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New ways of looking at synapses

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Abstract Current concepts of synaptic fine-structure are derived from electron microscopic studies of tissue fixed by chemical fixation using aldehydes. However, chemical fixation with glutaraldehyde and paraformaldehyde and subsequent dehydration in ethanol result in uncontrolled tissue shrinkage. While electron microscopy allows for the unequivocal identification of synaptic contacts, it cannot be used for real-time analysis of structural changes at synapses. For the latter purpose advanced fluorescence microscopy techniques are to be applied which, however, do not allow for the identification of synaptic contacts. Here, two approaches are described that may overcome, at least in part, some of these drawbacks in the study of synapses. By focusing on a characteristic, easily identifiable synapse, the mossy fiber synapse in the hippocampus, we first describe high-pressure freezing of fresh tissue as a method that may be applied to study subtle changes in synaptic ultrastructure associated with functional synaptic plasticity. Next, we propose to label presynaptic mossy fiber terminals and postsynaptic complex spines on CA3 pyramidal neurons by different fluorescent dyes to allow for the real-time monitoring of these synapses in living tissue over extended periods of time. We expect these approaches to lead to new insights into the structure and function of central synapses.

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Introduction

Synapses, particularly those on dendritic spines, are the major sites of neuronal plasticity in the central nervous system. In current concepts of fundamental brain processes such as learning and memory, synapses and plastic changes in their structure and function play an important role. For instance, it has been shown that long-term potentiation (LTP), a widely studied form of synaptic plasticity and a model of learning and memory processes, is associated with the formation of new dendritic spines (Engert and Bonhoeffer 1999). Other studies have indicated that LTP is accompanied by changes in the structure of synaptic contacts (Toni et al. 1999). Changes in synapse structure associated with functional changes point to an important role of the structural organization of synapses in the function of a neuronal network.

What are the structural components of a synapse as known from previous fine-structural studies? First, there is the presynaptic bouton with synaptic vesicles containing the neurotransmitter. Pre- and postsynaptic elements are separated by a widening of the extracellular space, the synaptic cleft. The adjacent membrane specialization on the presynaptic side is regarded as the release site of the transmitter that diffuses to the postsynaptic membrane specialization where transmitter receptors are located. Postsynaptic elements mainly include dendritic spines but also dendritic shafts, cell bodies, and axon initial segments. While all these principal structural components of synapses have been known for quite some time and made their way to text books (Peters et al. 1991; Matus and Frotscher

2005), important details are still missing. For example, it may not be justified to lump all synaptic vesicles, as physiological studies have provided evidence for different vesicle pools, i.e., a "readily releasable pool", a "recycling pool", and a "reserve pool" (Schikorski and Stevens 2001; Hallermann et al. 2003; Rizzoli and Betz 2005). Previous conventional electron microscopic studies have not allowed one to determine these different vesicle pools. Tissue fixation by aldehydes, such as glutaraldehyde and paraformaldehyde, may result in alterations of the distribution of these different vesicle pools. Similarly, the dimension of the synaptic cleft, an important parameter in transmitter diffusion from the presynaptic to the postsynaptic side, is subject to shrinkage associated with fixation and dehydration procedures. Finally, electron microscopy which unequivocally allows for the identification of synaptic contacts, cannot be used to study morphological changes in the shape and number of synaptic components over time. Real-time microscopy studies of green fluorescent protein (GFP)-labeled neurons in culture, on the other hand, allow for the imaging of spines and the de novo formation of these postsynaptic structures over time but do not enable the investigator to unequivocally identify synaptic contacts. Thus, questions have remained as to the nature of newly formed dendritic protrusions following the induction of LTP.

In this section, we will discuss novel approaches to the morphological analysis of synapses. First, we will discuss high-pressure freezing (HPF, Studer et al. 1989, 2001) of nervous tissue in the absence of aldehyde fixation as a novel method to overcome some of the limitations of conventional transmission electron microscopy. Second, we will show that real-time microscopy of differently labeled pre- and postsynaptic elements may provide a powerful tool to study structural plasticity at central synapses. A major issue in the studies described here is that they are performed on identified, characteristic synapses in the CNS, the synapses between granule cell axons, the mossy fibers, and large, thorny excrescences on pyramidal neurons in hippocampal region CA3.

