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3−Dimensional Reconstruction of Parallel fiber−Purkinje Cell Synapses Using High−Voltage Electron Microscopy

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고압전자현미경을 이용한 소뇌 평행섬유-조롱박세포 간 신경연접의 3차원 재구성

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ABSTRACT

Synapses are contact points where one neuron communicates with another. The morphological change of synapses under various physiological or pathological conditions has long been hypothesized to modify their functional properties. 3 dimensional (3 D) reconstruction of synapses with serial ultrathin sections has contributed to the understanding of ultrastructural dimensions and compositions of synapses. The 3 D reconstruction procedures, however, require a great amount of expertise as well as include prohibitively time consuming processes. Here, we introduce efficient 3 D reconstruction technique using high voltage electron microscopy (HVEM). Primarily, we established an optimal section thickness and staining condition to observe synaptic structures in detail under HVEM. The result showed that synaptic profiles were preserved at the section thickness of 250 nm without the overlapping of synaptic ultrastructures. An increase in the reaction time of *en bloc* staining was most efficient to enhance contrast than the extension of postembedding staining or the addition of uranyl acetate during dehydration. Then, 3 D reconstruction of parallel fiber Purkinje cell synapses in the rat cerebellum was carried out with serial HVEM images and reconstruction software. The images were aligned and the contours of synapses were outlined on each section. 3 D synapses were finally extracted from the section files by grouping all the synaptic contours. The reconstructed synapse model clearly demonstrated the configuration of pre and postsynaptic components.

These results suggest that 3 D reconstruction of synapses using HVEM is much efficient and suitable for massive quantitative studies on synaptic connectivity than conventional TEM approach using numerous ultrathin sections.

Key words : Synapse, 3 D reconstruction, HVEM, Purkinje cell, Cerebellum

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INTRODUCTION

Synapses are specialized junctions between neurons where signals are propagated from one cell to another. Most excitatory synapses consist of presynaptic axon terminals and postsynaptic dendritic spines in a mammalian central nervous system. The structure and number of synapses are dramatically changed under various physiological or pathological conditions such as development (Cohen-Cory, 2002), environmental enrichment (Rampon et al., 2000), learning (Muller et al., 2002), hormonal states (Woolley, 1998), alcohol exposure (Klintsova et al., 2002), ataxia (Rhyu et al., 1999), and mental retardation (Zhang et al., 2001). Since the structural modification of synapses could modulate their functional properties, the morphological investigation of synapses is crucial to understanding of synaptic transmission, synaptogenesis, and synaptic plasticity.

Purkinje cells, a main efferent neuron in the cerebellar cortex, have numerous synapses although synaptic density varies within the dendritic field of a neuron. The proximal dendrites of an individual Purkinje cell are innervated by a single climbing fiber, while distal branchlets are innervated mainly by parallel fibers. Long-term depression (LTD), a model of synaptic plasticity that is involved in certain types of learning associated with cerebellum, is induced at parallel fiber-Purkinje cell synapses. It has been also reported that the parallel fiber-Purkinje cell synapses exhibit changes in number after motor skill learning (Kleim et al., 1998; Kim et al., 2002; Federmeier et al., 2002). The parallel fiber-Purkinje cell synapses are, therefore, a valuable candidate to investigate the functional implication of morphological synaptic plasticity.

TEM of ultrathin sections has produced high-resolution images of synaptic profiles although the access to 3-D information is limited. The 3-D reconstruction of synapses using serial ultrathin sections has provided more comprehensive information on the structural dimension and composition of synapses. Recently, several studies reported ultrastructural characteristics of synapses in hippocampus CA1 area and in cerebellum using the reconstruction of serial TEM sections (Harris & Stevens, 1988; Shepherd & Harris, 1998; Ventura & Harris, 1999; Fiala et al., 2002). The 3-D reconstruction of synapses using TEM, however, includes prohibitively time-consuming processes requiring tools and expertise. HVEM provides an increase in resolution, specimen penetrating capability, and depth of information (Bozzola & Russell, 1992). Consequently, HVEM has been effectively applied to the studies of neuronal structures by using thick sections (Hama et al., 1977, 1989; Lee et al., 2004). In this context, we have considered the application of HVEM images based on serial thick sections for the 3-D reconstruction of synapses. If thick sections are used for reconstruction, it would be possible to reduce time-consuming processes such as a construction of numerous serial sections and an operation of stacks of images on a computer program. It should be noted, however, that the contrast of specimens is somewhat lessened under HVEM. Therefore, it is necessary to enhance contrast through the modification of staining condition ahead of 3-D reconstruction of synapses using HVEM.

