

Three-Dimensional Synapses and Astroglia in the Hippocampus of Rats and Ground Squirrels: New Structural–Functional Paradigms on the Functioning of the Synapse

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Abstract—Literature data and our own data on the synaptic plasticity and remodeling of synaptic organelles in the central nervous system were generalized. Modern techniques of confocal laser scanning microscopy and serial thin sectioning for *in vivo* and *in vitro* studies of dendritic spines were discussed, including the relationship between morphological changes and the synaptic transmission efficiency, in particular, using a model of long-term potentiation. Various categories of dendritic spines and postsynaptic densities, and also the role of filopodia in spine genesis were analyzed. The serial thin sectioning technique was shown to be most efficient for unbiased quantitative stereological analysis and three-dimensional reconstructions. Using a refined technique of serial thin sectioning and a computer program, giant mitochondria in dendrites of neurons in various regions of the hippocampus were demonstrated. Smooth endoplasmic reticulum was shown to form a continuum with the outer membrane of the mitochondrial envelope. It was assumed that such a continuum ensures calcium ion tunneling, thus favoring the functioning of the intracellular signal transduction system during synaptic transmission. Data were presented on the existence of gap junctions ("electric synapses") at mammalian brain synapses, and also between glial processes and glial cells and neurons. Using our own and literature data, we showed that glial cell processes form a glial network, which could modulate the functioning of the neuronal network. The connection of dendritic spines with the glial network was shown by the example of three-dimensional reconstructions of neuropil volumes in the CA1 area of the hippocampus of ground squirrels in three functional states: in the normothermia, in 2.5 h after the beginning of awakening from hibernation, and in cold torpor at a brain temperature below 6°C. Our own data were discussed on the formation of more than five synapses both on mushroom dendritic spines of CA1 neurons and on thorny excrescences of CA3 neurons. The results of the analysis of the data presented suggest new paradigms of organization and functioning of synapses.

Key words: hippocampus, neuron, glial cell, synapse, gap junction, dendritic spine, postsynaptic density, serial thin sectioning, three-dimensional reconstructions

In 2000, Vinogradova published a review [1] regarding the status of neural science at the turn of the millennium and a change of paradigms of the relation-

ships both between neurons and between neurons and glial cells. We considered it necessary to supplement this information with recent data on synapses ensuring the functioning of the mammalian brain and on the role of glial cells in these processes, which was the purpose of this paper.

Abbreviations: GFP, green fluorescence protein; Arc, activity-regulated cytoskeleton-associated protein; GABA, γ -aminobutyric acid.

In the context of the development of computer technologies and easy access to the Internet, by the present time, a new science—informatics—has practically been established. Achievements in informatics together with advances in biology have enabled establishment of a new branch of natural sciences: bioinformatics, in particular, neuroinformatics. Two main components of the nervous system, namely, neurons and glia, interact via synaptic and other mechanisms involved in the system of controlling behavior, memory, and forgetting. In this context, neuroinformatics is a collection of modern information technologies for studying various levels of the functioning of the nervous system, including a set of computer programs for performing quantitative stereological analysis of synaptic structures and their reconstruction. Unfortunately, the results of quantitative analysis and modeling of various subcellular structures using single sections often lead to misunderstandings because of too large an error [2]. In the literature [1–14], both the significance of unbiased quantitative stereological analysis and the necessity of three-dimensional reconstructions were substantiated in detail. In recent years, IBM PC programs for producing three-dimensional reconstructions were developed, which are relatively simple; are accessible to all; and enable one not only to construct three-dimensional models but also to estimate their quantitative parameters, e.g., the surface area and the volume [4, 5].

Problems of training, memory, and forgetting are conventionally related to the neuronal plasticity, which is based on changes in synapses. The term *synapses* (from the Greek word *synapsis*, which means junction) was coined by Foster and Sherrington [15] and referred to connections between neurons. According to Bailey *et al.* [16, 17], the plasticity can conventionally be divided into two phenomena, one of which is a change in already existing synapses without changing neuronal connections [18], and the other is a change in interneuronal connections proper, which is due to both *de novo* formation and vanishing of synapses [19]. In the late forties, Hebb introduced the concept of the modifiable *chemical synapse*; the chemical synapse is the region of contact of a dendrite and the presynaptic part or *presynaptic boutons* of an axon, which contains vesicles filled with a neurotransmitter, whose release into the synaptic gap activates the postsynapse or a spine. Dendritic spines were discovered more than a century ago [18, 19]. Over the century that has elapsed since then, using various

light and electron microscopy techniques, an enormous body of data on dendritic spines was obtained; however, many aspects of the organization, genesis, and functioning of spines remain unclear. In the Russian literature, publications on this subject are now few; therefore, we considered it important to fill this gap using literature data and our own data.

The hippocampus, which is the most convenient brain structure for studying spines, belongs to the archecortex (old cortex), where the organization of interneuronal connections and the functioning of synapses, in principle, differs little from those in the neocortex (new cortex). As electrophysiological and biochemical studies showed, this is the best studied brain structure, all regions of which are readily identified at the morphological level. Long-term hippocampal sections (slices) are widely used models in neurobiological studies. The hippocampus can be rapidly and easily isolated from the brain, and the identified region can further be used for organotypic cultures [20] and cultures of isolated neurons [21].

In the central nervous system, three types of dendrites are usually divided: spiny dendrites, sparsely spiny dendrites, and smooth or nonspiny dendrites [22]. Dendrites of the first and second types are often difficult to distinguish by light microscopy because of both a small size of spines and a high variability of their number in various regions of extended dendrites.

In recent years, dendritic spines have been investigated in more and more works [8, 11, 23–31]; e.g., in 2000, the entire issue of the journal *Hippocampus* (vol. 10, no. 5) was devoted to reviews of studies of dendritic spines in the hippocampus.

In many works, dendritic spines are still analyzed by the silver or chromium method for neuron staining by the Golgi technique and its modifications [26]. In the late seventies, Peters and Kaiserman-Abramof [32] generalized light and electron microscopic data and proposed a relatively simple classification of spines, which includes three main categories: (1) thin spines, whose length exceeds their diameter; (2) mushroom spines, which have a long or short stem and a large head; and (3) stubby spines, whose length and diameter virtually coincide. In many studies, similarly to this classification, four categories of synapses are distinguished: (1) thin spines; (2) mushroom spines; (3) stubby spines; and (4) shaft spines, where the presynaptic bouton directly contacts the

dendritic shaft. Later, one more category was proposed, which was called the branched spines: two thin spines at the same stem [33]. Sometimes, a simple classification is used [34], which distinguishes only two categories: sessile spines, which correspond to stubby spines; and pedunculated spines, which include thin and mushroom spines. Such terms as axodendritic synapses (corresponding to shaft synapses) and axospine synapses are often used. This is because the central nervous system contains neurons whose dendrites form unique spines, e.g., dendrites of CA3/CA4 and, partially, CA2 hippocampal pyramidal neurons, along with spines of ordinary categories, form the so-called thorny excrescences or simply thorns. According to Chicurel and Harris [3], about 90% of dendritic spines in the proximal region of apical dendrites of pyramidal neurons in the CA3 area of the rat hippocampus are thorny excrescences. Our produced three-dimensional reconstructions of such spines in the CA3/CA4 areas of the ground squirrel hippocampus, these thorny excrescences are short branches of an apical dendrite with ordinary spines on these branches. The fact that they are branches of an apical dendrite is confirmed by the presence of mitochondria in them, whereas mitochondria are always absent from spines of neurons from other regions of the mammalian hippocampus. Owing to such an organization, a large number of spines gather within a limited volume and simultaneously contact several, e.g., five, presynaptic boutons formed by various moss fibers going from dentate fascia neurons (as determined by our own observations using three-dimensional reconstructions of thorny excrescences on the basis of thin sections [35]). Such a specific organization of synapses in the CA3/CA4 areas is consistent with the concept [35] that it is these areas that provide the comparator properties of the hippocampus and the possibility of information processing also at the level of a small part of a dendrite.

Note that the cerebellum also contains small neurons, on whose dendrites the so-called coralline excrescences form, which are spines in the shape of numerous and often long processes [37]. On dendrites of cerebellar Purkinje cells, there is a population of spines, which is relatively homogeneous in shape; spines of this population can conventionally be assigned to mushroom spines [38].

