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# Three-Dimensional Reconstruction of Synapses and Dendritic Spines in the Rat and Ground Squirrel Hippocampus: New Structural-Functional Paradigms for Synaptic Function

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Published data are reviewed along with our own data on synaptic plasticity and rearrangements of synaptic organelles in the central nervous system. Contemporary laser scanning and confocal microscopy techniques are discussed, along with the use of serial ultrathin sections for *in vivo* and *in vitro* studies of dendritic spines, including those addressing relationships between morphological changes and the efficiency of synaptic transmission, especially in conditions of the long-term potentiation model. Different categories of dendritic spines and postsynaptic densities are analyzed, as are the roles of filopodia in originating spines. The role of serial ultrathin sections for unbiased quantitative stereological analysis and three-dimensional reconstruction is assessed. The authors' data on the formation of more than two synapses on single mushroom spines on neurons in hippocampal field CA1 are discussed. Analysis of these data provides evidence for new paradigms in both the organization and functioning of synapses.

**KEY WORDS:** hippocampus, neuron, glial cell, synapse, gap junction, dendritic spine, postsynaptic density, serial ultrathin sections, three-dimensional reconstruction.

Vinogradova has published a review [1] addressing the state of neuroscience at the change of one of millennium to the next and the paradigm shift in relation to the interactions both between neurons and between neurons and glial cells. We believe there is a need to supplement this information with further data on synapses, which underlie the operation of the mammalian brain, and this is the aim of the present article, which reviews recently published data and our own work.

Traditionally, the questions of learning, memory, and forgetting are linked to neuronal plasticity, which is based on changes in synapses. The term “synapse” (from the Greek *synapsis*, meaning junction) was introduced by Foster and Sherrington at the end of the 19th century [22] and means “connection.” The term subsequently acquired wide use in the contemporary sense to identify connections between neurons [47]. According to [5, 6], plasticity can arbitrarily be divided into two categories: 1) changes in already-existing synapses without changes in neuronal connections [71] and 2) changes in interneuronal connections due to the *de novo* formation and disappearance of synapses [52]. The “chemical synapse” is an area of contact between a dendrite and the presynaptic part or “presynaptic bouton” of an axon and contains vesicles containing neurotransmitter, whose release into the synaptic cleft activates the postsynaptic membrane, for example a spine membrane.

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Dendritic spines were discovered more than 100 years ago. What is the function of the dendrite? Why is there such a wide variety of dendrites? Why do dendrites have such complex shapes? Why do dendrite surfaces bear spines of complex shape? Ramon-y-Cajal [52] was the first investigator to try to address these and other questions. The use of a variety of light and electron microscopy methods over the last 100 years has led to the accumulation of extensive data on dendritic spines, though many aspects of the organization, genesis, and direct functions of spines thus far remain unclear.

In recent years, dendritic spines have received ever-increasing attention [2, 3, 11, 13–19, 23–25, 27–31, 33–35, 38, 41, 45–50, 55, 56, 61–70, 72, 74–77].

At the end of the 1970s, Peters and Kaiserman-Abramof [47] reviewed data obtained by light and electron microscopy and suggested a relatively simple classification of spines, identifying three main categories: 1) fine spines, in which the spine length was greater than spine diameter; 2) mushroom spines, with a long or short, “stem” with a large head; 3) stubby spines, in which the length and diameter are virtually identical. Many studies based on this classification identify four categories of synapses: 1) fine; 2) mushroom; 3) stubby; and 4) shaft synapses, where the presynaptic bouton makes direct contact with the dendritic stem. A fifth category was subsequently proposed [58], so-called branched spines, consisting of two fine spines borne by a single stem. A simpler classification is sometimes used [35], identifying only two categories: 1) spines without stems (sessile spines), corresponding to stubby spines, and 2) spines borne on a stem (pedunculated spines), which correspond to fine and mushroom spines. Terms such as “axodendritic” synapses (corresponding to stem synapses) and “axospinous” synapses are often used, because the CNS contains neurons whose dendrites form unique spines: for example, the dendrites of pyramidal neurons in hippocampal fields CA3/CA4 and particularly CA2, along with the usual categories of spines, form so-called thorny excrescences [13, 54].

