Neuroscience 150 (2007) 575–584

ULTRASTRUCTURAL LOCALIZATION OF ACTIVE ZONE AND SYNAPTIC VESICLE PROTEINS IN A PREASSEMBLED MULTI-VESICLE TRANSPORT AGGREGATE

J.-H. TAO-CHENG*

NINDS EM Facility, NIH, Building 49, Room 3A50, Bethesda, MD 20892-4477, USA

Abstract—Although it has been suggested that presynaptic active zone (AZ) may be preassembled, it is still unclear which entities carry the various proteins to the AZ during synaptogenesis. Here, I propose that aggregates of dense core vesicles (DCV) and small clear vesicles in the axons of young rat hippocampal cultures are carriers containing preformed AZ and synaptic vesicle (SV) components on their way to developing synapses. The aggregates were positively labeled with antibodies against Bassoon and Piccolo (two AZ cytomatrix proteins), VAMP, SV2, synaptotagmin (three SV membrane proteins), and synapsin I (a SV-associated protein). Bassoon and Piccolo labeling were localized at dense material both in the aggregates and at the AZ. In addition to the SV at the synapses, the SV membrane proteins labeled the clear vesicles in the aggregate as well as many other SV-like and pleiomorphic vesicular structures in the axons, and synapsin I labeling was associated with the vesicles in the aggregates. In single sections, these axonal vesicle aggregates were ~0.22 by 0.13 μm in average dimensions and contain one to two DCV and five to six small clear vesicles. Serial sections confirmed that the aggregates were not synaptic junctions sectioned en face. Labeling intensities of Bassoon and Piccolo measured from serially sectioned transport aggregates and AZ were within range of each other, suggesting that one or a few aggregates, but not individual DCV, can carry sufficient Bassoon and Piccolo to form an AZ. The present findings provide the first ultrastructural evidence localizing various AZ and SV proteins in a preassembled multi-vesicle transport aggregate that has the potential to quickly form a functional active zone. Published by Elsevier Ltd on behalf of IBRO.

Key words: Bassoon, Piccolo, VAMP, SV2, synaptotagmin, synapsin I.

Synaptic transmission takes place at the active zone (AZ) where synaptic vesicles (SV) fuse with the plasma membrane and release their transmitter contents. The process of synaptogenesis, including AZ formation, remains a subject of intense interest (reviewed in Matteoli et al., 2004; Zhen and Jin, 2004). Ahmari et al. (2000) conducted a salient study combining live imaging of green fluorescent protein (GFP)–VAMP [a SV membrane protein fused with GFP], and double labeling of other presynaptic proteins (including SV markers, calcium channel, and endocytosis-related protein). They proposed that these presynaptic proteins may be preassembled in a packet and transported together. Their retrospective EM analysis showed that GFP-VAMP transport packets coincide with a mixture of entities including pleiomorphic vesicles, tubulo-vesicular structures, and dense core vesicles (DCV), but few SV. Some of the mobile GFP-VAMP transport packets became endo/exocytosis-competent within 1 h of contact between axon and dendrite, consistent with the advantage offered by a pre-assembled presynaptic packet—poised for action and functional in a short time. However, they did not identify which particular structures in the packet carried VAMP or other synaptic proteins. Immunolabeling at the EM level for the various proteins could provide more specific answers at the ultrastructural level.

In addition to the SV proteins and various other presynaptic proteins, the AZ has a specialized cytomatrix structure (Phillips et al., 2001) consisting of numerous proteins (reviewed in Schoch and Gundelfinger, 2006), with Bassoon and Piccolo being two stable components of the AZ cytomatrix (Tao-Cheng, 2006). Immunolabeling of Piccolo at light microscopy (LM) and EM levels (Zhai et al., 2001) as well as live imaging of GFP-Bassoon (Shapira et al., 2003) combined with fractionation/immunolabeling on antibody-coated magnetic beads, identified a type of DCV which is ~80 nm in size and termed as Piccolo/Bassoon transport vesicle (PTV). It was suggested that PTVs carry a comprehensive set of AZ materials, and that AZ is formed by unitary assembly of two or three PTVs (Shapira et al., 2003). Furthermore, it was also proposed that DCV in the GFP-VAMP packet (Ahmari et al., 2000) may be the same as the PTV identified as the precursor vesicle for AZ (reviewed in Bonanomi et al., 2006). However, due to the challenging nature of EM immunolabeling, the micrographs of Piccolo-labeled neuronal cultures were of limited resolution (Zhai et al., 2001).