In this study, we propose an optimal condition for the observation of synapses under HVEM and describe practical procedures for the HVEM-based 3-D reconstruction of parallel fiber-Purkinje cell synapses in the rat cerebellar cortex.

MATERIALS AND METHODS

High-voltage electron microscopy

The tissue preparation for HVEM was based on routine sampling procedure for TEM, with slight modification of staining methods to offset low contrast (Table 1). All experimental manipulations were performed within the guide for animal experiment edited

Table 1. Various staining conditions conducted to enhance contrast and the decision of section thickness to avoid the overlapping of synaptic ultrastructures under HVEM.

Staining condition	Reaction time
Pre-embedding *En bloc* staining	90 min Overnight
Dehydration	EtOH 1% Uranyl acetate in EtOH
Post-embedding	Uranyl acetate for 20 min Lead citrate for 5 min Uranyl acetate for 1 h Lead citrate for 15 min
Section Thickness	250 / 500 / 1000 nm

by Korean academy of medical sciences. Briefly, 4 months old male, Sprague-Dawley rats were anesthetized with sodium pentobarbital (100 mg/kg) and intracardially perfused with 2% paraformaldehyde/2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The cerebellum was removed and stored in the same fresh fixative overnight at 4°C. Sagittal sections of each cerebellum were washed, post-fixed in 2% osmium tetroxide for 3 h. *En bloc* staining was carried out with 2% uranyl acetate for 90 min or overnight. The samples were then dehydrated through an ascending series of ethanol with or without 1% uranyl acetate and then embedded with Epon mixture. Semi-thin sections were obtained to identify the location of molecular layer under the light microscope, and a series of thick sections (250, 500, 1000 nm) were taken using Ultracut-E (Reichert-Jung) to evaluate the optimal section thickness preserving the ultrastructures of synapses (Table 1). 25∼30 consecutive serial sections were then mounted on formvar-coated slot grids (2×1 mm, Synaptek Co.), stained with 6% uranyl acetate for 20 min or 1h and then Reynold's lead citrate for 5 or 15 min. The sections were observed at the accelerating voltage of 1250 kV under JEM-ARM 1300S installed in Korea Basic Science Institute (KBSI, Daejeon, Korea). From each section, same spot was taken serially at the magnification of 10,000-20,000X. The negatives on HVEM film were scanned at 1000 dpi using Epson perfection 3200 photo scanner and saved as TIFFs for further computer-assisted reconstruction processes.

Computer-assisted 3-D reconstruction

Since each image of synapses was taken separately in the microscope, the images must be brought back together to understand the synaptic structure of the whole tissue. For this purpose, Reconstruct software, which is a Windows® application for aligning, tracing, and reconstructing objects from serial section images, was used in this investigation (available at http://synapses.bu.edu/tools). The scanned image files were imported into Reconstruct software using a multi-file import operation, and converted to sequence files. The alignment of serial images was carried out by putting a few points into correspondence between adjacent sections. This can be done by entering point traces alternately on the same organelle (for instance, mitochondria or microtubule) of adjacent sections. The membranes of presynaptic parallel fibers and postsynaptic dendritic spines of Purkinje cells were then manually outlined on each aligned section. The serial contours of each synapse previewed in an Open Graphics Language (OpenGL)-based 3-D scene window. The 3-D images of synapses were finally saved as a Virtual Reality Modeling Language (VRML) files.