On the surface of spines, thin processes of the postsynaptic membrane, the so-called spinules, are

often found, which penetrate deep into the presynaptic bouton. Our own data and literature data [39] suggest that such spinules can be both in postsynaptic densities and at the periphery of spine heads. The possible function of spinules is usually discussed from the standpoint of their contribution to the synaptic transmission efficiency owing to an increase in the area of contact between the pre- and postsynaptic membranes, in particular, in the formation of new spines [40]; however, no experimental facts have hitherto been presented that would confirm this role of spinules in the synaptic transmission [39]. Note that, in ground squirrel in cold torpor, when the electrical activity of the brain is virtually undetectable, spinules penetrating into the presynaptic bouton can be encountered even on the surface of the dendritic shaft [35].

Specially interesting structures are filopodia, thin processes of the dendritic membrane, which are up to 10 μm long and are filled with actin microfilaments [41]. Filopodia play an important role in the formation of new spines [41–44]. It was shown [43] that, in the CA1 area of the rat hippocampus *in vivo*, filopodia form on the surface of dendrites in the first two weeks of the postnatal period and ensure the search for presynaptic boutons to form new postsynaptic densities with the subsequent transition of filopodia into protospines to eventually form new thin spines. It was assumed [41, 43, 45, 46] that, in the growth and retraction of filopodia in their transition into spines, the cytoskeleton actively participates, whose main component is actin. A detailed electron microscopic analysis of filopodia in the postnatal development of synapses in the CA1 area of the rat hippocampus [43] showed that, in the first two weeks after birth, filopodia are an intermediate in the formation of new dendritic spines. In this context, the main paradigm of neurobiology, which is the active search for a target by the axon (efferent fibers), is supplemented with a new provision, namely, the concept that dendrites are also capable of searching for axons. Unique cases are known where mutant mouse strains were characterized by spines with postsynaptic densities on dendrites of cerebellar Purkinje cells in the absence of granular cells, which, in the norm, form presynaptic boutons [47, 48]. Note also that the introduction of substance P into the cerebral ventricles was reported [49] to have no effect on dendritic spines of Purkinje cells, which contained postsynaptic densities in the absence of contacts with presynaptic boutons of parallel fibers of granular cells. Filopodia are usually

rarely seen on dendrites of mature neurons in the normal brain; however, in organotypic cultures and cultures of isolated neurons, they are easily identifiable by electron and confocal microscopy [27, 28, 31, 50–56]. Surprisingly, there is evidence that the axon is also capable of forming structures similar to dendritic spines [57].

The main paradigm in studying memory mechanisms is related to the phenomenon of long-term potentiation, which was discovered in the late sixties of the XX century by Bliss and Lomo [58] for neurons of dentate fascia of the rabbit hippocampus *in vivo*. The long-term potentiation model is interesting in the context of memory storage: (1) after brief electrical tetanic stimulation of perforant pathway axons, the increased synaptic transmission efficiency at granular cell synapses persists for several days; (2) the long-term potentiation simultaneously requires both the presynaptic activation and the sufficient postsynaptic depolarization; (3) late long-term potentiation also manifest itself in several hours in inducing protein synthesis and are likely to require the transcription of new gene products [30, 59]. Cellular and molecular mechanisms of long-term potentiation are as yet unclear. Currently, three main mechanisms of the induction of long-term potentiation are considered: (1) changes in the state of already existing molecules at the synapse, e.g., changes in the state of phosphorylation of synaptic proteins; however, in this case, it is unclear how posttranslational modifications of synaptic proteins persists for many hours and days; (2) in the induction of long-term potentiation, there can be changes in the molecular composition of already existing synapses; e.g., there is evidence that some receptors are involved in the induction of long-term potentiation, but not in the induction of long-term depression; (3) the induction of long-term potentiation can be related to changes in the morphology of synapses, e.g., caused by changes in the size and shape of dendritic spines, or by the retraction or formation of new dendritic spines.

In the induction of long-term potentiation, a crucial role is played by the neurotransmitter glutamate. Glutamate receptors are usually divided into two basic groups: (1) metabotropic receptors, which are coupled through GTP-binding proteins to the intracellular signal transduction system; and (2) ionotropic receptors—ligand-controlled cation channels, which, according to their main agonists *N*-methyl-D-

aspartate, amino-3-hydroxy-5-methyl-4-isoxazole, and kainate, are divided into NMDA, AMPA, and kainate receptors, respectively. Between glutamate receptors, there is complex cooperation [60].

Note that any modifications or formation of new synapses is directly related both to postsynaptic density proteins [25, 61–63], and to receptors and channels that ensure synaptic transmission and require continuous renewal; an important role in it is played by the spine stem thickness [64] (Figs. 1, 2). One can distinguish three main pathways in which the renewal of the key proteins is possible: (1) synthesis on mRNA located near a synapse [30, 59]; (2) synthesis in the neuronal soma, relatively uniform distribution over all dendrites, and introduction into synapses using special capture mechanisms [65]; and (3) synthesis in the neuronal soma and introduction into specialized organelles—endosomes, which are selectively transported toward synapses [66].

All these pathways do not rule out one another and require special transport systems. For vertebrate cells, two such systems are widely known: (1) movement along actin microfilaments (F-actin) of motor proteins, e.g., dynactin; and (2) movement along tubulin microtubules using such motor proteins as kinesins and dyneins. It is known that motor proteins move owing to ATP or GTP hydrolysis and can transport various proteins packaged into membrane vesicles (the so-called vesicular transport). Both actin microfilaments and tubulin microtubules are characterized by “polarity,” according to which the ends of these filamentous structures are divided into minus ends and plus ends. Typically, the plus end corresponds to the growth zone of these structures. The growth of microtubules is initiated by proteins located at microtubule-organizing centers (centrioles). The microtubule end attached to a centriole is called the minus end, and the opposite end, where the microtubule is assembled, is referred to as the plus end. There is evidence that motor proteins of the dynein family usually move from the plus end to the minus end of microtubules, whereas kinesins move in the opposite direction [67]. The third candidate for a transport system can be intermediate filaments (neurofilaments). However, the role of neurofilaments in the neuronal transport has hitherto been studied poorly.

The next aspect of renewal of synapses is related to the organization of protein-synthesizing systems in