The dendrites of cerebellar Purkinje cells contain a population of spines which is relatively homogeneous in shape, and these can arbitrarily be identified as mushroom spines [67].

Spine surfaces often have fine outgrowths of the postsynaptic membrane, so-called spinules, which penetrate deeply into the presynaptic bouton. Data have been obtained [61, 62] showing that these “spinules” can be present both in the area of the postsynaptic densities (PSD) and around the periphery of the heads of spines. The possible functions of “spinules” are usually assessed in terms of their contribution to the efficiency of synaptic transmission due to increases in the areas of contacts between the pre- and postsynaptic membranes, especially when new spines form [23, 25], though experimental data supporting this role for “spinules” in synaptic transmission have yet to be obtained [61]. We note that in the state of cold-induced torpor in the ground

squirrel, when brain electrical activity is minimal, “spinules” penetrating into the presynaptic bouton can also be found on the surfaces of dendritic stems [2, 3].

“Filopodia” represent a special structure, these consisting of fine, actin microfilament-filled outgrowths of the dendritic membrane up to 10  $\mu\text{m}$  long [58]. Filopodia play an important role in forming new spines [15, 18, 21, 41, 55, 56, 60, 76, 77]. In hippocampal field CA1 of the rat, *in vivo* studies have demonstrated [18] that filopodia form on the surfaces of dendrites in the first two weeks of the postnatal period and that they support the searching for presynaptic boutons forming new PSD with subsequent conversion of filopodia into “protospines,” ultimately leading to formation of new fine spines. Studies have suggested [18, 36, 39, 68] that the cytoskeleton, whose major component is actin [20, 60], plays an active role in the growth and retraction of filopodia during their conversion into spines. Detailed electron-microscopic analysis of filopodia during the postnatal development of synapses in field CA1 of the rat hippocampus [18] showed that filopodia are an intermediate stage in the formation of new (*de novo*) dendritic spines in the first two weeks after birth. In this regard, the main paradigm of neurobiology – the active search by axons (efferent fibers) for “targets” – is supplemented by the new proposal that dendrites can also “seek” axons. Isolated cases of mutant mouse strains are known, with spines with PSD on the dendrites of cerebellar Purkinje cells in the absence of the granule cells which normally form presynaptic boutons [64]. Data have also been obtained [7] showing that injection of substance P into the cerebral ventricles had no effect on the dendritic spines of Purkinje cells, which contained PSD without making contacts with the presynaptic boutons of parallel fibers of granule cells. Filopodia are generally rarely seen on the dendrites of mature neurons in normal brains, though in organotypic cultures and cultures of isolated neurons they are easily identified by electron and confocal microscopy [16, 41, 55, 56, 76]. Data have also been obtained [4] on the ability of axons to form structures similar to dendritic spines.

We note that any modification or formation of new synapses is directly associated with both proteins in the postsynaptic density [32, 36, 40, 55] and receptors and channels which support synaptic transmission and need constant replenishment; the thickness of the base of the spine plays an important role in this [68].

Changes in the cytoskeleton of the dendritic spine are directly associated with changes in PSD proteins, whose existence is the sole reliable criterion for identifying synapses at the electron-microscopic level. There are two main types of PSD: 1) asymmetrical and 2) symmetrical; symmetrical PSD are thinner than asymmetrical. Round presynaptic vesicles, usually 30–50 nm in diameter, are generally located near asymmetrical PSD in the presynaptic parts of synapses; symmetrical PSD are characterized by being associated with flattened (oval) vesicles. It has been

suggested [8, 12, 48] that presynaptic vesicles located close to asymmetrical PSD generally contain the excitatory neurotransmitter/mediator glutamate, while vesicles located close to symmetrical PSD contain inhibitory neurotransmitters – gamma-aminobutyric acid (GABA) or glycine, as well as neuromodulator peptides.

