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Research article

Effective *synaptome* analysis of itch-mediating neurons in the spinal cord: A novel immunohistochemical methodology using high-voltage electron microscopy

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HIGHLIGHTS

- Here we report an effective *synaptome* using high-voltage electron microscopy (HVEM).
- HVEM tomography is useful for studying three-dimensional (3-D) synaptic structures.
- Our new approach made it possible to examine many itch-mediating synapses in rats.
- The 3-D methodology can be widely and easily applied in multiple ways.

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ABSTRACT

Transmission electron microscopy (TEM) is used for three-dimensional (3-D) analysis of synaptic connections in neuroscience research. However, 3-D reconstruction of the synapses using serial ultrathin sections is a powerful but tedious approach requiring advanced technical skills. High-voltage electron microscopy (HVEM) allows examination of thicker sections of biological specimens due to the increased penetration of the more accelerated electrons, which is useful to analyze the 3-D structure of biological specimens. However, it is still difficult to visualize the neural networks and synaptic connections in 3-D using HVEM because of insufficient and non uniform heavy metal staining in the membranous structures in semi-thin sections. Here, we present the successful chemical 3-D neuroanatomy of the rat spinal dorsal horn at the ultrastructural level as a first step for effective *synaptome* analysis by applying a high-contrast *en bloc* staining method to immune-HVEM tomography. Our new approach made it possible to examine many itch-mediating synaptic connections and neural networks in the spinal cord simultaneously using HVEM tomography. This novel 3-D electron microscopy is very useful for the analysis of synaptic structure and the chemical neuroanatomy at the 3-D ultrastructural level.

1. Introduction

Three-dimensional (3-D) analysis of synaptic connections (*synaptome*) is important in neuroscience research. Because synapses are a small structure (submicron order) in neurons, they are only able to be analyzed using either transmission electron microscopy (TEM) or scanning electron microscopy (SEM), and thus, the 3-D organization of synaptic connections revealed by serial ultrathin sections requires a time-consuming analysis in both TEM and SEM [7,8,14]. Although a combination of immunohistochemistry (IHC) and confocal laser scanning microscopy allows imaging and quantification of the 3-D organization of biological specimens, the ultrastructural morphology of the cells, including synaptic connections, remains obscure due to the insufficient spatial resolution of light microscopy [15]. The poor visualization of the structures remains a problem even if fluorescent visualization methods are used, and unlabeled (non-fluorescing) domains cannot

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be observed [1,11,12]. Because normal TEM has a limitation of specimen thickness which is generally less than a hundred nanometers, the structural information in ultrathin sections is mostly 2-D [6,19]. It is, therefore, difficult to use this approach for visualization of 3-D structures in biological specimens at either the cellular and organotypic levels. On the other hand, high-voltage electron microscopy (HVEM) has higher penetration power of the electrons due to the increased accelerating voltage (~1000 kV) and enables us to examine thicker sections of biological specimens [16,18]. Thus, HVEM should be particularly useful to analyze the 3-D ultrastructures of micrometer-sized tissues at the nanometer level [volume electron microscopy (EM)] [6,19].

Important findings have recently demonstrated that spinal itch transmission is independent of pain transmission and relies on gastrin-releasing peptide (GRP)/GRP receptor signaling in the dorsal horn (DH) of the spinal cord [22,23], as well as in the trigeminal sensory system in the medulla oblongata [25]. These new findings are a major breakthrough in the studies on the molecular basis of itch [22,23]. At the ultrastructural level, the spinal DH contains numerous synaptic connections locally that are important in conveying somatosensory inputs from the periphery, although the chemical neuroanatomy of the synapses involved in the itch sensation remains elusive [24]. In this study, using an immune-HVEM tomography with a high-contrast *en bloc* staining method, we successfully describe the chemical 3-D neuroanatomy of the rat spinal DH revealed by IHC at the ultrastructural level. This new approach also attempts an effective *synaptome* analysis and is a novel method of 3-D EM.

2. Materials and methods

2.1. Animals

Adult male Wistar rats were obtained from the Charles River Laboratories Japan (Yokohama, Japan). All experimental procedures have been authorized by the Committees for Animal Research, Okayama University and Kyoto Prefectural University of Medicine, Japan.