A synapse to remember–the mossy fiber synapse in the hippocampus

Axons of the granule cells of the dentate gyrus are thin, unmyelinated fibers that originate from the basal pole of the cell body, invade the hilus, and terminate in hippocampal region CA3. The synapses that they establish with the CA3 pyramidal cells show a number of unique structural characteristics. First, the thin mossy fiber axon enlarges to form a giant expansion of more than 5 μm in diameter. These large terminal boutons are densely filled with clear, round synaptic vesicles intermingled with a few dense-core vesicles. It is this enormous number of synaptic vesicles which allows for the rapid identification of these boutons at low magnification in the electron microscope. The postsynaptic elements, spines on proximal dendrites of CA3 pyramidal neurons, are similarly characteristic. These spines are much larger than regular spines on hippocampal pyramidal cell dendrites and are often branched. They protrude deeply into the presynaptic mossy fiber bouton. As a result, many sections of mossy fiber boutons appear as large areas full of vesicles with some embedded dendritic spines. Synaptic contacts on these spines are asymmetric in nature, i.e., the postsynaptic density is wider than the presynaptic membrane specialization (Gray 1959). Reconstruction of a mossy fiber bouton from ultrathin serial sections revealed some 37 release sites formed by an individual mossy fiber terminal (Chicurel and Harris 1992). In addition to these synaptic contacts, mossy fiber boutons establish *puncta adhaerentia* with dendritic shafts. While the latter contacts do not show a widening of the extracellular space, the cleft at synaptic sites is clearly widened when compared to non-synaptic membrane contacts. All these characteristic features of mossy fiber synapses have been described soon after transmission electron microscopy was established as a method of high-resolution structural analysis of nervous tissue (Blackstad and Kjaerheim 1961; Hamlyn 1962).

A regular feature of the large spines is the presence of a spine apparatus, a characteristic organelle in forebrain dendritic spines consisting of sacs of endoplasmic reticulum intervened by electron-dense plates (Gray 1959; Spacek 1985; Spacek and Harris 1997; Frotscher and Deller 2005). At the base of the spines, often polyribosomes are observed suggesting local protein synthesis (Steward and Levy 1982). Besides the giant mossy fiber expansions, thin, finger-like protrusions have been described which were found to establish contacts exclusively with GABAergic interneurons (Acsády et al. 1998). Occasionally, the giant expansions also establish synaptic contacts with dendritic shafts of GABAergic cells in addition to contacting pyramidal cell dendritic spines (Frotscher 1985, 1989; Frotscher et al. 1994, 2006).

Traditionally, the mossy fiber synapse is regarded as the second station in the so-called trisynaptic excitatory pathway of the hippocampal formation. This circuit involves the entorhinal synapses on dentate granule cells (first station), the mossy fiber synapses on CA3 pyramidal cells (second station), and the synapses formed by the axons of CA3 pyramidal cells (Schaffer collaterals) on pyramidal neurons in the CA1 region (Andersen et al. 1971). Studies over the last 30 years have provided evidence for a more complex circuit of the hippocampus involving mossy fiber synapses. Thus, the granule cell axons give rise to numerous contacts with hilar neurons, particularly mossy cells which project backwards and

innervate the proximal dendritic segments of the granule cells (Frotscher et al. 1994).

Functionally, the mossy fiber synapse has been implemented in the overall function of the hippocampus in the processing, storage and recall of spatial information (Squire et al. 2004). More specifically, the mossy fiber synapse has been viewed as a teacher synapse involved in the completion, separation, and linkage of patterns in the dentate gyrus—CA3 network (Bischofberger et al. 2006). For a more detailed discussion of mossy fiber synapses under normal and pathological conditions and of their putative function, the reader is referred to two recent reviews (Bischofberger et al. 2006; Frotscher et al. 2006). Here it may suffice to point out that the synapse formed by giant mossy fiber expansions is of a unique structure allowing for their unequivocal identification in light and electron microscopic studies.

Freeze—but no ice, please!

It is a fundamental question in electron microscopic studies of the central nervous system to what extent the structural characteristics observed reflect the in vivo situation. Chemical fixation by aldehydes and dehydration in an ascending series of alcohol may result in uncontrolled tissue alterations. Numerous attempts have been made to overcome the adverse effects of chemical fixation and dehydration. In recent years, high-pressure freezing (HPF) has proven to provide the investigator with hitherto unknown details of fine-structural organization of a variety of tissues (Studer et al. 1995, 2001). In brief, fresh tissue samples are placed on specimen carriers and shock-frozen within milliseconds under high pressure. The tissue water is then substituted by acetone, and the blocks are embedded in resin such as Epon or in Lowicryl.

