on the postsynaptic membrane are of greater significance in this respect. However, due to the strong influence on the SSU shape PSD determines the function of postsynaptic membrane under normal and pathological conditions [2,3,7,10,16,47].

The PSD has a mixed fibrillary and granular composition. The basic element of all SSU is a filamentous network associated with the postsynaptic membrane [27,63]. The fibrillary/granular composition of PSD in synapses may considerably differ from one part of the brain to another [43,45]. Generally, component *a* of the PSD consists of filaments 4 nm in diameter and component *b* of filaments up to 9 nm; the network of these thicker filaments extends to 100 nm into the postsynaptic portion of the synapse and is structurally associated with its cytoskeleton and organelles [27,34,47,62]. Subunits 18 nm in diameter were identified in a PSD by negative staining [64]. Between the subunits consisting of thin (4 nm) filaments, thicker and short branching fibers forming a net-like structure of the SSU are present, as well as filament-free islands of material composed of fine granules [64,81] containing biologically active substances (protein kinases, calmodulin, receptor proteins) [24,27]. In the PSD area, the postsynaptic membrane carries discrete intramembrane anchor particles that occur within receptor systems. Each particle is connected to fibers of the PSD network [27,46,51,77]. A 43 kD protein mediates immobilization and attachment of receptor molecules to the subsynaptic cytoskeleton [25]. PSD fibers fix and limit the movement of postsynaptic membrane receptors and render the entire SSU more rigid, the degree of rigidity depending on conformational state of filamentous proteins in PSD [47,49].

Thus, the presence of discrete intramembrane structures, which were demonstrated by freeze-fracturing [77], in the postsynaptic membrane and their association with PSD filaments via special proteins attest to structural continuity of the PSD and postsynaptic membrane.

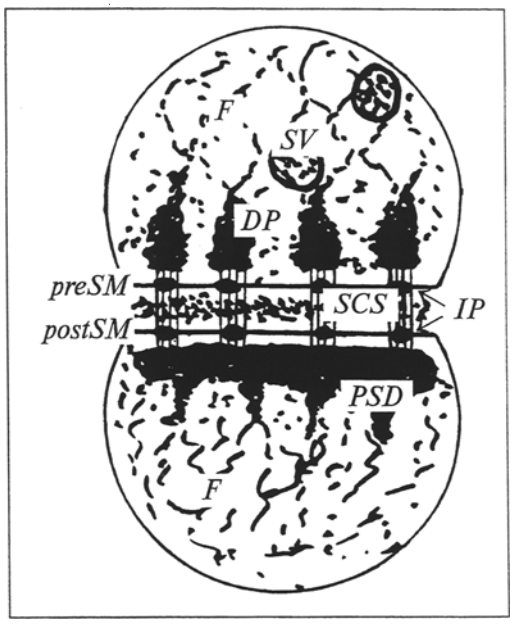

Fig. 1. A scheme showing organization of subsynaptic units in an interneuronal chemical synapse. *DP*) dense projections; *PSD*) postsynaptic density; *SCS*) synaptic cleft substance; *preSM*) presynaptic membrane; *postSM*) postsynaptic membrane; *SV*) synaptic vesicle; *F*) filaments of the synaptic cytoskeleton; *IP*) intramembrane anchor particles.

An individual PSD consists approximately of 15 major and 10 minor proteins [23,47]. These proteins are: basic 50 kD protein, actin, tubulin, phosphoprotein I, high-molecular-weight microtubular protein, cAMP- and calmodulin-dependent kinases, adenylate cyclase, phosphodiesterase, and various glycoproteins [33,39,43,45,52,61,64,66,69]. The basic protein is probably a subunit of calmodulin-activated protein kinase [20,42,60,61]. The contents of these proteins vary in a wide range in PSD of different synapses. For instance, the content of basic protein in the cerebellar cortex synapses is 5-fold lower that in PSD of cerebral cortical synapses [45].

The pre- and postsynaptic filamentous regions of the SSU are spatially integrated by hexagonally arranged intramembrane anchor particles of the pre- and postsynaptic membranes and by glycolipid and lipoglycoprotein molecules in the glycocalyx of contacting membranes (substance of the synaptic cleft) (Figs. 1 and 2); of special importance are gangliosides [2,3,16]. On transverse sections stained with phosphotungstic acid, the synaptic cleft substance is 11.1±0.2 nm thick [48]. The structural bond formed by fused elements of pre- and postsynaptic glycocalyx is very strong, it is not disintegrated by ultracentrifugation, and is preserved in synaptosomes [3,16].