Analysis of three-dimensional (3D) reconstructions of PSD on serial ultrathin sections usually identifies three main categories: 1) “macular” PSD; 2) perforated PSD; and 3) segmented PSD [12, 16, 17, 22–24, 37, 56, 59–61, 63, 64]. Macular PSD are usually located on relatively small spines, are round, and do not contain perforations. Segmented PSD represent a special case of perforated PSD, which on three-dimensional reconstructions usually consist of several densities, of which at least one is perforated.

Winter hibernation of the Yakutsk ground squirrel *Citellus undulatus* provides a unique model for studying synapses. Bioelectrical activity in hibernating animals in, for example, the neocortex, is suppressed when brain temperature is below 20°C; structures of the limbic complex in deep sleep at brain temperatures of less than 7°C show regular activity. The hippocampus, a key structure of the limbic complex, plays a special role in hibernation processes, constituting a “guard station” for controlling the CNS during sleep. Mutual inhibition of the hippocampus and the reticular activatory system functions on the positive feedback principle, accelerating entry into sleep and exit from this state [9, 32, 40, 59]. Hibernation of ground squirrels is not an uninterrupted process, but consists of a sequence of cycles (bouts) lasting from one to 3–4 weeks, with relatively short – up to one day – periodic wakings. Bouts in experimental conditions are quite simple to monitor [51].

In vivo, the process of hibernation can yield nervous tissue in two alternative functional states: 1) normothermia, when the functioning of the ground squirrel nervous system is little different from that in other mammals, for example, rats; 2) cold-induced torpor, when brain temperature in the animals can drop to 1–6°C, which is associated with virtually complete suppression of synaptic activity [2, 25, 32, 48–50]. Ground squirrels removed from their nests in the state of cold-induced torpor wake at room temperature, making it possible to obtain a complete set of states of synapse activation during warming of the brain. The state of cold-induced torpor is associated with transition of nucleoli into an inactive state, degradation of free and membrane-bound polyribosomes to individual subunits, break-up of the Golgi apparatus, and retraction of dendritic spines. The protein-synthesizing apparatus and dendritic spines recover by 2.5 h after the onset of waking [2, 26, 49–51]. Unlike estrogen-regulated retraction of dendritic spines in estrus in rats [74, 75], reversible spine retraction in ground squirrels is associated with other as yet poorly studied mechanisms. A surprising feature of hibernating animals is the large scale of oscillations in the functioning of vital body systems – making these animals a unique model for studying plasticity,

for example of the central nervous system, especially in relation to learning, memory, and forgetting [43].

Previous studies have demonstrated [26, 49–51] that the entry of ground squirrels from normothermia to the state of hypothermic torpor is accompanied by retraction of the dendritic spines of pyramidal neurons in field CA3. Spine recovery starts by 2.5 h after the onset of waking from hibernation. These results provided the first demonstration of the rates of these processes in vivo [49–51].

Three types of dendrite are usually identified in the central nervous system: 1) dendrites covered with spines (spiny dendrites); 2) dendrites with rare spines (sparsely spiny dendrites); and 3) dendrites without spines or smooth dendrites (non-spiny dendrites) [47, 48]. It is often difficult to distinguish dendrites of types 1 and 2 at the light microscopic level because of the small size of the spines and because of the great variability in the numbers of spines on different parts of long dendrites.