The aim of the present study was to identify the specific cargo carriers for Bassoon and Piccolo in developing axons by EM immunolabeling. Different antibodies were tested with different fixation conditions in order to improve structural preservation and signal levels. One particular type of vesicle aggregate, rather than individual DCV, was identified to be the carrier of Bassoon and Piccolo. Measurements of labeling intensities of aggregates were compared with those from AZ of immature synapses to see if one aggregate is sufficient to form an AZ. Since these

*Tel: +1-301-496-0579; fax: +1-301-480-1485.
E-mail address: chengs@ninds.nih.gov (J.-H. Tao-Cheng).

Abbreviations: AZ, active zone; CGA, chromogranin A; DCV, dense core vesicle; GFP, green fluorescent protein; LM, light microscopy; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PSD, postsynaptic density; PTV, Piccolo/Bassoon transport vesicles; p65, synaptotagmin; SV, synaptic vesicle.

0306-4522/07$30.00+0.00 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2007.09.031

aggregates also contain SV-like vesicles, three SV membrane proteins (VAMP, SV2 and synaptotagmin (p65)) and one SV-associated protein (synapsin I; reviewed in Bonanomi et al., 2006) were also selected for EM immunolabeling analysis. The Bassoon/Piccolo transport aggregates identified here are smaller than the GFP-VAMP packet (Ahmari et al., 2000), but more complex than the PTV (Zhai et al., 2001). These aggregates that contain various AZ and SV proteins may represent a preassembled transport unit that is sufficient to form a stable presynaptic AZ.

EXPERIMENTAL PROCEDURES

Antibodies

Mouse monoclonal antibody (mAb) against Bassoon (1:100, clone SAP7F407) and p65 (1:250, clone ASV30) were from Stressgen (Victoria, BC, Canada). Rabbit polyclonal antibody (pAb) against Piccolo (1:400) and mouse mAb against synapsin I (1:250, clone 46.1) were from Synaptic Systems (Gottingen, Germany). Guinea-pig polyclonal antibody against Piccolo (1:100) was a gift from Dr. Eckart Gundelfinger (Leibniz Institute for Neurobiology, Magdeburg, Germany). Mouse mAb against VAMP (1:100, clone SP10) and SNAP-25 (1:250, clone SP14) were from Chemicon (Temecula, CA, USA). Mouse mAb against SV2 (1:500, clone 10H3) was a gift from Dr. Erik S. Schweitzer (UCLA, Los Angeles, CA, USA). Rabbit pAb against chromogranin A (CGA, 1:250) was a gift from Dr. Lee Eiden (NIMH, Bethesda, MD, USA). A summary of labeling patterns for each antibody is listed in Supplementary materials.

Disassociated hippocampal cultures

The animal protocol used in this study was approved by the NIH Animal Use and Care Committee and conforms to NIH guidelines, minimizing the number of animals used and their suffering. Hippocampal cells from 21-day embryonic Sprague–Dawley rats were dissociated and grown either on top of a feeder layer of glial cells (for details, see Lu et al., 1998) or without the feeder glial cells for 3–11 days. No difference in labeling pattern was observed between the two types of cultures for any of the antibodies. Cultures older than 7 days already had numerous mature looking synapses. Thus, most of the sampling of immature synapses and developing axons is from 3 to 6-day-old cultures. Samples of 3-week-old cell cultures from a previous study (Tao-Cheng, 2006) were also examined for comparison.