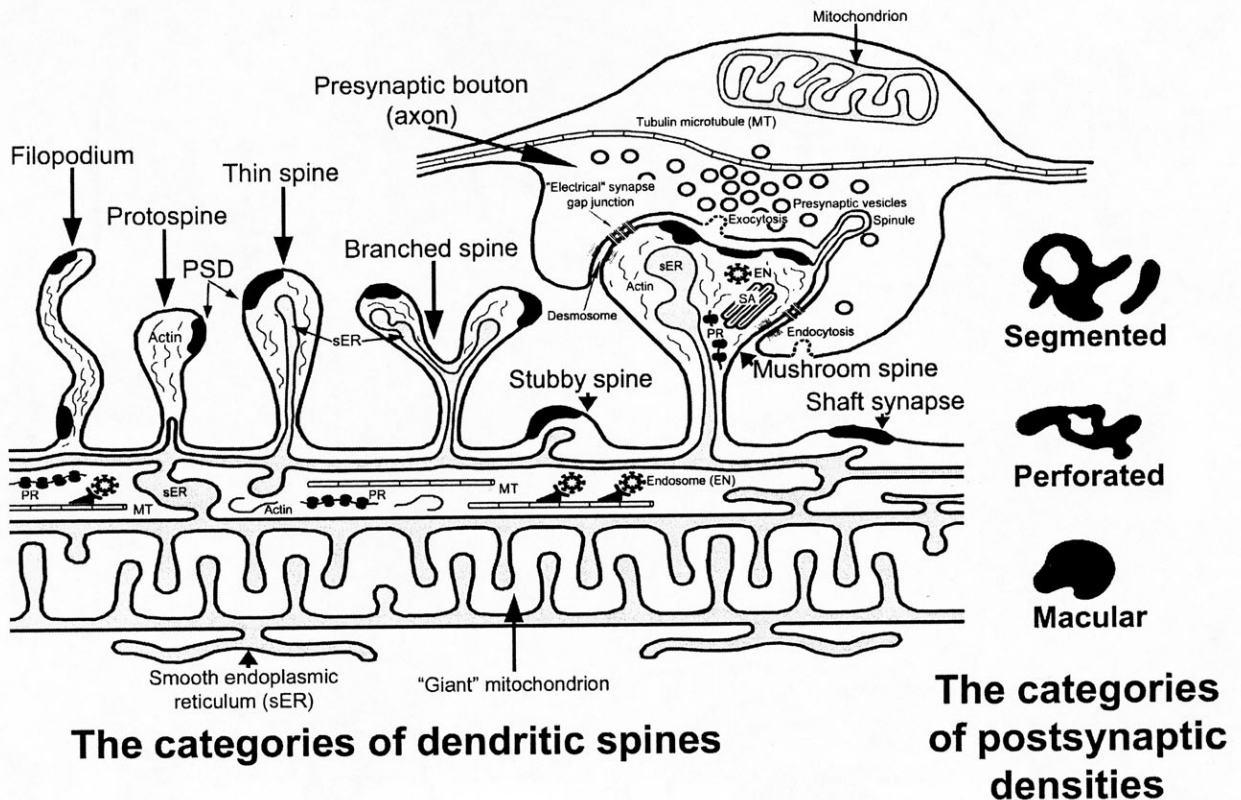


Fig. 1. Organization of various categories of dendritic spines and postsynaptic densities, including the main organelles of synapses: SR, smooth reticulum; MT, microtubules; PR, free polyribosomes; PSD, postsynaptic density; SA, spine apparatus; and EN, endosome.

the neuron. There are two types of polyribosomes, where proteins are synthesized, one of which is free polyribosomes/free polysomes, and the other is polyribosomes connected to endoplasmic reticulum membranes to form such an organelle as the rough endoplasmic reticulum. There is as yet no accurate information on which proteins are synthesized on free polysomes; it is only assumed that they are water-soluble cytoskeletal proteins, which require no maturation in the Golgi apparatus. In recent years, the main attention has been focused on the rough endoplasmic reticulum; on it, "membrane" proteins (e.g., subunits of receptors, channels, etc.) are synthesized, which eventually become integral proteins of the neuronal membrane. Great progress in the understanding of the role of polysomes on the rough endoplasmic reticulum in the synthesis of membrane proteins has been achieved owing to the development of a new technique in Lippincott-Schwartz's laboratory [68, 59]: by the example of the widely known glycoprotein ts045 incorporated in the coat of the vesicular stomatitis virus mutant ts045, in COS cells, a genetic structure was created, which allowed one to attach green fluorescence protein (GFP) to the cyto-

plasmic tail of the glycoprotein and, by confocal microscopy, to track the synthesis region and pathways of transport of a newly synthesized membrane protein in the living cell. The results of these studies are directly consistent with the idea of vesicular protein transport [70, 71], according to which the "membrane" proteins are synthesized on the rough endoplasmic reticulum by incorporating into its membrane, whereupon this domain of the rough endoplasmic reticulum membrane is budded off as a coated vesicle and is transported toward the *cis* compartment of the Golgi apparatus along microtubules, e.g., using the minus end-directed dynein-dynactin motor complex. After maturation in the Golgi apparatus, the "membrane" protein leaves the *trans* compartment of the Golgi apparatus as a part of a coated vesicle and is transported using the dynein-dynactin complex along a microtubule toward the plasma membrane [70]. This mechanism is likely to be universal for all types of mammalian cells in which the "membrane" proteins are synthesized. Dynactin is one of the main components of synapses [72]. For neurons, the main problem is the fact that synapses are relatively far from the soma, where the protein-synthesizing apparatus is

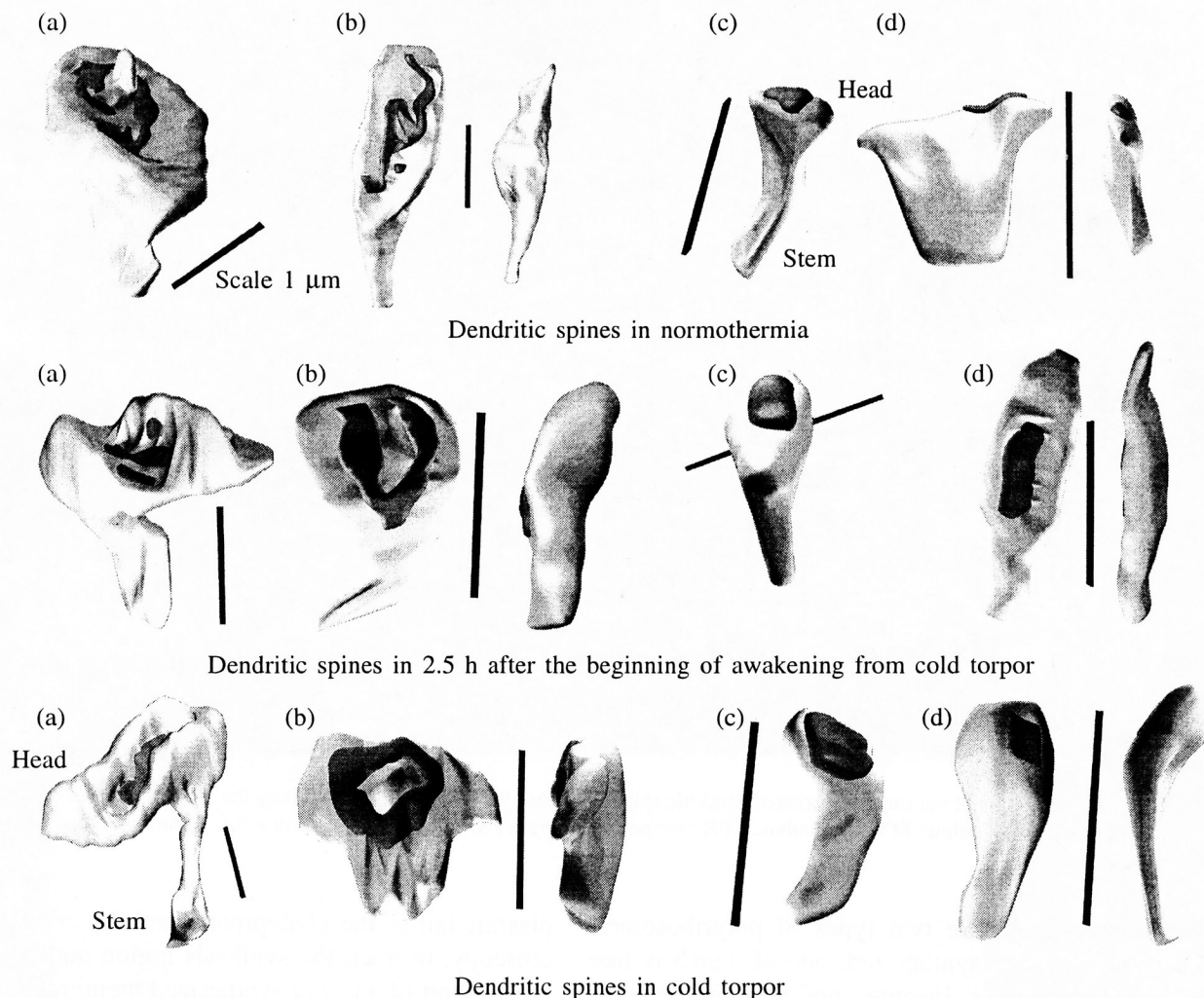


Fig. 2. Three-dimensional reconstructions of mushroom and thin spines on dendrites of pyramidal neurons in the CA1 area of the hippocampus of ground squirrel during hibernation. Typical examples of both (a, c) “bulk” and (b, d) “plane” mushroom and thin spines, respectively. Shaded regions on the spine surface are postsynaptic densities (PSD). The scale for all the spines is 1 μ m. The brain temperature: in the normothermia between bouts of hibernation, 36–37°C; in 2.5 h after the beginning of awakening from cold torpor, 33–34°C; and in cold torpor, 2–6°C.

located, which includes the following organelles: the nucleus, the nucleolus, polyribosomes, the endoplasmic reticulum, and the Golgi apparatus [73–78]. Theoretically, the “membrane” proteins can be transported by lateral diffusion along the continuous cell membrane from the soma into various membrane compartments of axons and dendrites, but no proof has hitherto been presented for such a mechanism of renewal of synaptic proteins.