The dendroplasm and spines are known to contain polyribosomes [28–31, 45, 63, 65, 69]. Two possible variants of the renewal and recycling of synaptic “membrane” proteins have received extensive discussion in the recent literature. According to [69], the synthesis of cytoskeletal proteins of postsynaptic densities occurs directly on the polyribosomes in the dendritic spines; neurons have a mechanism whereby newly synthesized mRNA undergoes controlled “movement” to active synapses, where it supports the local synthesis of synaptic proteins. The role of the “early” gene “Arc” (activity-regulated cytoskeleton-associated protein) has been discussed; like other “early” genes [69], this protein controls the activity of neurons; expression of the “Arc” gene, transport of mRNA to dendrites, and synthesis of proteins occur during the several hours after tetanic stimulation, virtually simultaneously with the synthesis of both synaptic proteins and synaptic modifications, mostly of PSD proteins. These data are in good agreement with the possibility that cytosol-soluble PSD proteins, which do not require rough endoplasmic reticulum, subsequent “maturation,” or release from the trans compartment of the Golgi apparatus, are synthesized on free polyribosomes [42].

Special methods have been developed over recent years for studying dendritic spines in living tissues. One method is that of local superfusion of field CA1 pyramidal neurons in organotypic cultures of the hippocampus using the fluorescent stain calcein followed by generation of three-dimensional images using two-photon laser scanning confocal microscopy [16]. Experiments of this type showed that induction of long-term potentiation [10], unlike its blockade, induces significant increases in the numbers of new spines [16]. Another in vivo method for studying the behavior of dendritic spines is based on the expression of the GFP protein in neurons. Experiments reported in [41] used the neurotropic recombinant coated RNA-containing Sindbis virus as a vector to carry the GFP gene; this was used to infect CA1 pyramidal neurons in organotypic cul-