Fixation and pre-embedding immunocytochemistry

Cells were fixed with different fixatives for optimal immunolabeling for different antibodies (assessment of optimal fixation conditions for each antibody is listed in Supplemental materials): (1) 4% paraformaldehyde in phosphate-buffered saline (PBS) for 45–60 min for all antibodies, (2) 4% paraformaldehyde and 0.02–0.1% glutaraldehyde for 30–60 min for the SV2 antibody, and for 30–45 min for the guinea-pig Piccolo antibody, (3) 2% acrolein in PBS for 1 min followed by 4% paraformaldehyde in PBS for 30–60 min for the guinea-pig Piccolo and the SV2 antibodies, and (4) 4% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 h for samples without immunolabeling. Fixed cells were washed and permeabilized/blocked with 0.1% saponin/5% normal goat serum in PBS for 1 h, incubated with primary antibody for 1–2 h, incubated with secondary antibody conjugated to 1.4 nm gold particles (1:250, Nanogold from Nanoprobes, Yaphand, NY, USA) for 1 h, silver enhanced (HQ silver enhancement kit, Nanoprobes) for 10–15 min, treated with 0.2% OsO_4 in phosphate buffer for 30 min, followed by 0.25% uranyl acetate at 4 °C overnight, dehydrated in ethanol and embedded in epoxy resin (Tanner et al., 1996). Controls for specificity of immunolabeling include omitting the primary antibody and using the different primary antibodies as controls for each other.

Morphometry

Sampling. Every encountered synapse, labeled vesicle aggregates, and individual DCV within randomly selected grid openings was photographed at 40,000× on the microscope with a CCD digital camera system (XR-100 from AMT, Danvers, MA, USA) and measured at a final magnification of 150,000×. Synapses are characterized by at least two of the following three features: the uniform synaptic gap between the apposed pre- and post-synaptic membranes, the postsynaptic density (PSD), and the presence of clustered SV. Labeled vesicle aggregates are characterized by a mixture of vesicles closely clustered and are associated with more than two labeling grains.

Synaptic AZ measurement. Length of AZ was measured by tracing the presynaptic membrane opposite the PSD. An area at the AZ, 67 nm from the presynaptic membrane (cf. Tao-Cheng, 2006), was shown to preferentially contain ~80% and 55% of Bassoon and Piccolo (the rabbit antibody) labeling, respectively. This area was marked to measure all the AZ-associated labeling grains.

Measurements of transport vesicle aggregates. Each labeled vesicle aggregate was traced with a pen to mark the border defined by the outer edge of the associated vesicles. The aggregates are typically irregular in shape, and their size was expressed as two linear dimensions: the length of the marked area along its long axis, and a second measurement perpendicular to the first line at the midpoint. Within each marked aggregate, the number of DCV, clear vesicles and labeling grains was counted. The mean diameter of DCV and small clear vesicles was measured as the average of two measurements: the maximum diameter and a second measurement taken at the midpoint of the first.

Statistical analysis (KaleidaGraph by Synergy Software, Reading, PA, USA) was carried out by Student's *t*-test (two-sided, unpaired data with unequal variance) with confidence level set at $P<0.01$ unless otherwise indicated.

RESULTS

Bassoon localization at AZ and vesicle aggregates

Among all of the antibodies used here, Bassoon had the most consistent and selective localization at the AZ of immature synapses (double arrow in Fig. 1A, enlarged in 1B) and at a particular type of vesicle aggregate (arrow in Fig. 1A, enlarged in 1C). Thus, Bassoon labeling offered an exceptional opportunity to examine developing synapses in these young (3–6 days *in vitro*) neuronal cultures, and to unequivocally characterize this novel vesicle aggregate.

Bassoon labeling at immature synapses was concentrated within 70 nm from the presynaptic membrane (double arrowheads in Fig. 1B, D, E), a pattern similar to mature synapses (cf. Tao-Cheng, 2006). Some of the young synapses at 4 days *in vitro* already have many SVs (Fig. 1D), and in synapses where SVs were scarce (Fig. 1B, E) it was apparent that Bassoon signals were associated with dense material at the AZ. Regardless of the amount of SV present, in 3 to 4-day-old samples, only 17% (14/84) of the synapses had any DCV within 200 nm of the AZ. The frequency of seeing DCV within 200 nm of the AZ