It is well known that polyribosomes are located both in the dendroplasm and in spines [8, 12, 13, 23, 24, 29, 30, 33, 59, 65, 66, 77]. Recently, two possible variants of renewal and recycling of the synaptic “membrane” proteins have been discussed. According

to Steward *et al.* [30, 59], the synthesis of at least cytoskeletal proteins of postsynaptic densities takes place immediately on polysomes in dendritic spines. In neurons, there is a mechanism by which newly synthesized mRNA directionally move toward active synapses, where they ensure the local synthesis of synaptic proteins on already existing polyribosomes. The role of early Arc (activity-regulated cytoskeleton-associated protein) gene was examined, which, similarly to other early genes, regulates the neuronal activity [30, 59]. Arc gene expression, mRNA delivery to dendrites, and protein synthesis take place for several hours after tetanic stimulation; this time virtually coincides with the time of both the synthesis of

synaptic proteins and the synaptic modification of mainly postsynaptic density proteins. These data are well consistent with the possibility of the synthesis of cytosol-soluble postsynaptic density proteins on free polyribosomes, whose synthesis requires neither rough endoplasmic reticulum, nor subsequent maturation in the Golgi apparatus, nor departure from the *trans* compartment of the Golgi apparatus [54].

Long-term potentiation lasting many hours requires that, to the postsynaptic membrane of the synapse, both integral and secretory proteins should be delivered [78]. An electron microscopic analysis of the organization of various mammalian neurons showed the smooth endoplasmic reticulum extends from the soma both to dendrites and to spines [8, 12, 23, 24, 66, 79, 80]. There is evidence that the smooth endoplasmic reticulum forms a single continuous network [79]. Apparently, the rough and smooth endoplasmic reticula form a single continuous intracellular network, at least, between the soma and dendrites, which is extremely important to intracellular calcium regulation [81]. In such a continuous network, owing to the lateral mobility, integral proteins can locally migrate along a dendrite into spines and, eventually, can directly contact postsynaptic densities [65].

The question of the transport of integral proteins, which are newly synthesized in the soma, from the *trans* compartment of the Golgi apparatus as a part of endosomes along a dendrite toward synapses remains open. In the dendroplasm near spines, the rough endoplasmic reticulum and coated vesicles can often be found, which also occur in dendritic spines [66], which hypothetically does not rule out the synthesis of integral proteins in the dendroplasm with the subsequent maturation of them in the spine apparatus as a certain analog of the Golgi apparatus [82]. However, there has hitherto been no data on the nature and function of the spine apparatus, which is located primarily in mushroom spines.

Another possibility the renewal of receptors, which does not require their synthesis *de novo*, is related to recycling. The presence of coated vesicles and omega-shaped profiles on the membranes of dendrites and spines have lately been discussed in the context of the recycling of postsynaptic receptors in the activation of synapses [66]. The omega-shaped profiles are indicative of endo- and exocytosis. For example, the postsynaptic endo- and exocytosis are known to play an important role in the induction of

both long-term potentiation and long-term depression [83–88]; block of exocytosis prevents the induction of long-term potentiation, whereas block of endocytosis prevents the induction of long-term depression. According to the available data on the mechanism of the synaptic plasticity after stimulation of synapses [84], glutamate receptors can, for several minutes, both enter and leave the postsynaptic membrane, which suggests that, near synapses, there are organelles serving as storage for glutamate receptors. The recycling of glutamate receptors involves such organelles as endosomes, the smooth endoplasmic reticulum, coated vesicles, omega-shaped profiles, vesicles, tubular membrane compartments, multivesicular bodies and tubular membrane complexes bound to multivesicular bodies [66]. A model of the relationship between endosomes and the recycling of glutamate receptors was proposed, according to which coated vesicles and the spine apparatus are uncoupled and the spine apparatus is regarded as a system of smooth endoplasmic reticulum membranes, which ensures calcium regulation.

Studies of the molecular mechanisms of long-term potentiation revealed a certain sequence of events: the calcium ion influx into a spine activates a number of protein kinases and the subsequent phosphorylation of synaptic proteins activates a cascade of processes in the neuronal nucleus, which triggers the synthesis of new synaptic proteins [78, 83, 86, 89] and, eventually, leads to changes in the shape and density of dendritic spines [27, 28]. A number of studies showed that the main components of the dendritic spine cytoskeleton are actin and postsynaptic density proteins [25, 45, 61–63, 87, 90]. It is known that dendritic spines contain neither neurofilaments nor tubulin microtubules [9, 45, 91]. Kaeck *et al.* [92] using high-speed confocal microscopy directly demonstrated the actin dynamics using GFP in a culture of isolated neurons. It is also significant that intense stimulation of glutamate receptors causes disassembly of filamentous actin [45]. A detailed analysis of the organization of the actin cytoskeleton is available in the literature [25, 45, 63, 87].

Changes in the actin cytoskeleton are directly related to changes in postsynaptic density proteins, which are the only reliable criterion for identification of synapses at the electron microscopic level. There are two main types of postsynaptic densities: asymmetric and symmetric; symmetric postsynaptic

densities are thinner than asymmetric postsynaptic densities. Near asymmetric postsynaptic densities in the presynaptic part of the synapse, there are presynaptic vesicles, which are round and are usually about 30 to 50 nm in diameter; and near symmetric postsynaptic densities, there are flattened oval vesicles. It was assumed [93] that presynaptic vesicles near asymmetric postsynaptic densities usually contain the excitatory neurotransmitter glutamate, whereas vesicles near symmetric postsynaptic densities contain the inhibitory neurotransmitter γ -aminobutyric acid (GABA) or glycine and also neuromodulatory peptides. There is evidence that, in the visual region of the cat brain cortex, 84% of synapses are asymmetric and only 14% of them are symmetric. Most (about 79%) of asymmetric postsynaptic densities are located on dendritic spines; fewer (21%) of them, immediately on dendritic shafts; and only about 0.1% of them, on neuronal somata. Most (62%) of symmetric postsynaptic densities are located on dendritic shafts; 31% of them, in dendritic spines; and only 7% of them, on neuronal somata. Thus, only 7% of symmetric postsynaptic densities are located on dendritic spines, whereas 93% of them are located on the somata of neurons of the cat visual cortex [94].

When analyzing three-dimensional reconstructions of postsynaptic densities, on serial thin sections, macular, perforated, and segmented postsynaptic densities are usually distinguished [3, 8, 9, 11, 13, 29, 40, 43, 80, 95–99]. Macular postsynaptic densities are typically located on relatively small spines, are round, and, unlike perforated postsynaptic densities, contain no perforations. A particular case of perforated postsynaptic densities is segmented postsynaptic densities, which in three-dimensional reconstructions usually consist of several densities, at least one of which is perforated.

The above results of studies of dendritic spines are schematically generalized in Fig. 1.

It is known that some dendritic spines of pyramidal neurons of the mammalian hippocampus are connected to the presynaptic part by desmosomes [12, 13, 38], whose main function in various types of cells is likely to ensure the mechanical strength of the tissue. In many types of tissues, including the brain, there are also *electrical synapses* or gap junctions, which ensure the direct intercellular exchange of ions and compounds with a molecular weight to 1000 Da [47, 48]. Electrical synapses have lately been discovered not

only in, e.g., at GABAergic synapses of interneurons [100], but also on dendritic spines of pyramidal neurons [101–103]. According to the data [101–103] obtained by the freeze-fracture-etching technique, at mammalian brain synapses, each gap junction usually does not exceed 100 nm and contains only few connexons (channels). The size of gap junctions is comparable to the thickness (70–100 nm) of typical thin sections, which immensely complicates their identification. To identify gap junctions in the dentate fascia and the CA1 area of the rat hippocampus, we used thinner (10- to 20-nm-thick) sections; the data obtained indicated that electrical synapses are located only on mushroom spines [35].