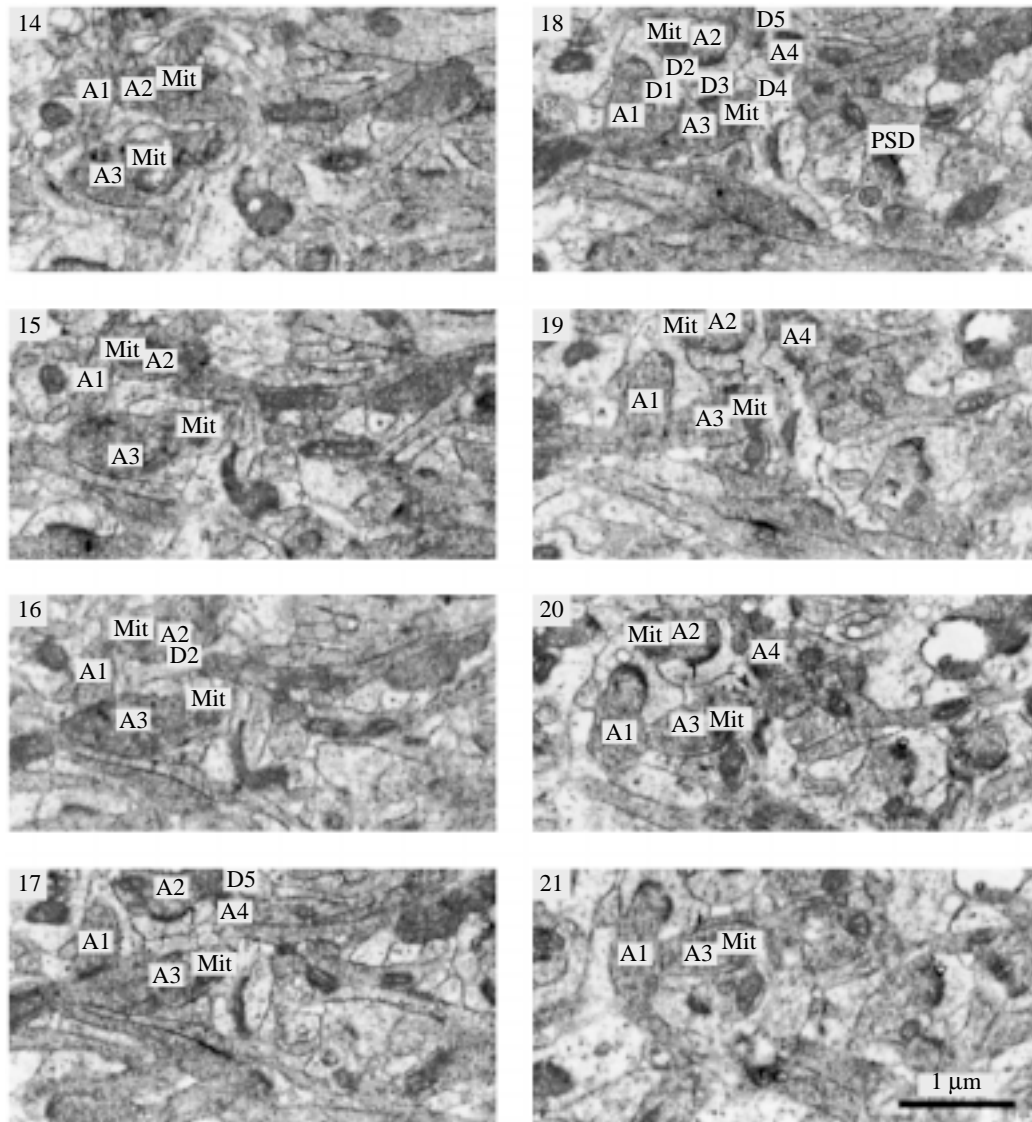


Fig. 1. Eight serial sections after "smoothing," demonstrating the ultrastructure of the neuropil of ground squirrel hippocampal field CA1 2.5 h after waking from the state of hypothermic torpor. The method is described in detail in [19, 51]. A1, A2, A3, and A4 show presynaptic boutons of four independent axons; D1, D2, D3, D4, and D5 show dendritic spines; Mit = mitochondria; PSD = postsynaptic densities; numbers identify the sequence of microphotographs of this series. The scale bar is 1  $\mu\text{m}$ .

tures of hippocampal slices. The virus concentration was selected to infect from one to 10 neurons per slice. These experiments showed that infected neurons retained activity for at least three days. Hippocampi were collected from rats on postnatal day 7, as the survival rate of neurons obtained from adult rats was extremely low. Two-photon scanning laser confocal microscopy was used to analyze the three-dimensional organization of dendritic spines at depth of 50–200  $\mu\text{m}$  in hippocampal slices. Filopodia were found to arise on dendrites only when the stimulating electrode was located at a distance of about 30  $\mu\text{m}$  from the zone containing the study dendrites. The author interpreted the presence

of "heads" on these structures as supporting the formation of new mature dendritic spines in response to post-tetanic stimulation and activation of ionotropic NMDA receptors. These results correlate directly with data reported in [16] from superfusion of neurons using fluorescent stains.

Along with undoubted advantages such as the ability to investigate subcellular structures in living tissues, all light microscopic techniques have the disadvantage of relatively low resolving ability, which cannot theoretically be greater than half the wavelength of the light used. Even in the best case, use of special computer programs and two-photon laser confocal microscopy, resolution cannot exceed

300–400 nm. In electron microscopy, use of ultrathin sections generally gives resolution of better than 2–3 nm. Possible artefacts resulting from chemical fixation of brain specimens can be avoided by parallel use of cryofixation and cryoultramicrotomy of rapidly frozen nerve tissue.