Application of HPF to the study of the central nervous system has remained difficult. Reasons for that include the unavoidable damage to fresh brain tissue during preparation and rapid hypoxic changes occurring in the highly vulnerable nerve cells and their processes. One recent study (Rostaing et al. 2006) applied HPF to acute brain slices as used in electrophysiological studies. However, for the preparation of acute slices, the tissue has to be removed from the skull and has to be sectioned on a vibratome or tissue chopper. Numerous neuronal cell bodies, dendrites, and axons are cut by the slicing procedure, and swelling and shrinkage of tissue components may occur side by side (Frotscher et al. 1981).

Organotypic hippocampal slice cultures offer a variety of advantages for electron microscopic studies using HPF. Following preparation, slice cultures are allowed to recover from the tissue damage for a couple of days which includes the removal of tissue debris and the re-growth of severed processes. As sections and not dissociated cells are cultivated, the neurons are kept in their normal environment allowing for an organotypic differentiation of the tissue. Numerous studies have shown that not only cell layers and cellular characteristics but also the normal arrangement of fiber layers and the structural and functional properties of synapses are retained under these culture conditions (e.g. Frotscher et al. 1995; Gähwiler et al. 1997; Förster et al. 2006). In particular, giant mossy fiber synapses on CA3 pyramidal neurons are nicely preserved and display all characteristics as known from numerous in vivo studies (Frotscher and Gähwiler 1988).

We have recently started a comprehensive study on the fine structure of mossy fiber synapses after high-pressure freezing and cryosubstitution. Slice cultures of hippocampus are prepared on postnatal day (P) 6 and incubated for one week. Thereafter, they are taken to the recording chamber, and CA3 pyramidal cells are patched and mossy fiber synapses stimulated by a stimulating electrode. Following recording, the same slice cultures are then subjected to HPF.

Figure 1 shows a hippocampal mossy fiber bouton (MFB) as it appears after HPF, cryosubstitution, and embedding in Epon. All characteristic features of mossy fiber synapses as described above are seen, and many structural details are better visible and better preserved than after perfect fixation using aldehydes. Membranes of synaptic vesicles are not homogeneous but show rosette-like particle accumulations. Often long tethers are attached to the vesicles. Postsynaptic densities give rise to unusual, long extensions protruding deeply onto the postsynaptic element. The widening of the synaptic cleft is conspicuous. Spines are filled with fuzzy material, and dendrites contain numerous tubules. Our current studies are aimed at quantifying various structural components of mossy fiber synapses in complete series of thin sections of boutons in HPF-treated material. We expect from these studies not only novel insights into the fine-structural organization of an identified central synapse but also more realistic data on the spatial dimensions of these structures which are needed for a better correlation of physiological and morphological findings. For instance, realistic estimates of the membrane surface of a synaptic vesicle are a prerequisite for the determination of the total number of released vesicles during synaptic transmission from measurements of the capacitance change of a bouton (Hallermann et al. 2003). Rapid freezing within milliseconds may provide a powerful tool in the study of subtle structural changes at synapses associated with forms of synaptic plasticity such as long-term potentiation and long-term depression (LTD).