Basic organization of SSU described above attests to structural continuity and reciprocal relationships between presynaptic cytoskeleton and DP, presynaptic membrane, synaptic cleft substance, post-

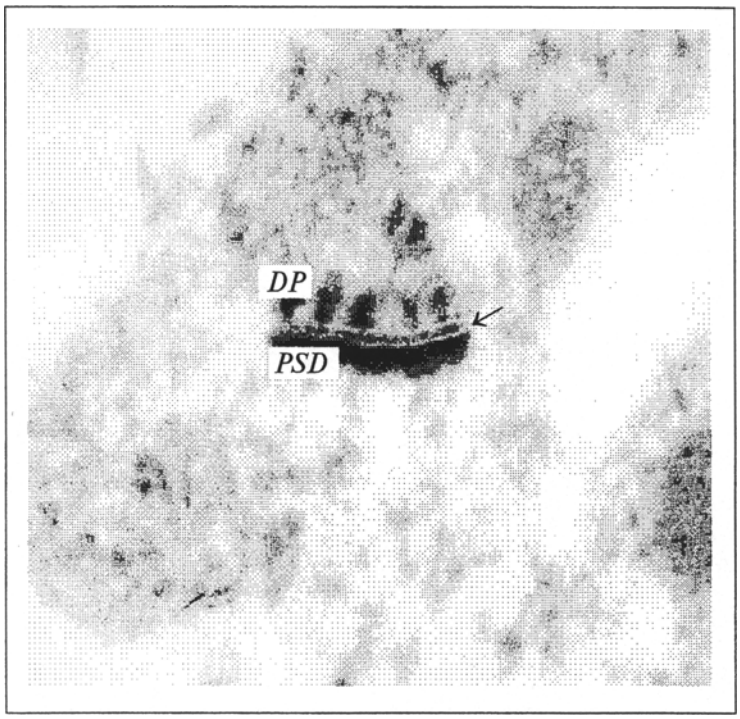

Fig. 2. The system of subsynaptic units in an axospike synapse of the cerebral cortex after staining with phosphotungstic acid; ×56,000. *DP*) dense projections; *PSD*) postsynaptic density. The arrowhead points to synaptic cleft substance.

synaptic membrane, PSD, and postsynaptic cytoskeleton. The shape of SSU is determined by the condition of PSD as the largest and stable "felt-like" structure the bulk of which is in a gel-like state [7,10,47].

Two groups of SSU have been identified: SSU of axospike synapses with thick PSD in the cerebral cortex and SSU of axospike synapses with thin PSD in the cerebellar cortex. In each group, all synapses can be divided into small, medium, and large depending on the SSU size (<200, 200-600, and >600 nm in diameter, respectively); according to the shape of the SSU, they can be divided into disk-like, ring-like, and horseshoe-like synapses and those with a more complex perforated SSU [7,24, 34-36,47]. Modifications in the functional state of synapses are manifested physically as alterations of the height and shape of DP as well as type and degree of curvature of the junctional plane, while plastic reorganization of synapses involves an increase in the SSU diameter and shape change [7-13,27,34-36, 47,79,80,82].

2. Spatial recombination and hyperplasia of SSU as a basis for the functioning of synapses and for qualitative changes in interneuronal relations in health and disease

Pathological and functional states of the synapse are reflected in particular shape of SSU, and transition from one functional state to another is accompanied by conformational changes in all components of this system [7,10,47,79]. The life cycle of a synapse can be divided into the following stages: 1) formation of presynapse, 2) functional maturation and differentiation, 3) functioning in the ordinary mode with no alteration in the SSU volume, 4) reorganization after activation with possible formation of autonomous synapses on the basis of functional hypertrophied junctions, 5) conservation as a result of considerable elevation of the activation threshold, and 6) destruction [7,24,32,34,36,71,74,75,79]. Each stage is associated with specific alterations of SSU [7,10,34-36].

At the stage of presynapse formation (1), the elements of SSU can be detected only after completion of adhesive processes occurring between pre- and postsynaptic membranes. Dot-like structures of PSD appear at first; later they expand [7,34,36,47]. The growth of the PSD is paralleled by the emergence of presynaptic paramembrane filaments, and the junction then acquires a desmosome-like symmetrical appearance [35]. In the brain of adult mammals, presynapses constitute up to 30% of all junctions [7,35]. The stage of functional maturation and differentiation (2) involves the formation of distinct DP in the presynaptic zone. Dense projections are of low height in the least mature synapses and may reach a height of 50-60 nm in fully differentiated synapses. Transsynaptic information transfer occurs only in synapses with functionally mature SSU [7, 35,47]. The processes of stages 1 and 2 proceed continuously, being most active during maturation of the brain in ontogeny and during the period of enhanced compensatory and reparative reactions oc-

curring after partial damage to the mature brain [7,24,29,34-36,47].