It is known that connexins (connexon proteins) in gap junctions between glial cells are immunologically different from those on dendritic spines [101–103]. The special role of electrical synapses in the functioning of neuronal networks is as yet unclear.

In recent years, unique methods of intravital analysis of dendritic spines have been developed. One such method is the local superfusion of the fluorescent dye calcein into CA1 pyramidal neurons in organotypic hippocampal cultures with the subsequent production of three-dimensional images using a two-photon confocal laser scanning microscope [51]. It was shown that the induction of long-term potentiation, unlike its block, causes a reliable increase in the number of new spines [51]. Another method of intravital analysis of dendritic spines is GFP expression in neurons. As a vector carrying GFP gene, Maletic-Savatic *et al.* [54] used the Sindbis virus, a neurotropic recombinant envelope RNA virus. CA1 pyramidal neurons in an organotypic culture of hippocampal sections were infected with the Sindbis virus, whose concentration was chosen so that one to ten neurons per slice were infected [54]. It was found that the infected neurons remained active for at least three days. In the experiments, the hippocampi of rats on the seventh postnatal day were used, since adult rat neurons survived very poorly. Using two-photon confocal laser scanning microscopy, the three-dimensional organization of dendritic spines at depths of 50 to 200 μ m in hippocampal sections was analyzed. It was found that, on dendrites, filopodia emerge only when a stimulating electrode was at a distance of about 30 μ m of the regions of dendrites under investigation. The presence of heads on these structures was interpreted in favor of the formation of new mature

dendritic spines in response to the postsynaptic stimulation and activation of ionotropic NMDA receptors. These results directly correlate with the data [51] on the superfusion of fluorescent dyes into neurons.

Although light microscopy has the undoubted advantage of the possibility of intravital study of intracellular structures, an essential disadvantage of any light microscopy technique is the relatively low resolution, which theoretically cannot be better than the half of the wavelength of the visible light used. Even at best, using special computer programs, in two-photon confocal laser scanning microscopy, the resolution is no better than 300–400 nm, whereas in electron microscopy, using the thin section method, the resolution is usually better than 2–3 nm. Possible artifacts in the chemical fixation of brain samples can be obviated when simultaneously using the cryofixation and cryoultratomy of the rapidly frozen nerve tissue.

Figure 2 presents our data on the ultrastructure of mushroom spines (Figs. 2a, 2b) and thin spines (Figs. 2c, 2d) by the example of three-dimensional reconstructions of dendrites of pyramidal neurons in the CA1 area of the hippocampus of ground squirrels in three functional states: in the normothermia (active winter ground squirrels with the brain temperature $t_b = 35\text{--}36^\circ\text{C}$), in 2.5 h after the beginning of awakening from hibernation (once $t_b = 32\text{--}34^\circ\text{C}$ is attained) and in deep cold torpor ($t_b = 1\text{--}6^\circ\text{C}$). It was previously shown that the transition of ground squirrel from normothermia to cold torpor is accompanied by retraction of dendritic spines of CA3 pyramidal neurons [76, 78, 104–106]. In 2.5 h after the beginning of awakening, spines are restored. These results were the first to demonstrate the rate of *in vivo* processes [76, 104–106]. Hibernation of ground squirrels is not a continuous process but a sequence of cycles (bouts) lasting one to three or four weeks and relatively brief, up to a day, periodic awakenings. Bouts under experimental conditions are quite easy to control [106].

In hibernating animals, the bioelectrical activity of, e.g., the neocortex, is suppressed even at t_b below 20°C , whereas in the limbic complex, the regular activity is detected even in deep torpor at t_b below 7°C . The hippocampus, which is the key structure of the limbic complex, plays a special role in hibernation, being a particular kind of a point of control of hibernation by the central nervous system. The mutual inhibition of the hippocampus and the reticular

activating system functions as a positive feedback, thus accelerating the process of going into hibernation and awakening from hibernation (Stark, 1973; Heller, 1979; Beckman *et al.*, 1982; cited from [104–106]).

The process of going into hibernation enables one to track *in vivo* two alternative functional states of the nerve tissue: (1) in the normothermia, where the functioning of the nervous system of ground squirrels differs little from that in ordinary warm-blooded animals, e.g., rats; and (2) and in cold torpor, where t_b of animals can decrease down to $1\text{--}6^\circ\text{C}$, which is related to the almost complete suppression of the synaptic activity [76, 104–106]. Ground squirrel, which is removed from its burrow in cold torpor, at room temperature wakes up, and in this period, it is possible to detect a complete set of states of activation of synapses during the brain warm-up [73, 76, 104–106]. In cold torpor, the nucleolus becomes inactive, free and membrane-bound polyribosomes break up into monosomes, the Golgi apparatus is disassembled, and dendritic spines are retracted. And in as little as 2.5 h after the beginning of awakening, the protein-synthesizing apparatus and dendritic spines are restored [73, 76, 104–106]. Unlike the estrogen-regulated retraction of dendritic spines in the estrus in rats [107–109], the reversible spine retraction in ground squirrels occurs by other, as yet unknown mechanisms. A surprising peculiarity of hibernating animals—a large amplitude of oscillations of the functioning of the body's systems—allows one to use these animals as a unique model for studying the plasticity of, e.g., the central nervous system, in particular, training and memory [110]. We hope that, with time, this line of investigation will be duly developed.

Figure 2 presents typical examples of mushroom (Fig. 2a) and thin (Fig. 2c) spines on dendrites of pyramidal neurons in the CA1 area of the ground squirrel hippocampus. Mushroom spines generally have a stem and a head, on which there are perforated and segmented postsynaptic densities. In particular, as Fig. 2b shows, in hibernating ground squirrels at the center of a perforated postsynaptic density, there is a spinule. Along with “bulk” mushroom spines, “plane” mushroom spines can also be detected in all the functional states of ground squirrel (Fig. 2b). A similar situation also takes place for thin spines: along with “bulk” spines (Fig. 2c), there are also “plane” spines (Fig. 2d); on thin spines, we observed only macular postsynaptic densities. Literature data on the presence