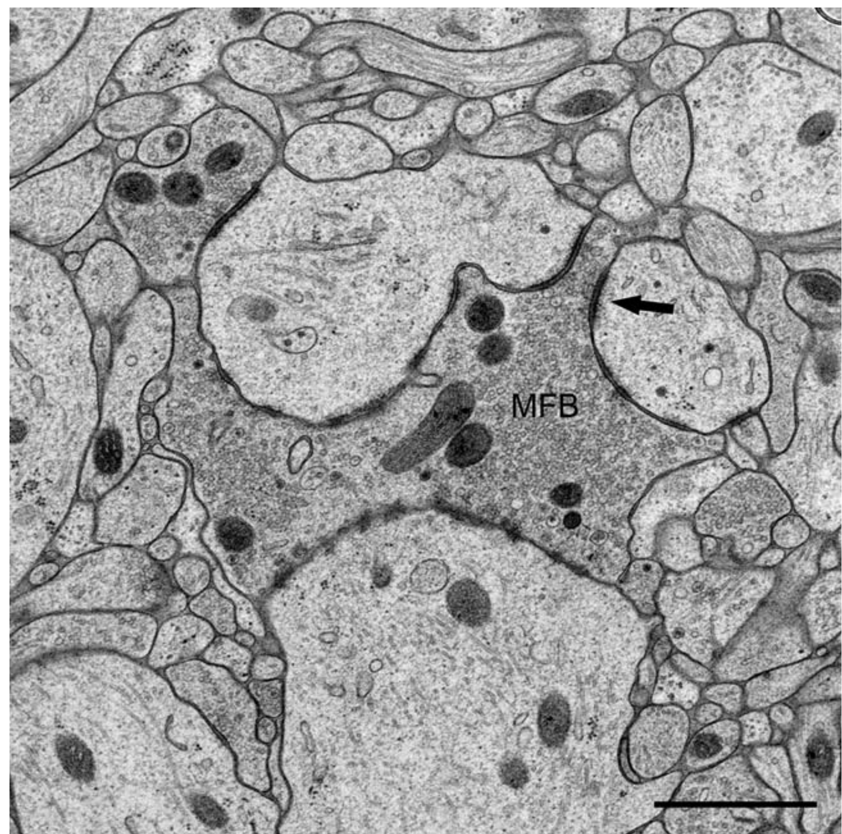

Fig. 1 Synaptic fine structure after high-pressure freezing. Mossy fiber bouton (MFB) in a slice culture of hippocampus after high-pressure freezing in the absence of aldehyde fixation. Note good preservation of fine-structural details. The MFB is densely filled with clear synaptic vesicles intermingled with a few dense-core vesicles. Synaptic contacts (one labeled by *arrow*) are established with large spines protruding into the presynaptic terminal. Scale bar: 1 µm

A limiting factor in studies of this kind is the size of the tissue samples. The tissue must be shock-frozen, and the tissue water is to be vitrified. Larger tissue samples which cannot be frozen rapidly will show the development of ice crystals associated with obvious tissue destruction (Studer et al. 1995). Taken together, we think that slice cultures of hippocampus, and in particular an easily identifiable synapse such as the mossy fiber synapse on CA3 pyramidal neurons, will provide a most useful tool in studies combining electrophysiology, high-pressure freezing, and ultrastructural analysis.

Different colors for pre and post in real-time microscopy of mossy fiber synapses

Problems with the identification of synaptic structures have remained a major issue in studies aimed at monitoring structural changes at synapses over time by using life microscopy approaches. One example is the de novo formation of dendritic spines after LTP induction (Engert and Bonhoeffer 1999). Are the outgrowing protrusions really dendritic spines? Do they get in contact with presynaptic boutons? When are synaptic contacts formed and how stable are they?

As a rule, real-time microscopy studies take advantage of genetically engineered mutants expressing fluorescent proteins, such as green fluorescent protein (GFP). Alternatively, living cells in acute slices or slice cultures are intracellularly stained by fluorescent dyes, for instance Lucifer Yellow or calcein. In both cases, the staining is mainly used for the identification of cell bodies, dendrites, and dendritic spines since axons and terminals are often only weakly stained. The labeled neurons are monitored over time to follow changes in the position and shape of their cell bodies and processes. This way, it has been possible to monitor the modes of migration of cortical neurons from their site of origin to their destinations in the cortical plate (Nadarajah and Parnavelas 2002, review). Using GFP labeling, remodeling of the actin cytoskeleton and structural changes in dendritic spines have been documented under various experimental conditions (see Matus 2000, review). While these latter studies, like the ones on the de novo formation of dendritic spines after LTP induction, pointed to an unexpected plasticity of spines, they could not resolve the role of presynaptic axonal structures in these plastic processes. Attempts to identify presynaptic boutons by labeling for synaptic vesicle proteins such as synaptophysin were hampered by the fact that even the close apposition of synaptophysin-positive elements to GFP-labeled dendritic structures did not unequivocally demonstrate the presence of a synaptic contact.