We compared the responses of two above-mentioned structural/functional types of SSU to anoxia and subsequent recovery of systemic circulation. As the objects for comparison, we chose functionally mature axospike synapses of the molecular layer in the cerebral and cerebellar cortex. The chosen synapses of each type were similar regarding their function and location on dendritic tree and differed from those of the other type by the shape of SSU, internal molecular organization, and the ratio between components *a* and *b* of PSD [7-13]. The reactions of SSU at stages 3, 4, 5, and 6 have been identified.

During anoxia, prolonged (>10 min) "activation" in most brain synapses results from depolarization of pre- and postsynaptic membranes [21,50,53], while recovery of systemic circulation led to repolarization of synaptic membranes and restoration of the initial state of synapses [7,10]. This can be regarded as a modeling of the functional cycle of mature synapse considerably extended in time. Our results indicate that there is a great variety of SSU organization, and the responses of axospike synapses of cerebral and cerebellar cortex to anoxia are different [7,10,13]. The character and degree of the curvature of the junctional plane directly depend on the shape of the SSU and on the relative volume of pre- and postsynaptic material in the SSU [10]. The curvature varies widely from positive to negative in synapses with thick and uneven PSD of the sensorimotor cortex, while in synapses with thin and smooth PSD of the cerebellar cortex the curvature variations were positive [10]. This indicates that the stage of synaptic functioning (3) includes phases of flat → positively curved (the degree of curvature of a junction depends on the diameter of the SSU) → negatively curved → flat junctions in axospike synapses of the cerebral cortex, but only the phases of flat → positively curved → flat junctions in those of the cerebellar cortex [7,10]. Such differences are of major significance for synaptic functioning because the phase of negative curvature involves approximation of DP tops, which results in blocked transmission of the next pulse in the zone of negative curvature [79], and corresponds to activation of postsynaptic area [12].

Presumably, the forces generated when filamentous proteins of PSD undergo conformational changes in cerebral cortical axospike synapses upon the appearance of a postsynaptic potential and anoxic depolarization, impart to the SSU a shape in which the release of subsequent synaptic vesicles is blocked in the area of negatively curved contact. In synapses of the cerebellar cortex, however, this additional mechanism of transsynaptic exchange regulation does not operate so that the amount of transmitter substance released when synapses are activated is determined only by the SSU diameter and practically does not depend on the state of the postsynaptic portion of this system [10]. In such synapses, the postsynaptic portion remains in the activated state longer, and conditions are created favoring excitotoxicity of excitatory neurotransmitters released in excess during brain anoxia. The absence in cerebellar synapses of the above-mentioned mechanism blocking the excess release of excitatory neurotransmitters may account for the high susceptibility of Purkinje's neurons to damage during the postresuscitation period [10].

Thus, changes only in the shape of SSU provide for considerable variability of the axospike synapse functioning and striking differences in their response to brain anoxia. Therefore, the predominance of SSU of particular shape in synapses of afferent inputs may be a cause of a low activation threshold of neurons and their selective vulnerability during postischemic or postresuscitation period.

Functional or compensatory/restorative activation of neurons occurring in various states of the brain transfers synapses from the ordinary mode of functioning (stage 3) to that (stage 4) when interneuronal relations are reorganized by the mechanisms responsible for hyperplasia and recombination of SSU elements [7-13,24,47]. The ordinary mode of synaptic functioning (stage 3) is accompanied by conformational changes in filamentous proteins of pre- and postsynaptic zones with subsequent changes in the synaptic junction curvature, while the SSU volume remains unchanged. This functional cycle of synapses includes the following morphological stages: 1) high-threshold resting state characterized by a flat junction and a gel-like state of the SSU throughout its volume, 2) lowering of the threshold for synapse activation, which involves a slight positive curvature of the junctional plane with part of the external volume of DP passing to sol-like state, 3) exocytosis of synaptic vesicles characterized by greater positive curvature, more profound conformational changes in filamentous proteins, and local pressure elevation promoting directed movement of synaptic vesicles, and 4) activation of the postsynaptic part in synapses with thick PSD accompanied by transition of component *b* to an sol-like state and by initial restoration of the gel-like state in DP; the only change in synapses with thin PSD is a decrease in the positive curvature, and 5) restoration of gel-like conformation in the entire SSU and of the high-threshold resting state. If activation of neurons continues, stage 5 is not completed and the synapse will enter stages 2 and 3 and remains at these stages until exhaustion or hyperplasia and recombination of SSU [7,10,12,

32,58,79]. Neuronal inactivation leads to stabilization of the gel-like conformation of filamentous proteins in the SSU (flat junction) and to their transition to a state of long-lasting "conservation".

