DOI: 10.1017/S1431927605501363
Microsc Microanal 11(Suppl 2), 2005

Ultrastructure of the Synaptic Junctions in Mouse Brain Slice after High Pressure Freezing and Freeze Substitution

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Nerve cells communicate with each other at specialized intercellular junctions named synapses. Although synaptic structure has been studied for decades, many important questions addressing function in terms of synaptic architecture have yet to be answered. Traditional chemical fixation based electron microscopy has proved to be too slow to capture the constantly changing structure, such as the fusion of the synaptic vesicle with the plasma membrane [1]. Using rapid freezing techniques, mainly slam freezing, to study synaptic structure yields only about 10-20 μm of good ultrastructural detail. Any cellular structure beyond this depth range will be subject to extensive distortion caused by ice crystal damage [2]. Therefore, an alternative method has being developed. The intact tissue is frozen under high hydrostatic pressure resulting in an increased layer of vitrification. High pressure freezing (HPF) followed by freeze substitution (FS) has produced excellent preservation of the subcellular structure of various biological samples. However, the brain tissue is among the most difficult tissue to cryofix due to its delicate structure and high percentage of water content. One solution to this problem would be to "mild" aldehyde fix and apply cryoprotection prior to high pressure freezing. However, this approach not only suffers the disadvantage of chemical fixation, but also introduces a new potential osmotic problem. It is unlikely that the morphology achieved by this method represents the near-nature state in vivo.

In order to overcome the above problems, we have developed a method of rapidly freezing the tissue from a vibratomed living brain slice (200 μm). The slice was constantly incubated in artificial cerebral spinal fluid (ACSF) equilibrated with 95%O_2/5%CO_2 at 37 ºC. The cerebral cortex was quickly punched out and immediately frozen in the Leica EM PACT high-pressure freezing machine followed by freeze substitution in a Leica AFS apparatus using a medium containing acetone and osmium tetroxide, then infiltrated and embedded in EMbed 812 resin [3]. The 60 nm thin sections were cut and stained with uranyl acetate and lead citrate prior to EM observation. To compare the effect of sample preparation, we also examined brain tissue that had been pre-fixed and cryoprotected prior to freezing.

Here, we present two sets of experimental results. Group I is the directly frozen living brain tissue (Figs.1A, 2A); Group II is pre-fixed and sucrose cryoprotected tissue that underwent the same HPF and FS process as Group I (Figs.1B, 2B). In general, Group II yields better morphology characterized by the smoothness of plasma membrane, and minimal ice damage to the cellular structure. However, the contrast for synaptic vesicles was poor and the number of the vesicles at the presynaptic terminal is less than that in Group I, suggesting the loss the vesicles during the process. Conversely, Group I clearly demonstrates more structural detail, such as synaptic vesicles and their associated microfilaments. This result is comparable to freeze-etching results from early studies [4]. In some areas, however, it is difficult to distinguish new structural features from possible ice crystal damage.

To our knowledge, this is the first HPF experiment on a "live" brain slice. The advantages of such a procedure are the following: 1) Replaces the biopsy gun, which has difficulty obtaining a specific brain region specimen and may stimulate neurons during the process; 2) This approach is the same as preparing for electrophysiological recording on a brain slice, and the ultrastructural features can be correlated with the physiological data; 3) Since pre-freezing treatment is eliminated, it would mostly

likely capture the near-nature state of synaptic organization and be theoretically capable of arresting the transit event that happens at the synaptic junction; 4) Such preparation also will allow us to examine the response of synapses after stimulation and pharmacological manipulation. Certainly, the classical questions remain to be answered: the ability to distinguish newly revealed features from the ice crystal damage is the major challenge for us. Nevertheless, immunoelectron microscopic labeling on direct frozen sections may provide insight into how synaptic proteins are organized at the active zone and their possible role in the synaptic exocytosis.

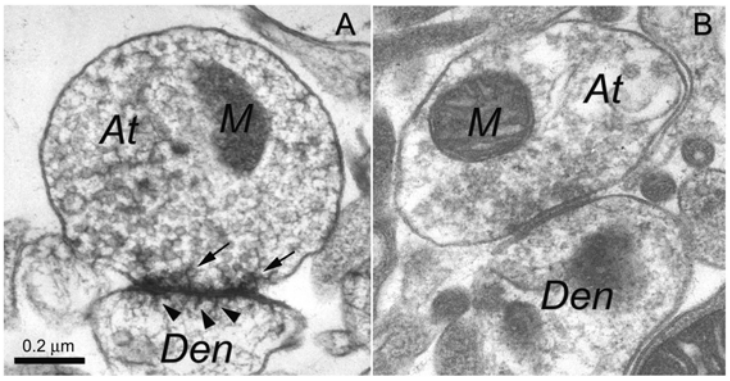

Figure 1. Synapses between axon terminal (At) and dendritic spines (Den)
A.: Direct freezing result. B.: Pre-chemical fixed and cryo-protected prior to freezing. In A, the synaptic vesicles are well distinguished from the cytoplasm and are clustered at the contact zone, the fine filaments extending from the presynaptic plasma membrane link to the vesicles that are at the vicinity (arrows). Note that short filaments associate the vesicles together. At the postsynaptic side, the micro-filaments are attached to postsynaptic density (arrowheads), joint the cytoskeleton network of the spine. This feature normally is missing in the chemical fixed sections. M stands for mitochondria.

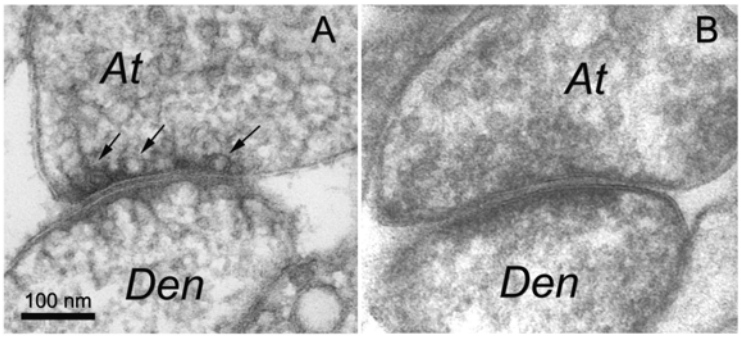

Figure 2. Enlarged view of the synaptic vesicles and contact zones
A. When freezing directly without any pre-treatment, we were able to capture the vesicles (arrows) that docked on the plasma membrane. These vesicles were undergoing the process of releasing neuro-transmitter. Note the structural detail of the docked vesicles. B. The general plasma membrane is well preserved, but it lacks of information regarding synaptic vesicle and its relation with other components.

Acknowledgements:
We wish to thank Kent McDonald and Elaine Humphrey for their help with the protocols of freeze substitution and their useful comments on the experimental results.

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https://doi.org/10.1017/S1431927605501363 Published online by Cambridge University Press