It should be noted that the roles played by synapses of the four types and changes in their morphology remain unclear [55, 56]. This question applies equally to PSD. Because of their small size, these are virtually inaccessible to study by light microscopic methods. In addition, along with purely technical problems, studies of both living tissue and cultured slices by one- and two-photon scanning laser confocal microscopy usually involve analysis of dendritic spines only in the surface layer of the hippocampus to a depth of 25–75  $\mu\text{m}$ , which is close to the zone of damage occurring during preparation [76]. Thus, the neurons whose dendritic spines are being examined are subjected to the actions of neurotransmitters and various enzymes released from cells damaged during slice preparation. The situation is no less complicated when cultured neurons are used: firstly, neuron cultures are made from newborn animals, in which synaptogenesis is still incomplete; secondly, neurons lose axons and dendrites during isolation of cells from intact tissues, and these cells become rounded in culture medium. Although synapses form between neighboring neurons during the first week in culture, it is extremely difficult to identify the origin of the neuron being studied. Both in organotypic cultures of the hippocampus and in cultures of isolated neurons, *de novo* synaptogenesis and formation of filopodia occurs virtually constantly, and they may reach lengths of up to 10  $\mu\text{m}$  [15, 60, 77]. Data have been obtained showing that the hippocampus slices usually used also show synaptogenesis [28, 38, 55, 56, 61–63, 76]. As demonstrated, comparative analysis of dendritic spines of cultured neurons and intact hippocampus showed that about half the excitatory synapses *in vitro* are located on dendrites, while *in vivo* most are located on spines [11]. In addition, the number of spines on day 5 of cultivation was found to be significantly greater than the corresponding measure *in vivo*, on postnatal day 5. Thus, it is difficult to relate data on changes in synapses obtained *in vitro* to events occurring *in vivo*.

Our data allow us to assess plastic changes occurring in the spine apparatus *in vivo*, using the generalized rearrangements in the brains of hibernating animals as an example. Analysis was based on serial ultrathin sections (more than 100 sections per series), which were studied by electron microscopy with subsequent processing of the resulting microphotographs by computer-based methods. Figure 1 shows a series of eight microphotographs of synapses in hippocampal field CA1 in a ground squirrel 2.5 h after the onset of waking (brain temperature had reached 32–34°C) from hibernation (brain temperature 1–6°C). This state was not selected randomly, as exit of ground squirrels from hypothermic torpor involves activation of virtually all brain

processes. Thus, this state represents a model of “active synapses.” Only the serial section method allows detection of the actual disposition and three-dimensional organization of different synaptic structures and glial processes. Thus, analysis of serial microphotographs, individual segments of which are shown in Fig. 1, identifies five spines separated by distances of no more than 15 nm. These spines form synapses with four axons, separated by distances of no more than 15 nm. This fact cannot be explained from the classical point of view based on the electrical properties of the axon membrane, as these processes separated by these distances should experience mutual influences, while the passive properties of the dendrite membranes do not impose any limits on their closeness. The close location of axons and dendritic spines may also be important from the point of view of the “spillover” hypothesis – which is when an “excess” of neurotransmitter is able to diffuse across the intercellular space and activate neighboring synapses [53]. Analysis of the serial sections in Fig. 1 shows that astroglial processes are located close to dendritic spines and axons.

Recent years have seen active discussion of the role of presynaptic boutons in the mechanisms of memory, these forming synapses with two or more dendritic spines, i.e., so-called multiple-synapse boutons [3, 24, 58, 72]. It would be logical to suppose that activation of synaptic transmission on exit from the state of hypothermic torpor would be accompanied by the formation of multiple synapses on presynaptic boutons. However, comparative analysis of three-dimensional reconstructions of axons and dendritic spines in ground squirrels in the states of normothermia and hypothermic torpor showed that these multiple synapses on axons were seen in all states of the hibernating animal. In addition, our analysis demonstrated that multiple synapses could also be detected on dendrite spines (Fig. 2).

Figure 2*a* shows eight serial sections of a mushroom spine and two axons making contact with it. Three-dimensional reconstruction of these two axons and the dendritic spines making contact with them is shown in Fig. 2*b*. This shows that axon 1 forms synapses with three dendritic spines, two mushroom and one fine. Axon 2 forms two synapses, one with a mushroom spine and one with a fine spine. The three-dimensional organizations of all these spines are shown separately in Fig. 2*c*. Figure 2*d* shows that the presynaptic boutons contain a population of isolated mitochondria. It is known [58] that mitochondria do not necessarily have to be present in presynaptic boutons. Figure 2*e* shows the three-dimensional organization of three types of PSD: perforated, segmented, and macular. Figure 2*f* shows a stereoscopic pair of images of a mushroom spine forming two synapses with two axons.