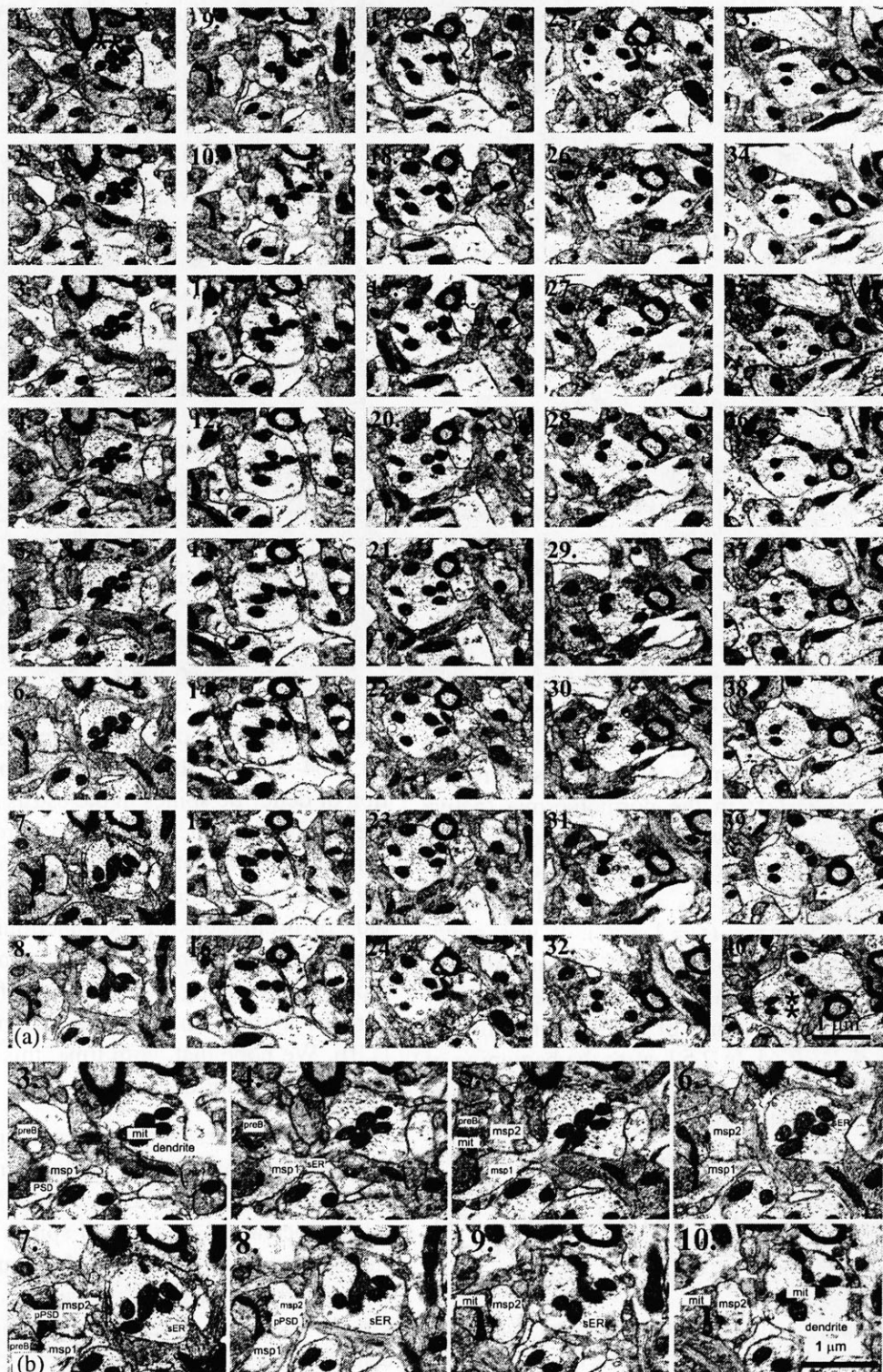


Fig. 3. Structure of a dendrite and mitochondria on serial thin (70-nm-thick) cross sections by the example of the CA1 area of the hippocampus of ground squirrel in 2.5 h after the beginning of awakening from cold torpor. The technique used was described in detail in the literature [34, 88]. (a) A series of 40 sections. The numbers are section nos. At the first section, five mitochondrial cross sections are seen (stars), whereas at the 40th section, there are only two mitochondrial cross sections on a dendritic cross section (stars). (b) The organization of two mushroom spines and synaptic organelles on eight dendritic cross sections: SR, the smooth reticulum; ms1, mushroom spine 1; ms2, mushroom spine 2; Mit, mitochondrial cross section; pPSD, perforated postsynaptic density; and PB, presynaptic bouton with vesicles. The numbers are section nos. corresponding to those in the panel (a). The scale is 1 μ m.

of plane mushroom and thin dendritic spines in the rat hippocampus are as yet unavailable. The results of our *in vivo* studies of dendritic spines in various functional states of the hippocampi of ground squirrel (hibernation) and rat (long-term potentiation) suggest that the reversible spine retraction is characteristic only of populations of thin, stubby, and shaft synapses containing only asymmetric postsynaptic densities [35].

The data in Fig. 2 vividly demonstrate advantages of three-dimensional reconstructions on the basis of serial thin sections for revealing the fine morphology of spines over light microscopy techniques. Note that the roles that synapses of each of the four categories and changes in their morphology play in the synaptic transmission are as yet unclear [27, 28]. This question also equally applies to postsynaptic densities. Because of their small size, postsynaptic densities are virtually impossible to be studied by light microscopy techniques. Moreover, along with purely technical problems, in one- and two-photon laser scanning microscopy studies of both long-term and cultivated slices, dendritic spines are usually analyzed only in the surface layer of the hippocampus at a depth of 25 to 75 μm , which is near the zone of damages caused by the preparation of the slices [31]. Consequently, neurons whose dendritic spines are examined are subject to nonspecific actions, e.g., of neurotransmitters and various enzymes released from cells destroyed in the preparation of the slices. The case of cultivated neurons is no less complex: firstly, a culture of neurons is isolated from newborn animals, in which the synaptogenesis has not yet been completed [43]; secondly, when cells are isolated from the intact tissue, neurons lose axons and dendrites, and such cells in a cultural medium are round. Although for the first week of cultivation, synapses between neighboring neurons form, it is extremely difficult to determine the origin of a neuron under investigation [21]. Both in organotypic hippocampal cultures and in a culture of isolated neurons, there are virtually continuous *de novo* synaptogenesis and formation of filopodia, whose length can reach 10 μm [41, 42, 44, 50]. There is evidence that, in commonly used long-term hippocampal slices, there is also synaptogenesis [8, 9, 27–29, 31, 98, 99]. A comparative analysis of dendritic spines of cultivated neurons and the intact hippocampus showed that, *in vitro*, approximately half of excitatory synapses were located on dendrites, whereas *in vivo*, most of them were located on spines [111]. Furthermore, it proved that the

volume of spines on the fifth day of cultivation considerably exceeded the corresponding *in vivo* parameter on the fifth postnatal day. Thus, it is difficult to collate the results of the *in vitro* studies of changes in synapses with the *in vivo* data.

An illustrative example of using serial sections for analyzing neuronal substructures may be given by the three-dimensional reconstructions of mitochondria in axons and dendrites. In this context, mitochondria are of particular interest, since they play a key role in the pathways of apoptosis signaling and maintenance of calcium homeostasis in neuronal cells [8, 23, 53, 55, 56, 79, 81, 112–115]. The question of whether cells contain a single mitochondrion or whether they contain several mitochondria remains topical [113]. It was assumed that there is a giant mitochondrion, whose inner membrane could serve as an “electrical cable,” along which there could be “energy transfer” toward various cellular compartments [116]. A no less important function of such a giant mitochondrion could be “calcium ion tunneling” within the cell [8, 112, 113]. Presynaptic boutons of axons are known to contain small mitochondria. From some presynapses, they can even be absent [80]. Our studies confirmed the data that the usual length of mitochondria does not exceed 2–3 μm [113]. In some cases, mitochondria to 4–5 μm in length can be encountered.

The organization of mitochondria in dendrites has been studied less thoroughly. Using a confocal laser scanning microscope and specific fluorescent dyes, Collins *et al.* [113] produced three-dimensional reconstructions of mitochondria in nerve cell cultures. It was shown that both neurons and other types of cells contain heterogeneous populations of numerous mitochondria, whose maximal length does not exceed 5 μm [113]. Our studies of the three-dimensional organization of mitochondria in dendrites of various neurons of the ground squirrel and rat hippocampi using serial thin sections (each series consisting of more than 100 sections) gave quite different results. The main conclusion is the fact that all apical dendrites of pyramidal neurons in the CA3 and CA1 areas and the dentate fascia of the hippocampus contain giant branched mitochondria no less than 20–25 μm in length. Figures 3 and 4 exemplify the use of serial sections for producing three-dimensional reconstructions of mitochondria. Figure 3a presents 40 serial photographs of cross sections of a dendrite of CA1 neuron of the ground squirrel hippocampus. At the