2.2. GRP IHC and EM

Male rats ($n = 9$) were overdosed with sodium pentobarbital and perfusion fixed with 4% paraformaldehyde, 0.1% glutaraldehyde, and 0.3% tannic acid in 0.1 M phosphate buffer. Cervical spinal cords were immediately removed and immersed in 4% paraformaldehyde in 0.1 M phosphate buffer for 3 h. Spinal sections (C3–C6 level; 30 μm in thickness) were prepared with a LinearSlicer® (Dosaka EM, Kyoto, Japan). The free-floating sections were thoroughly washed with phosphate buffered saline (PBS) and preincubated in PBS containing 0.05% Triton X-100, 1% normal goat serum, and 1% BSA for 30 min at room temperature to block nonspecific reactions. Sections were then incubated with the primary rabbit antiserum against rat $GRP_{20–29}$ (1:1000) (AssayPro St. Charles, MO, USA) overnight at room temperature with gentle agitation. The GRP antiserum used in this study have previously shown to be specific for rat GRP in the spinal cord [25]. Immunoreactive (ir) products were detected with a streptavidin-biotin kit (Nichirei, Tokyo, Japan), followed by diaminobenzidine development, as described previously [25]. Some immunostained sections were first viewed using a light microscope for reference after dehydration and clearing. The other stained sections were then subjected to special *en bloc* staining according to the protocol of the National Center for Microscopy and Imaging Research (NCMIR), University of California, San Diego, CA, USA with slight modifications [3]. The NCMIR method is widely used for serial block-face SEM (SBF–SEM), which was designed to enhance signal for backscatter electron imaging of epoxy-embedded mammalian tissue at low accelerating voltages [3]. This sample preparation method for effective heavy metal staining was applied to our *synaptome* analysis by HVEM tomography. Briefly, after being washed with 0.15 M cacodylate buffer, the sections were post-fixed for 1 h in 2% aqueous osmium tetroxide/1.5% potassium ferrocyanide in 0.15 M cacodylate buffer, for 20 min in a thiocarbohydrazide solution, and then for 30 min in a 2% osmium tetroxide solution. Sections were placed in 1% uranyl acetate overnight at 4 °C and then in a lead aspartate solution in a 60 °C oven for 30 min. The sections were dehydrated and flat embedded in epoxy resin (Quetol-812; Nisshin EM, Tokyo, Japan). For reference, some sections were embedded without the above *en bloc* staining and post-fixed with 1% osmium tetroxide for 2 h instead as conventional EM-IHC. Ultrathin sections (70 nm in thickness) containing the GRP-ir fibers in the DH were prepared and collected on mesh grids coated with a collodion film. First, the preparations were viewed and imaged with an H-7650 electron microscope (Hitachi, Tokyo Japan) without any additional heavy metal staining at an accelerating voltage of 80 kV. After the TEM observations, the same sections were stained with both uranyl acetate and lead citrate. These heavy metal-stained sections were then viewed and imaged again using the same TEM condition, and the images were compared with the former non-stained sections. Subsequently, semi-thin sections (1–2 μm in thickness) containing the GRP-ir fibers in the DH were then prepared and collected on mesh grids coated with a collodion film. Each grid-mounted semi-thin section was first selected using a light microscope (Olympus; BH-2, Tokyo, Japan). Selected grids or sections were observed using an HVEM (Hitachi H-1250 M; National Institute for Physiological Sciences, Okazaki, Japan) at an accelerating voltage of 1000 kV.

2.3. Tomography

The specimen was tilted from −60.0° to +60.0° and imaged at 1-degree steps (121 images per view; 0.0, ±1.0–60.0). The images were digitized and 3-D models were constructed using IMOD software [10,17]. Individual subcellular and organelle structures were observed by UCSF Chimera and manually segmented using the Amira® software package (FEI Visualization Science Group, Burlington, MA, USA) [17]. This software package was also used to generate the 3-D image figures.

3. Results

Using conventional light microscopy, IHC analysis of the EM preparations first showed that GRP-ir fibers were densely stained in the superficial layers of the cervical spinal DH (laminae I-II) in a similar way as reported previously [5,22,25] ($n = 3$) (*arrowheads* in Fig. 1A and B). After pre-embedding using the GRP IHC method, the stained sections were then flat-embedded in epoxy resin and observed by conventional light microscopy again (Fig. 1C). Numerous GRP-ir terminals were also visible in the spinal DH (laminae I-II) ($n = 3$) (*arrowheads* in Fig. 1C), whereas the IHC-stained and resin-embedded sections with NCMIR method did not identify any GRP-ir due to the heavy *en bloc* staining ($n = 3$) (Fig. 1D). *Arrowheads* in Fig. 1D indicate the region of laminae I-II, possibly including regions of numerous GRP-ir. The ultrathin sections (70 nm), including regions of laminae I-II (*arrowheads* in Fig. 1C) without any additional heavy metal staining, showed the presynaptic terminals containing the GRP-ir (*asterisk*) and the synaptic connection (*double arrows*) with low contrast using conventional TEM (Fig. 1E). On the other hand, when these sections were contrasted with the heavy metal staining, many GRP-ir vesicles were then clearly visualized in the presynaptic terminal (*asterisk*), and the electron-dense