RESULTS

To reconstruct parallel fiber-Purkinje cell synapses using HVEM, it was primarily required to establish an optimal condition to observe synaptic structures in detail under HVEM. First of all, we decided the appropriate section thickness to avoid overlapping, since synaptic ultrastructures tend to overlap on the thick sections by the increased penetration power of HVEM.

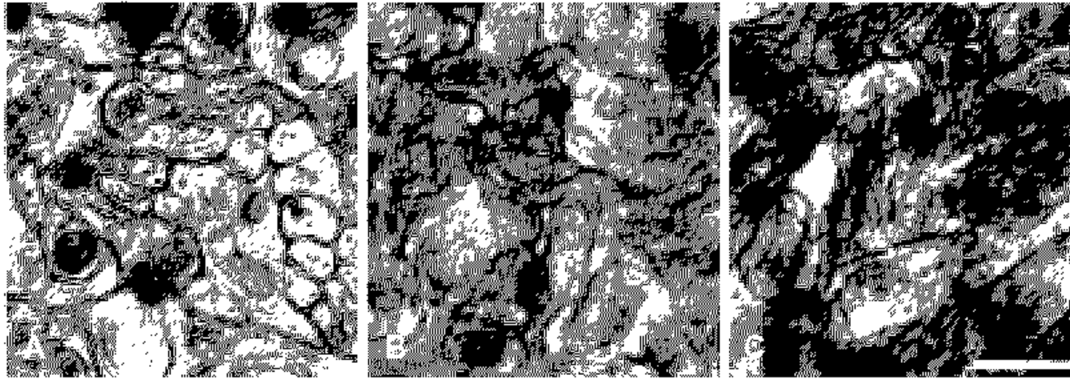

Fig. 1. The decision of an optimal section thickness for 3-D reconstruction of parallel fiber-Purkinje cell synapses using HVEM. The overlapping of synaptic profiles is observed in proportion to the increase in section thickness: (A) 250 nm, (B) 500 nm, (C) 1000 nm. Bar = 2 μm.