These results provide direct evidence that both the dendritic spine and the axon can form more than one synapse [2, 3]. In other words, the one spine-one synapse concept is replaced by a new paradigm – that one spine can form several synapses.

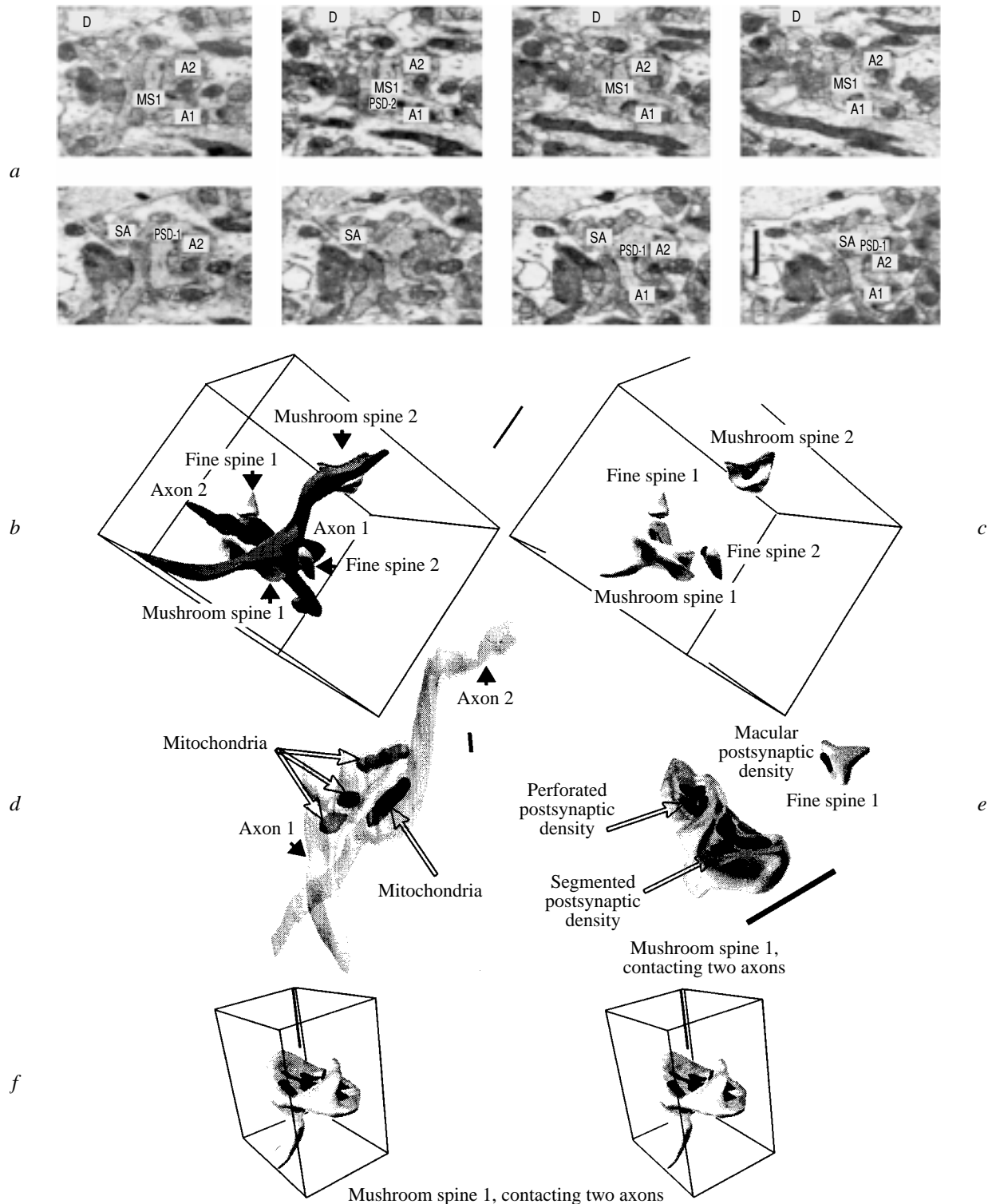


Fig. 2. Three-dimensional reconstruction of a mushroom spine in field CA1 of the ground squirrel hippocampus. The spine simultaneously forms synapses with two axons. Each of the axons also forms multiple synapses with dendritic spines. *a*) Eight microphotographs of a series consisting of 74 sections used for three-dimensional reconstruction; A1 and A2 are axons; D shows the dendrite bearing the mushroom spine (MS1) with perforated and segmented postsynaptic densities (PSD1 and PSD2 respectively); SA identifies the spine apparatus; *b*) three-dimensional reconstruction of axons and dendritic spines; *c*) three-dimensional reconstruction of dendritic spines forming synapses with two axons; *d*) three-dimensional reconstruction of axons and mitochondria; *e*) three-dimensional reconstruction of three types of postsynaptic densities; *f*) stereoscopic pair of images of a mushroom spine with postsynaptic densities. The scale bar shows 1  $\mu$ m.

The two main components of the nervous system – neurons and glial cells – interact via gap junctions, so-called electrical synapses, and other mechanisms involved in the control of behavior, memory, and forgetting, and including thought processes. In this regard, neuroinformatics provides a series of contemporary information technologies for studying different levels of operation of the nervous system. This includes a set of computer programs allowing quantitative stereological analysis of synaptic structures and their reconstruction. Unfortunately, quantitative analysis and modeling of various subcellular structures on the basis of single sections lead to misunderstandings because they introduce large errors [14]. A number of studies [14, 19, 28, 61–63, 73] have provided detailed grounds for the importance of both an unbiased quantitative stereological analysis and the need for 3D reconstruction. Recent years have seen the development of relatively simple and thus available programs for 3D reconstruction using IBM personal computers, which can be used to produce three-dimensional models along with simultaneous assessment of their quantitative parameters, such as the surface areas and volumes of study structures [19].