The transition of synapses to stage 5 after activation probably represents a structural form for a long-term storage of information recorded by the brain [7,24,47,79]. Stage 6 involves Ca^{2+}-dependent proteolysis of synaptic cytoskeleton, DP, and PSD, the loss of their contours and homogenization of internal structures, decrease in the height of DP, and the maximum positive curvature of junctional planes in the cerebellum axospike synapses [7,10,47,53,56,68].

Prolonged activation of synapses in stage 4 triggers the mechanisms leading to hyperplasia of their structural elements via Ca^{2+}-dependent incorporation of various protein kinases and stimulation of protein synthesis [21,31,40,73]. Our analysis of hypertrophic SSU with mixed type of curvature of the junctional plane showed that the number of synaptic vesicles that can be released at a time increases from 1 in synapses with SSU diameter <500 nm to 3-4 and 6-7, respectively, in synapses with SSU diameter <700 and <900 with a corresponding increase in the synapse efficiency. This illustrates the transition of quantity (increased volume of the SSU) into quality (increased synaptic efficiency) [7]. However, the volume of SSU in active synapses can increase through hyperplasia only to a certain (critical) value, after which a "qualitative leap" occurs, which is accompanied by recombination of SSU, and the system acquires a complex spatial organization. This may be due to the fact that during local conformational transitions of the SSU from gel-like to sol-like state, the vector of cohesive forces acting in pre- and postsynaptic areas of hypertrophic synapse is such that the more rigid gel fragments of the SSU are caused to diverge laterally, and several functionally active autonomous junctions are formed [7-9,34,36]. In brain hypoxia the probability of such recombinations increases considerably [7]. It should be noted that hyperplasia is an energy-dependent process, whereas no additional energy is required to the SSU to recombine [6,7]. Activation of recombination mechanisms leading to complex synaptic forms can be considered as a means of brain protection directed at lowering excessive local concentration of excitatory neurotransmitters occurring in ischemia [26] and thus preventing them from exerting their excitotoxic action.

Recombination of hypertrophic SSU involves the formation of complex synaptic structures (single and multiple perforated, combined, and independent synapses) and of qualitatively new interneuronal relations (enhanced divergence or convergence) [7,24, 34,47]. The time factor is an important characteristic of hyperplastic and recombinational processes. The former processes are of relatively long duration and energy-dependent, while the latter exhibit a rapid leap-like transition to a new quality without substantial quantitative or energy changes, but only in the presence of a hyperplastic process. As SSU becomes more hypertrophic, its structural stability decreases, leading to recombination. Such alterations of synapses illustrate some aspects of the recombination theory developed by Sarkisov [6] and confirm special contribution of recombinations to the emergence of qualitatively new characters in biological systems.

The functional significance of recombinations in SSU lies in the fact that at a given total volume, the area of the contact between presynaptic and postsynaptic neurons and the effectiveness of transsynaptic information transfer in perforated and more complex synapses is greater than in simple hypertrophied synapses [7,8,24,34,47]. Recombination provides favorable conditions for the formation of highly receptive channels for information transfer and, consequently, the establishment of stable functional and pathological brain systems. Furthermore, the abovementioned mechanisms of recombination and synapse cleavage expand more active afferent inputs and replace the less active ones, which substantially alters the way in which the neuron perceives afferent pulses. This is of particular importance in the pathogenesis of various encephalopathies in which no destructive changes are observed in neurons, although integrative and triggering activities of the brain are markedly impaired [7,8]. It is likely that the recombination of hypertrophic SSU and interneuronal relations by the abovementioned mechanisms is a structural equivalent of the information engram-fixed by the brain.

Thus, recombination of the SSU may have a compensatory and restorative significance or be of key importance in the establishment of pathological systems in the brain. In any case, it involves a leap-like transition to qualitatively new interneuronal relations in the brain. Through the joint interdependent actions of the mechanisms by which the elements of SSU become hyperplastic and recombine, the synaptic structure is constantly and automatically tuned to meet the specific functional needs of the brain. Changes in spatial organization of SSU do not affect its basic biological function, i.e., pulsed transsynaptic information transfer. Internal spatial organization of SSU but not the mode of information coding is altered, which may be the general biological system-forming role of the SSU.

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