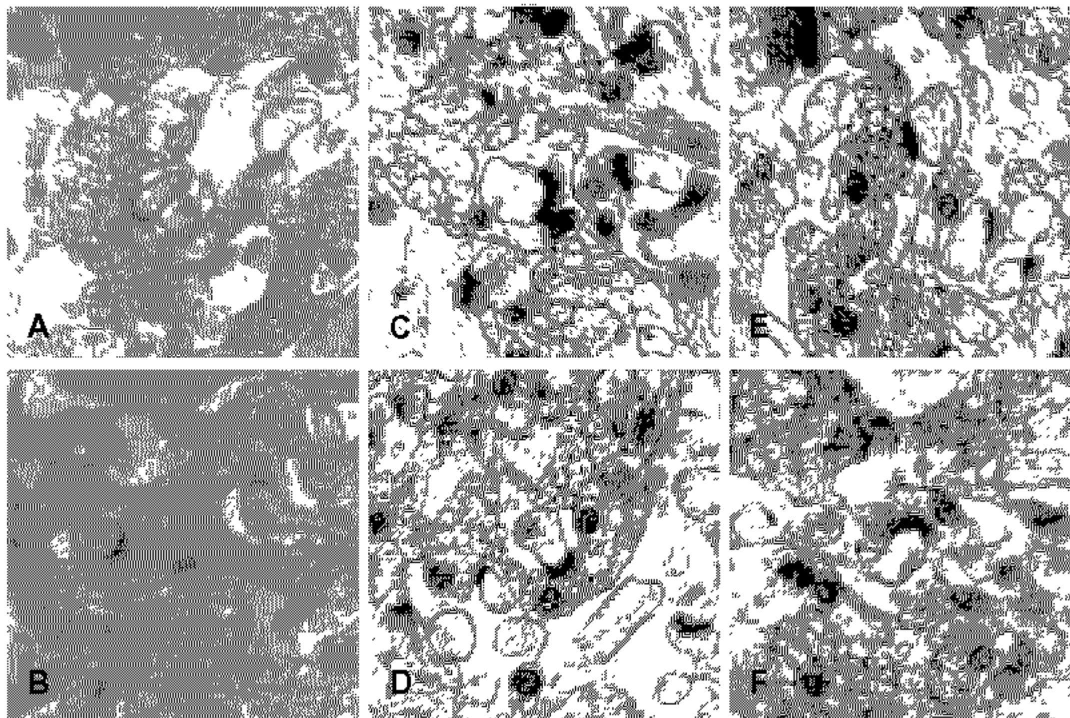

Fig. 2. An optimal staining condition for the observation of neuronal synapses under HVEM. The addition of uranyl acetate to dehydration was not enough to offset low contrast: A vs. B and C vs. D. The extension of postembedding staining didn't show a significant difference in overall staining quality: E vs. F. Prolonged *en bloc* staining was most suitable to enhance the contrast: A & B vs. C & D. (A) *En bloc* staining for 90 min, dehydration without uranyl acetate (UA), UA for 20 min followed by lead citrate (LC) for 5 min. (B) Same as A except the addition of 1% UA in dehydration. (C) Same as A except overnight *en bloc* staining. (D) Same as B except overnight *en bloc* staining. (E) Same as D. (F) Same as D except UA for 1 h followed by LC for 15 min. Bar = 1 μm.

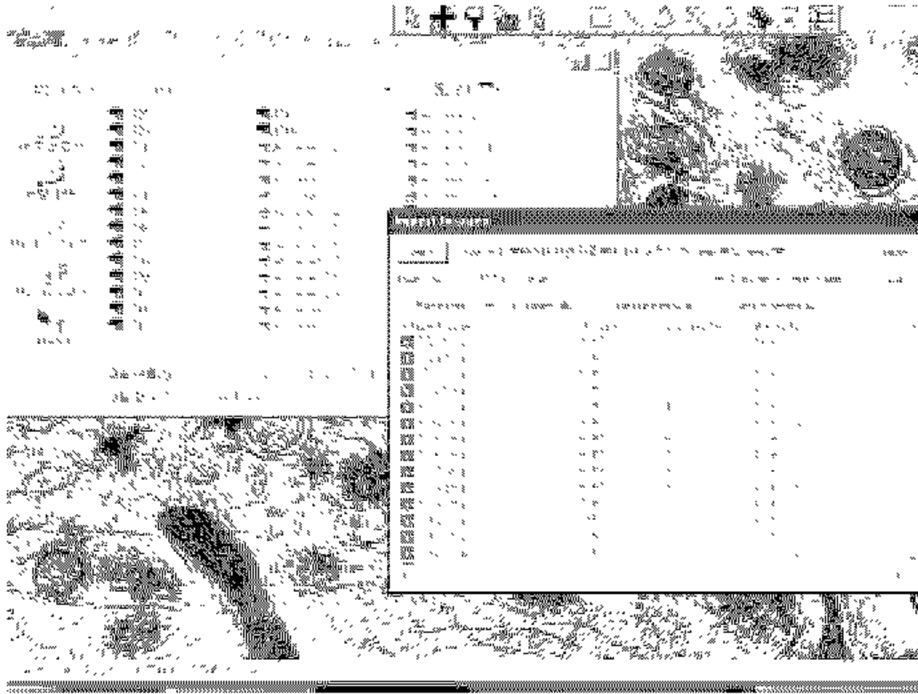

Fig. 3. A multi-file import operation for the conversion of HVEM images to sequence files.