Preparation of serial images from an identified area of the hippocampus is only one of the intermediate stages in producing three-dimensional reconstructions. The next step is alignment of the images. The “Alignment” computer program is used to align electron microscope images taken from a given part of a dendrite on different sections; 3D images are generated in the wrl/vrml format by constructing outlines for each structure using the “Trace” program [19], followed by transformation into the raw format and the commercial program “3D View Actify” to generate the overall model exemplified in Fig. 2.

We have also developed our own set of programs for aligning and constructing smoothed 3D objects, and also for quantitative analysis of the study structures and the distances between them. This set of programs is relatively simple to use and runs on IBM personal computers with Pentium 2 and above. This set has greater capabilities than programs used previously for 3D reconstruction.

## CONCLUSION

Chemical synapses play an important role in brain function and consist of presynaptic boutons containing neurotransmitters and postsynaptic zones comprising dendritic spines. The largest spines in the hippocampus are mushroom spines, which contain free polyribosomes probably involved in synapse renewal. It is significant that fine spines can retract to become stubby spines and then shaft synapses. This process of transition is reversible and may be a real mechanism of synaptic plasticity. In addition, all categories of spines can change their shapes, volumes, and surface

areas. Three types of densities are valid markers for synapses: macular, perforated, and segmented, and these can also change their shapes.

The “one spine-one synapse” paradigm is now, thanks to the use of serial ultrathin sections and three-dimensional reconstruction, refuted by the possible presence of several synapses on one mushroom spine. We have also observed examples of contacts of several (up to five) presynaptic mossy fiber boutons on “thorny excrescences” of pyramidal neuron dendrites in hippocampal fields CA2, CA3, and CA4 [2]. The advantage of having several synapses on one spine may be associated with the ability to process incoming information at the level of a small area of the dendrite – an individual spine. Our studies show that the proportion of these multiple synapses on mushroom spines amounts to no more than 0.1% of all mushroom spines, while the proportion of mushroom spines in rat and ground squirrel hippocampal field CA1 is usually about 12–14% of the whole synapse population.

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