The unique structure of mossy fiber boutons is advantageous in real-time microscopy studies of synapses. As

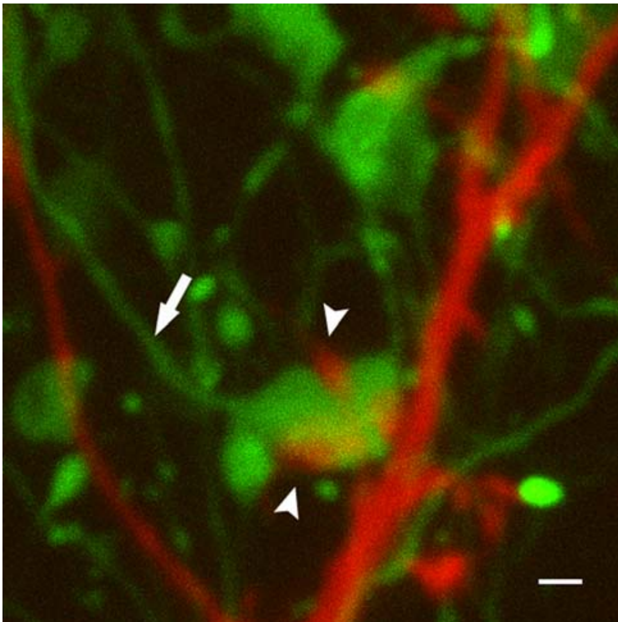

Fig. 2 Differential labeling of presynaptic and postsynaptic elements of a mossy fiber synapse in the hippocampus. Presynaptic mossy fiber axons (*arrow*) and their terminal expansions in a hippocampal slice culture are labeled by the green fluorescent dye Alexa 488. The postsynaptic pyramidal cell dendrite and its large spines protruding into the mossy fiber terminal (*arrowheads*) are intracellularly stained using the red fluorescent dye Alexa 594. Using this approach, mossy fiber synapses in living slice cultures can be monitored for extended periods of time. Scale bar: 1 μm

shown in Fig. 1, the large spines on proximal dendrites of CA3 pyramidal neurons deeply protrude into the presynaptic terminal and in sections appear as "islands" in the vesicle-filled mossy fiber expansion. This characteristic arrangement of pre- and postsynaptic elements can be visualized in the light microscope by labeling the presynaptic mossy fiber expansion and the large complex spines by different fluorescent dyes.

In the experiment illustrated in Fig. 2, we labeled mossy fibers in a hippocampal slice culture by extracellular injection of Alexa 488 dextran (green) into the mossy fiber termination zone (stratum lucidum) in CA3. Postsynaptic target cells were intracellularly filled with the red fluorescent dye Alexa 594 dextran by patching the cells in the whole-cell mode. The red fluorescent postsynaptic spines are embedded in a large mossy fiber expansion, reminiscent of the appearance of mossy fiber synapses in the electron microscope (Fig. 1). The characteristic emergence of a giant expansion from a thin fiber allows for the unequivocal identification of the mossy fiber terminal. Such identified mossy fiber synapses can be viewed for extended periods of time, and changes in spine shape and the occurrence of new spines can be monitored under various experimental conditions.

Conclusions and perspective

In the present study, a well characterized, easily identifiable synapse, the mossy fiber synapse in the hippocampus, was used as a model synapse in novel morphological approaches towards a better understanding of synaptic structure and function. Using high-pressure freezing, native tissue is fixed within less than a second. We hope to be in a position to monitor subtle structural changes at mossy fiber synapses associated with functional synaptic plasticity. A variety of questions as to fine-structural changes in vesicle pools, the number and extension of release sites, and structural changes in the spine apparatus can be addressed with this new approach, having in mind that in previous studies of these subcellular structures aldehyde fixation was applied, a process that lasts for minutes. Since the tissue is not fixed by aldehydes, HPF may offer new possibilities in electron microscopic immunocytochemical studies of molecules present in the tissue at low concentration.

Using life microscopy of differently labeled pre- and postsynaptic structures of mossy fiber synapses, we expect to learn more about plastic changes at an identified synapse. In future studies, we aim to label the presynaptic mossy fiber terminal and the postsynaptic CA3 pyramidal neuron by different calcium-sensitive dyes to follow sequential Ca^{2+} transients in the pre- and postsynaptic element. It is our ultimate goal to visualize the process of synaptic transmission at an identified synapse using these imaging techniques.

Taken together, we are facing an exciting new era of microscopy research. One particular example is the recent development of Stimulated Emission Depletion microscopy (STED microscopy) by Stefan W. Hell from the Max Planck Institute in Göttingen. A wealth of exciting new findings can be anticipated with this high resolution light microscopy technique that allows for the study of single molecules in synaptic vesicles (Willig et al. 2006).

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