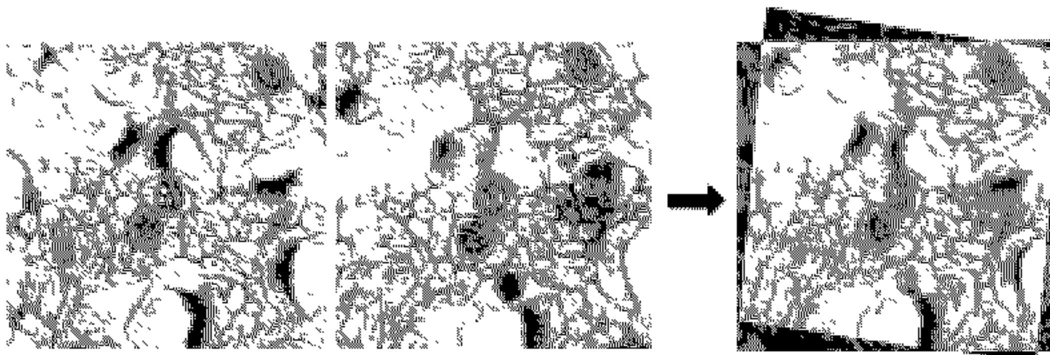

Fig. 4. The alignment of sections by entering point traces alternately on the same organelle of adjacent sections.

Thus the establishment of appropriate section thickness is essential to outlining the contours of synaptic membranes precisely. Although it was possible to observe synaptic ultrastructures in all sections prepared in this study (250, 500, and 1000 nm thick), the overlapping of cellular membranes, organelles, or synaptic vesicles was frequently observed in proportion to the increase in section thickness (Fig. 1). 250 nm thick sections showed well-preserved synaptic membranes in detail and was finally selected to avoid a complication in contouring synaptic membranes, which is caused by a pile of ultrastructures in a section. Then we slightly modified

Fig. 5. A series of synaptic contours previewed in an OpenGL-based 3-D scene window.