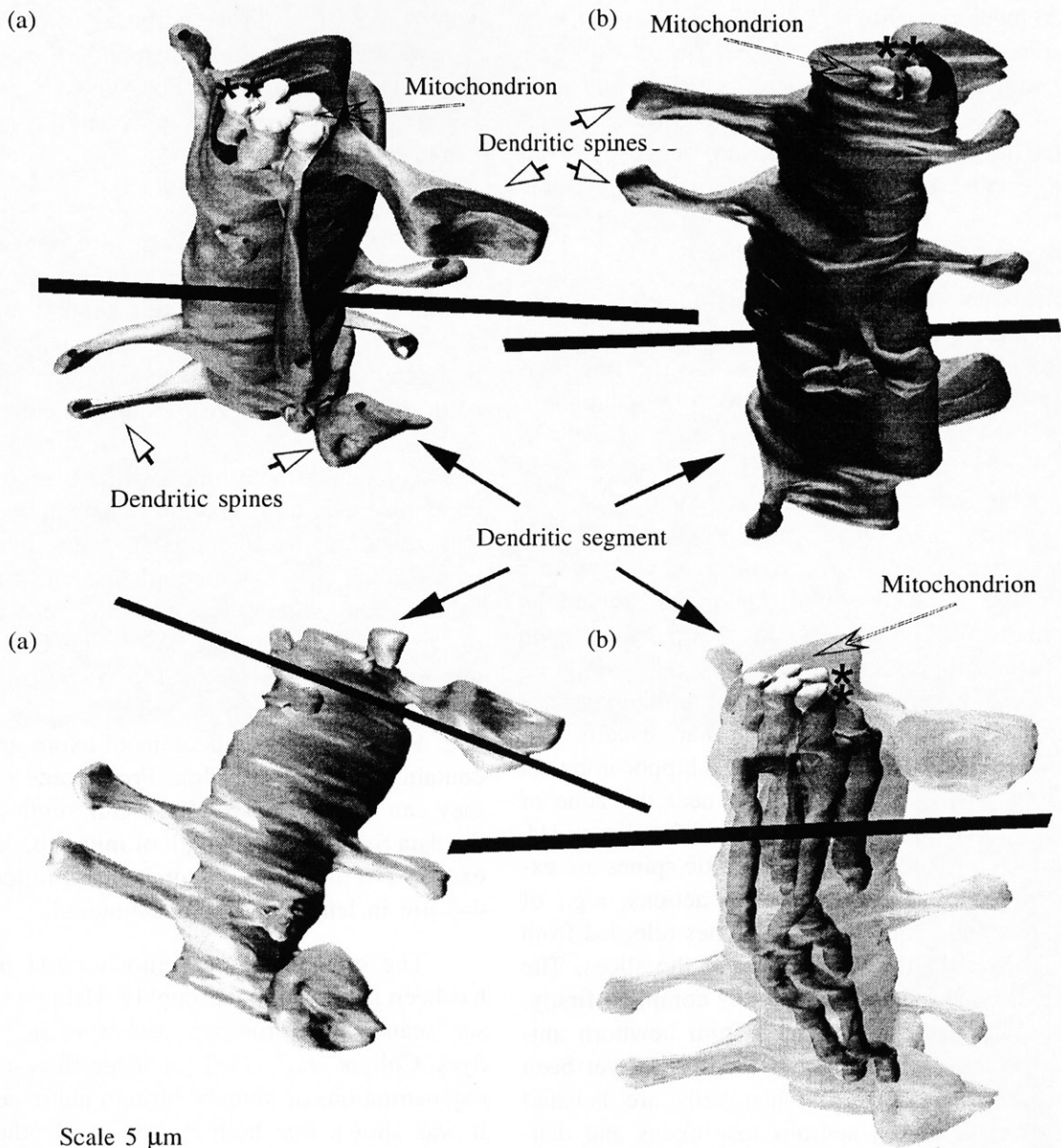


Fig. 4. Three-dimensional reconstruction of a dendritic segment of a neuron in the CA1 area of the ground squirrel hippocampus as constructed using 40 serial sections shown in Fig. 3a. (a) Five and (b) two mitochondrial cross sections at a dendritic cross section are shown, which correspond to images 1 and 40 in Fig. 3a, respectively.

first section, five mitochondrial cross sections are seen (stars), whereas at the 40th section, there are only two mitochondrial cross sections (stars). Eventually, the analysis showed that all of these sections belong to a single giant mitochondrion (see Fig. 4). Figure 3b shows some details of the organization of dendritic spines. It is seen that mitochondria do not penetrate even into the largest mushroom spines.

Electron micrographs of different sections of the same region of the dendrite were aligned using the computer program Alignment. To generate three-

dimensional images, in the wrl/vrml format, contours of each structure were constructed, and in the program Trace [35, 36] (<http://synapse.bu.edu>), individual files were created for them, which were further converted into the raw format and, in the commercially available program 3D View Actify, combined into a single model, whose example is given in Fig. 4. Thus, the three-dimensional reconstructions of a dendritic segment with spines and a complex mitochondria illustrate well the potential of serial thin sectioning. It is significant that details observable at these

sections cannot be obtained by light microscopy techniques. Moreover, these results showed that the number of mitochondrial cross sections per dendritic cross section varies from two (Fig. 3a, images 38–40) to six (Fig. 3a, images 16–23), whereas this dendritic segment contains only a single mitochondrion. Our studies of more than 100 proximal and distal dendritic segments in a series containing 70 to 130 serial sections, by and large, confirmed the possibility of their existence of giant mitochondria in all types of hippocampal dendrites. Axons and their presynaptic boutons contain a large number of small mitochondria, whose maximal length does not exceed 5 μm .

According to our hypothesis, the continuity of the mitochondrial system is ensured by the smooth endoplasmic reticulum, which is directly connected to the outer mitochondrial membrane (see the scheme in Fig. 1). It is not quite clear how signals from hundreds of synapses are transmitted into the soma to generate a single action potential; the coupled and continuous compartmentalization of mitochondria with the continuous smooth endoplasmic reticulum system [115] could ensure the directed “energy transfer” through the inner mitochondrial membrane, which would serve as an “electrical cable” [116], and also provided “tunneling of calcium ions” as the main link in the intracellular signal transduction system [81, 112].

It is conventionally considered that approximately 50% of brain cells are neurons, whereas the others are mainly glial and ependymal cells [117]. Astrocytes are known to form a functional syncytium, in which cells are connected by gap junctions [118]. Earlier studies showed that, on astroglial cells, depolarization emerges in response to an increase in the extracellular potassium concentration in the functioning of synapses [119, 120]. The *in vivo* investigation of astrocytes in the CA1 area of the hippocampus detected that, during electrical stimulation of Schaffer collaterals, the glutamate release from synapses causes an increase in the calcium concentration in astrocytes owing to stimulation of metabotropic glutamate receptors located in the astrocytic plasma membrane [121]. The data that the plasma membrane contains glutamate receptors responding to extracellular glutamate in long-term potentiation are also available for cerebellar Purkinje cells [122]. It has recently been shown that mature hippocampal astrocytes contain both metabotropic and ionotropic glutamate receptors [123]. It was also found that variation of the calcium ion

concentration within the physiological range of 84 to 140 nM stimulates glutamate release from astrocytes, which modulates interneuronal synaptic transmission [124]. Of particular interest are studies [125–128] that demonstrated the existence of gap junctions between glial cells and neurons. At the present time, this line of investigation is being intensely developed by electrophysiological and modern molecular biological methods using both cultures of glial cells and neurons, and the intact brain, including the hippocampus. The expedience of the presence of electrical synapses between glial cells and neurons is probably due to the fact that membrane potential oscillations at glial cells can play an important role in the modulation of synaptic transmission in neuronal networks [126, 127]. It was assumed that astrocytes are involved in the modulation of the neuronal activity in ontogeny [128, 129] in various brain pathologies [126, 127], including epilepsies [130] and brain ischemia [126].

The location of glial cells and their processes in the interneuronal space is very important to understand how interneuronal and astrocyte–neuron interactions participate in synaptic transmission mechanisms. The idea is being actively developed that the spillover of glutamate released from a synapse can migrate over the extracellular space and activate neighboring synapses [131, 132], because of which it is significant to determine the distance between neighboring synapses and their relationship with astrocytes [117, 132]. Three-dimensional reconstructions of a molecular layer in the CA1 area of the rat hippocampus allowed Ventura and Harris [117] to estimate the average distance between synapses at 0.65 μm . Close values were obtained by Hosokawa *et al.* [131], who estimated the linear distance between synapses at 0.436 μm ; if account was taken of their introduced tortuosity factor of the extracellular space, which was taken to be 1.34, the average distance was estimated at 0.584 μm .