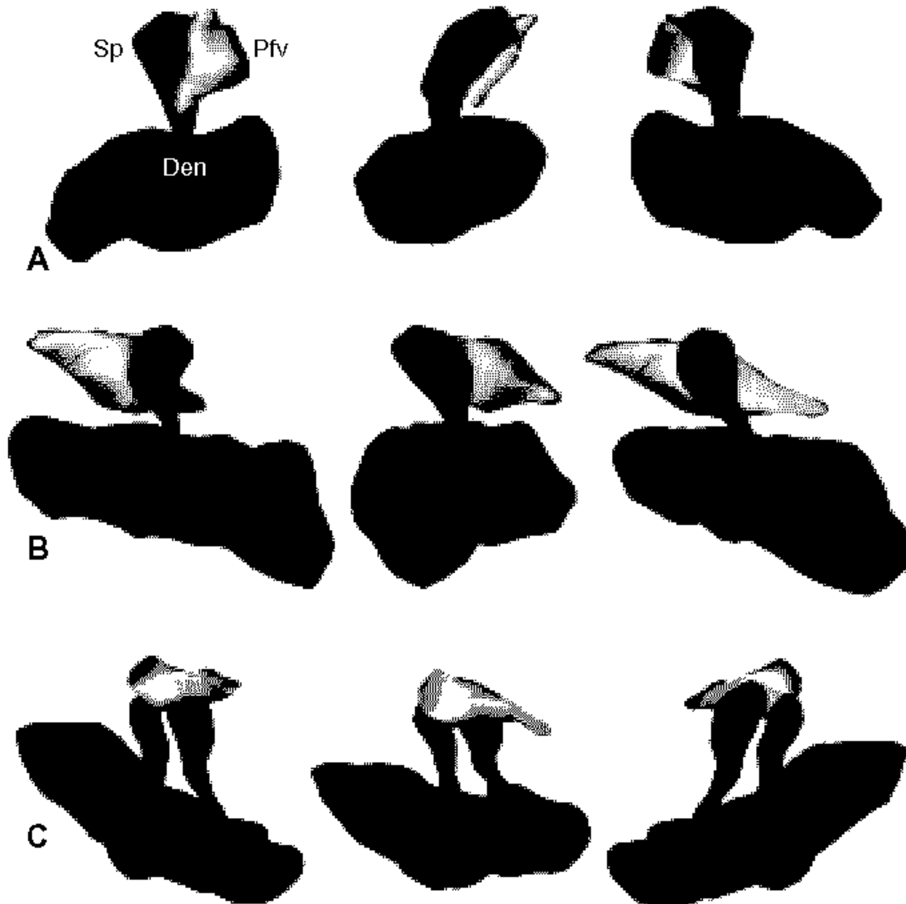

Fig. 6. A representative 3-D reconstruction of parallel fiber-Purkinje cell synapses. The synapses show single contact (A and B) and double contact (C) configuration. Note that two dendritic spines of double contact synapse originate from same dendrite. Den: dendrite, Sp: spine, Pfv: parallel fiber varicosity.

electron staining methods to enhance the contrast of HVEM specimens. The result clearly showed that an increase in the reaction time of *en bloc* staining is more efficient to enhance contrast than the extension of postembedding staining or the addition of uranyl acetate to dehydration step (Fig. 2). Thus the combination of 250 nm of section thickness and the prolongation of *en bloc* staining was adopted as the optimal condition and utilized for 3-D reconstruction of neuronal synapses in this study.

Practical 3-D reconstruction was conducted with parallel fiber-Purkinje cell synapses in the rat cerebellum. A multi-file import operation converted serial HVEM images to sequence files (Fig. 3). Alternate point traces on same organelle of adjacent sections resulted in the alignment of serial sections (Fig. 4). The accumulation of manually-traced synaptic contours revealed the entire 3-D morphology of synapses (Fig. 5). After surface rendering of serial traces, representative 3-D models of synapses clearly exhibited the configuration of pre and postsynaptic components (Fig. 6).

DISCUSSION

The synapses are specialized contact sites where neuronal information is transferred. Since the number and shape of synapses dynamically change in response to various physiological or pathological states, a detailed morphological investigation is critical to understanding of functional properties of the synapses and neuronal networks. In this context, the first focus of this study was to establish an optimal section thickness for 3-D reconstruction of synapses using HVEM and an appropriate electron staining to offset low contrast. Synaptic membranes, intracellular organelles, or synaptic vesicles showed a tendency to be overlapped in proportion to the increase in section thickness due to high penetrating capability of HVEM. Since it is crucial to avoid a complication in contouring complex structures like synaptic membranes, the section thickness should not be over 250 nm, in which synaptic membranes are well-preserved without overlapping. Through the modification of staining methods, the prolongation of *en bloc* staining exclusively revealed a significant difference in overall staining quality and the enhancement of contrast. The addition of uranyl acetate to dehydration or the prolonged post-embedding staining did not enhance the contrast sufficiently in this study. A previous study reported that blood vessels in 0.5~1 μm thick sections were adequately stained with 2% uranyl acetate for 1~4 h at 50°C and Reynolds lead citrate for 30~45 min for HVEM observation (Song et al., 1986). Several studies also recommended that the improvement of staining could be attained by raising staining temperature by 60°C or by coating the section with carbon following staining (Locke & Krishnan, 1971; Hama & Kamino, 1974). It is likely that an optimal condition for specimen observation under HVEM should be determined according to the purpose of experiment or the structural complexity of objects to be reconstructed. Our results clearly showed that the simple prolongation of *en bloc* staining is sufficient to enhance the contrast. Thus the combination of 250 nm thick sections and prolonged *en bloc* staining is supposed to be appropriate for 3-D reconstruction of neuronal synapses using thick HVEM sections.

The 3-D Reconstruction of biological specimens can be obtained by the computerized tomography using sequentially-tilted images or by putting continuous thin sections back together. Since the computerized tomography is basically applied to reconstruct the distribution of a substance inside the body of an object, this method is especially useful for the reconstruction of intracellular organelles such as endoplasmic reticulum and Golgi apparatus. The reconstruction of serial sections can be performed with TEM or confocal microscopic images. Although it is not required to realign numerous sections in the reconstruction using confocal microscopy, this method is not suitable to observe small

intrasynaptic organelles due to the limitation of resolution. The 3-D reconstruction using 50∼60 nm TEM sections has effectively applied for the quantitative studies on the structural dimensions and composition of synapses (Harris & Stevens, 1988; Shepherd & Harris, 1998; Ventura & Harris, 1999; Fiala et al., 2002; Ostroff et al., 2002). The investigation using ultrathin sections, however, includes time-consuming and labor-intensive processes requiring tools and expertise. To overcome these weak points of conventional approach, we have applied thick serial sections and HVEM to the 3-D reconstruction of synapses (Lee et al., 2005). The results revealed that the reconstruction of 4∼5 μm of Purkinje cell dendrite could be accomplished with only 16∼20 serial HVEM sections, while the reconstruction of same dendritic length would require roughly 80∼100 ultrathin sections in a conventional TEM approach using 50 nm sections. Thus our new reconstruction method could practically reduce time-consuming processes such as the construction of numerous ultrathin sections and the alignment of stacks of images.

In conclusion, this study suggests that 3-D reconstruction of synapses using HVEM is much efficient and suitable strategy for massive quantitative studies on synaptic connectivity. The present study will provide useful methodological information on the research of synaptic plasticity using HVEM.

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<국문초록>

신경연접은 신경세포 사이의 신호전달을 위해 형성되는 미세구조로 다양한 생리적, 병리적 상태에 반응하여 형태적, 기능적 변화를 보인다. 현재까지 투과전자현미경을 이용한 신경연접 미세구조의 2차원적 연구들이 많은 유용한 정보를 제공하여 왔으나 신경연접 구성요소들을 보다 정확하게 분석하고 전신경연접부위와 후신경연접부위의 정확한 연결관계를 이해하기 위해서는 신경연접의 3차원 재구성이 요구된다. 고압전자현미경은 고해상도와 시료투과력의 증가로 인해 두꺼운 절편의 관찰이 가능하며 이를 통해 미세구조의 3차원적 특성을 규명하는 것이 용이하므로, 신경연접의 3차원 재구성에 고압전자현미경을 응용하는 것은 많은 수의 연속절편 제작과 오랜 기간의 영상처리가 요구되는 기존의 재구성 방법의 난점들을 극복할 수 있을 것으로 생각된다. 이에 본 연구에서는 고압전자현미경을 이용하여 흰쥐 소뇌 평행섬유와 조롱박세포 간 신경연접의 3차원 재구성을 시도하였다. 3차원 재구성에 앞서 염색방법과 절편 두께의 조절을 통해 고압전자현미경 하에서 신경연접의 적절한 관찰조건을 확립하고자 하였다. 관찰 결과, 절편의 두께가 증가하면 신경연접의 막, 소포와 같은 미세구조들의 겹침 현상이 나타나기 때문에 용이한 3차원 재구성을 위해서는 250 nm 두께의 절편을 제작하는 것이 적합한 것으로 판단되었다. 또한 절편제작 이전의 *en bloc* 염색 반응시간을 증가시키는 것이 절편제작 후 염색시간을 조절하는 것에 비해 contrast 증가에 더 효과적이었다. 이상의 결과로부터, 고압전자현미경을 이용하여 일련의 두꺼운 연속 절편을 촬영하고 3차원 재구성 프로그램을 이용하여 이미지들을 정렬하였으며 각각의 이미지에서 신경연접 막의 윤곽선을 그린 후 모든 윤곽선을 쌓아 올려 최종적으로 3차원 신경연접을 재구성하였다. 본 연구를 통하여 신경연접의 3차원 재구성에 있어 고압전자현미경의 적용 가능성을 검증하였고 관찰 조건을 확립하였다. 또한 고압전자현미경을 이용한 신경연접의 재구성은 많은 수의 연속절편 제작이 요구되는 기존의 방법에 비해 효율적이며 신경연접 연결형태에 관한 대규모의 정량 분석에 유용할 것으로 생각된다. 본 연구가 향후 고압전자현미경을 이용한 신경연접의 가소성 연구에 유용한 방법적 정보를 제공하기를 기대한다.