Ready three-dimensional reconstructions have exact coordinates *X*, *Y*, and *Z* for each structure, using which many programs, e.g., Trace (<http://synapse.bu.edu>) or 3D View Actify (<http://www.actify.com/v2/index.htm>), can quantitatively estimate, e.g., the surface area, the volume, and the distances between these structures. Figure 5 presents three-dimensional reconstructions of glial processes and their contacting thin and mushroom spines of dendrites in the CA1 area of the hippocampus of ground squirrels in three



Fig. 5. Three-dimensional reconstructions of glial processes and (thin and mushroom) dendritic spines that directly contact the surface of astrocytes: (a) in the normothermia, (b) in forced awakening, and (c) in the middle of a bout of hibernation. The functional states of ground squirrels and the studied region in the CA1 area of the ground squirrel hippocampus are the same as in Figs. 2 and 3. In panels 1 are spines contacting the glial network; panels 2, the glial network, on which dendrites and presynaptic boutons (light regions) are located; and panels 3, thin and mushroom spines, on which postsynaptic densities (dark regions) are located. The scale of all the images is 1 μm .

functional states: in the normothermia (Fig. 5a), in 2.5 h after the beginning of awakening from hibernation (Fig. 5b), and in cold torpor (Fig. 5c). The reconstructions were produced using 50 serial sections for each of three samples so that approximately equal volumes (about 500 μm^3) of the nerve tissue were analyzed. Figures 5-1 show astrocytic processes (gray

regions), on whose surface lie thin and mushroom spines (white regions), on which both macular and perforated or segmented postsynaptic densities (black regions) are located. Figures 5-2 demonstrate astrocytic processes, which, using desmosomes and gap junctions, form a continuous network. Figures 5-3 present dendritic spines, between which both minimal and

Minimal and average distances between thin and mushroom dendritic spines that directly contact the surface of astrocytic processes in a molecular layer in the CA1 area of the hippocampus of ground squirrel in three functional states

Functional state	Dendritic spines		Postsynaptic densities	
	minimal distance, μm	average distance between all spines, μm	minimal distance, μm	average distance between all spines, μm
Normothermia	1.052 ± 0.071	3.968 ± 0.068 $N = 741$	1.052 ± 0.071	4.029 ± 0.070 $N = 741$
Awakening	0.822 ± 0.063	3.429 ± 0.064 $N = 703$	0.868 ± 0.059	3.428 ± 0.065 $N = 703$
Hibernation	0.940 ± 0.064	5.199 ± 0.071 $N = 1485$	0.996 ± 0.059	5.305 ± 0.072 $N = 1485$

Note: The minimal distances both between spines and between postsynaptic densities for all the functional states were not significantly distinguishable. The average distances both between spines and between postsynaptic densities for all the functional states were significantly distinguishable. $p < 10^{-8}$. The data were statistically processed using the Student's *t*-test.

average distances were determined. The table lists quantitative data, which imply that the minimal distance between spines is about $0.9 \mu\text{m}$ and this distance is virtually independent of the functional state of the nerve tissue. The data in Fig. 5 and the table describe only approximately 10% of the population of synapses (postsynaptic densities serve as markers) in the volume under investigation. The other 90% of synapses are connected to the astroglial network through dendrites and presynaptic boutons. Importantly, stereological analysis showed that the density of synapses/postsynaptic densities remains unchanged and, for the studied zone in the CA1 area, is about 170 synapses per $100 \mu\text{m}^3$. It was earlier shown that the process of going into hibernation is accompanied by the retraction of dendritic spines, in particular, dendrites in the CA3 area [76, 104, 105]. In as short as 2.5 h after the beginning of awakening, the retracted spines are restored [76, 104, 105]. It is significant that, in the CA1 area, the synapse density is virtually independent of the functional state of the nerve tissue. However, the process of going into hibernation induced a reliable almost 20% decrease in the proportion of thin spines owing to an increase in the proportions of stubby and shaft synapses. In 2.5 h, the proportion of thin spines was almost restored to its value in the normothermia. The proportion of mushroom spines remained constant in all the three functional state of ground squirrels. In this context, the fact that the average distance between thin and mushroom spines in cold torpor is consistently larger than that in the normothermia (table) can be explained by the retraction of thin spines, since stubby and shaft synapses in this study were not fixed. This explanation is

also confirmed by the data on a reliable decrease in the average distance between spines in 2.5 h after the beginning of awakening, when the previously retracted spines are actively being restored from the stubby and shaft synapses. Note also that the distances between the centers of mass of spines fully coincide with the distances between the centers of mass of postsynaptic densities (table), whose volume is more than an order of magnitude smaller than the spine volume. It is as yet unclear how the reversible retraction (the transition of thin synapses into stubby synapses and the transition of stubby synapses into shaft synapses) can be related to the activity of the astroglial network and how this network affects the electrical activity of the neuronal network in awakening of ground squirrel from hibernation, when in a relatively short time, the brain warms up from $1-6^\circ\text{C}$ (cold torpor) to $32-34^\circ\text{C}$. In awakening, in as short as tens of minutes, the protein-synthesizing apparatus is activated [73, 75, 76, 104, 105] and various compounds are intensely synthesized. At this temperature, ground squirrel responds to external stimuli, i.e., cortical and hippocampal synapses are activated and various neurotransmitters are likely to be released into the extracellular space.

CONCLUSION

Thus, the key link in the functioning of the brain is a chemical synapse involving a presynaptic bouton with neurotransmitters and a postsynaptic part (dendritic spine). In the hippocampus, the largest spines are mushroom spines, which contain free polyribosomes, the spine apparatus, the smooth

endoplasmic reticulum, and coated vesicles participating in the recycling of receptors. It is essential that the number of mushroom spines is constant, whereas thin spines can be retracted to form stubby spines and, further, shaft synapses. This transition is reversible and can be a real mechanism ensuring the synaptic plasticity. Moreover, all categories of spines can change their shape, volume, and surface area. A valid synapse marker is three main types of postsynaptic densities, namely, macular, perforated, and segmented postsynaptic densities, which also can change their shape.

A particular type of spine is found in the CA2-CA3-CA4 areas of the hippocampus. This is the thorny excrescence, a short dendritic branch on which there are numerous spines. Possibly, such an organization of spines in these areas is related to the comparator properties of the hippocampus [36], since it is these hippocampal regions that are potentiated by mossy nonmyelinated fibers/axons formed by granular neurons from the dentate fascia, which contains presynaptic boutons of axons from various brain structures.

Owing to insights from serial thin sectioning and three-dimensional reconstructions, the one spine—one synapse paradigm is currently replaced by examples that, on a single mushroom spine in the CA1 area of the hippocampus, there can be up to eight independent presynaptic boutons. Examples of contacts of several (up to five) presynaptic boutons of moss fibers were also found on thorny excrescences of dendrites of CA2-CA3-CA4 pyramidal neurons [35]. The expedience of the presence of multiple synapses on a single spine can be due to the possibility of processing of incoming information even at the level of a small part of a dendrite—at an individual spine. Our studies showed that the proportion of such multiple synapses on mushroom spines does not exceed 0.1% of all their population, whereas the proportion of mushroom spines in the in the CA1 area of the rat and ground squirrel hippocampi is usually about 12–14% of all synapse population.

Owing to chemical and electrical synapses, the neuronal networks are a real functional syncytium. Three-dimensional reconstructions showed that glial processes in various hippocampal regions form a single morphologically continuous network, which, owing to gap junctions between glial processes, is also a functional syncytium. Thus, already available our

own and literature data suggest an important electrophysiological role of the glia. Indeed, it is difficult to believe that the glia, whose cells amount to almost 50% of all the brain cells, performs only supporting functions. Consequently, in various areas of the hippocampus and, probably, the cortex, there are two coupled systems capable of generating electrical oscillations: slow oscillations in the astroglia and fast oscillations in the neuronal networks.

The results we have presented show that dendrites of almost all neurons in the *in vivo* hippocampus contain highly branched giant mitochondria both in proximal and distal regions. The continuous connection of smooth endoplasmic reticulum membranes to the outer membrane of the envelope of dendritic mitochondria is likely to suggest that the “passive properties of a dendrite” [1], more precisely, its plasma membrane, are implemented as active properties through the continuum formed by the smooth endoplasmic reticulum to the outer membrane of the mitochondrial envelope and ensure at least calcium regulation [89]. If the inner membrane of a giant mitochondrion is continuous, then, under Skulachev’s assumption [116], this inner membrane can serve as an “electrical cable,” ensuring the transmission of signals from various spines into the soma. At the same time, we already have examples of three-dimensional reconstructions of mitochondria in which the continuity of a giant mitochondrion is ensured only by the outer membrane, with which smooth endoplasmic reticulum membranes are connected. In this case, the inner membranes of individual mitochondria are not connected to one another; however, in such a system, the excitation transmission from various spines into the soma could take place owing to calcium currents on smooth endoplasmic reticulum membranes.

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