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Differentiation and Characterization of Excitatory and Inhibitory Synapses by Cryo-electron Tomography and Correlative Microscopy

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As key functional units in neural circuits, different types of neuronal synapses play distinct roles in brain information processing, learning, and memory. Synaptic abnormalities are believed to underlie various neurological and psychiatric disorders. Here, by combining cryo-electron tomography and cryo-correlative light and electron microscopy, we distinguished intact excitatory and inhibitory synapses of cultured hippocampal neurons, and visualized the in situ 3D organization of synaptic organelles and macromolecules in their native state. Quantitative analyses of >100 synaptic tomograms reveal that excitatory synapses contain a mesh-like postsynaptic density (PSD) with thickness ranging from 20 to 50 nm. In contrast, the PSD in inhibitory synapses assumes a thin sheet-like structure ~12 nm from the postsynaptic membrane. On the presynaptic side, spherical synaptic vesicles (SVs) of 25–60 nm diameter and discus-shaped ellipsoidal SVs of various sizes coexist in both synaptic types, with more ellipsoidal ones in inhibitory synapses. High-resolution tomograms obtained using a Volta phase plate and electron filtering and counting reveal glutamate receptor-like and GABAA receptor-like structures that interact with putative scaffolding and adhesion molecules, reflecting details of receptor anchoring and PSD organization. These results provide an updated view of the ultrastructure of excitatory and inhibitory synapses, and demonstrate the potential of our approach to gain insight into the organizational principles of cellular architecture underlying distinct synaptic functions.

Key words: correlative light and electron microscopy; cryo-electron tomography; neurotransmitter receptor; postsynaptic density; synaptic ultrastructure; synaptic vesicle

Introduction

Chemical synapses are basic functional units in neural circuits for information transmission, processing, and storage ( Eccles, 1964; Südhof and Malenka, 2008; Mayford et al., 2012). The brain’s remarkable computational power and cognitive capacity stem from the enormous number of synapses in the brain, the plastic-

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ity of each synapse, and the molecular and functional diversity across these synapses (Milner et al., 1998; Bi and Poo, 2001). Glutamatergic and GABAergic synapses, the two main types of central synapses, play opposite roles in excitation and inhibition. They have been shown by biochemical and electrophysiological studies to contain different sets of molecular and cellular components and to exhibit distinct functional properties and plasticity rules (Craig and Boudin, 2001; Südhof and Malenka, 2008; Vogels and Abbott, 2009; Sassoe-Pognetto et al., 2011). How are these different components organized into the intricate machinery to perform distinct synaptic functions? Electron microscopy (EM) has been a primary tool for addressing this question by enabling the visualization of the ultrastructure of various synapses (Sikou et al., 2007; Harris and Weinberg, 2012).

Classical EM uses chemical fixation, dehydration, and plastic embedding, followed by sectioning and heavy-metal staining to image brain tissues and cultured neurons. Such meticulous processing has enabled the use of electron beams to image various biological specimens at high contrast. Indeed, classical EM observations have shaped much of our current knowledge about synaptic ultrastructure (Sorra and Harris, 2000; Harris and Weinberg, 2012). For example, prominent subcellular features, such as the postsynaptic density (PSD) and synaptic vesicles (SVs), are well documented, especially for excitatory synapses (Gray, 1959; Colomnier, 1968; Schikorski and Stevens, 1997; Harris and Weinberg, 2012). The 3D resolving capability of electron tomography (ET) has yielded better views of synaptic ultrastructure (Harlow et al., 1998; Res et al., 2004; Buret et al., 2012). The improved structural preservation provided by high-pressure freezing with freeze substitution (HPF-FS; Tatsuoka and Reese, 1989) combined with ET (Rostaing et al., 2006) has allowed studies of the organization and dynamics of SVs (Sikou et al., 2007; Watanabe et al., 2013; Imit et al., 2014; Jung et al., 2016) and the 3D organization of macromolecular complexes in individual synapses, such as the PSD-95/glutamate receptor complex at the PSD (Chen et al., 2008, 2015).

It remains challenging to characterize the structure and organization of cellular and molecular machinery of specific synaptic types at higher resolution (Hurbain and Sachse, 2011; Tao et al., 2012), because damage or deformation from sample preparation procedures can complicate structural interpretation (Hurbain and Sachse, 2011). Cryo-electron tomography (cryo-ET), which aims to overcome this limitation, has been used to visualize distribution of SVs and other ultrastructural features in isolated synaptosomes and cryo-sections of brain tissues (Fernández-Busnadiego et al., 2010; Shi et al., 2014; Wilhelm et al., 2014; Perez de Arce et al., 2015). However, cryo-ET alone cannot unambiguously identify synapse types due to a lack of specific labeling, such as immunogold staining or photoconversion of diamino-benzidine for classical EM (Megias et al., 2001; Schikorski and Stevens, 2001; Rostaing et al., 2006; Harris and Weinberg, 2012).

One way to overcome this shortcoming is to take advantage of the molecular specificity of fluorescence labeling and sample preservation of cryo-ET in cryo-correlative light microscopy (LM) and EM (cryo-CLEM), as suggested previously (Lucic et al., 2007), although the ability of this approach to distinguish different synapse types is yet to be realized. In the current study, we developed an efficient cryo-CLEM platform to identify different types of synapses in cultured hippocampal neurons, and to define presynaptic and postsynaptic ultrastructural features of excitatory and inhibitory synapses in their native state. By high-resolution cryo-ET with cutting-edge direct electron detection (Li et al., 2013), Volta phase plate (VPP; Danev et al., 2014; Fukuda et al., 2015), and electron energy filter (Verbeek et al., 2004) technologies, we could also visualize putative glutamate receptors and GABAA receptors (GABAA Rs) and their organization at the postsynaptic membrane of excitatory and inhibitory synapses.

**Materials and Methods**

The overall workflow of experimental procedures is illustrated in Figure 1A. Primary neuronal cultures were grown on EM grids and then plunged into liquid nitrogen to perform cryo-EM imaging followed by 3D reconstruction. For some cultures transfected with constructs of fluorescent protein-tagged synaptic proteins, cryo-fluorescence microscopy was performed before cryo-ET for correlative imaging. All animal procedures were performed following the guidelines of the Animal Experiments Committee at the University of Science and Technology of China.

**Primary culture of hippocampal neurons.** Low-density cultures of dissociated embryonic rat hippocampal neurons were prepared as previously described (Bi and Poo, 1998) with modifications. Quantifori R2/2 gold EM grids (200 mesh with holey carbon film of 2 μm hole size and 2 μm spacing) or Quantifoil R2/R2 gold NH2 finder grids (100 mesh with holey carbon film of 2 μm hole size and 2 μm spacing) were plasma-cleaned with H2 and O2 for 10 s using a plasma cleaning system (Gatan), and sterilized with UV light for 30 min. These grids were then coated with poly-L-lysine (Sigma-Aldrich) overnight, followed by washing with HBSS and double-distilled H2O for ~12 h each. Hippocampi were removed from embryonic day 18 rats (without distinguishing sex differences) and were treated with trypsin for 15 min at 37°C, followed by washing and gentle trituration. The dissociated cells were plated on the poly-L-lysine-coated EM grids in 35 mm Petri dishes at a density of 40,000 – 60,000 cells/ml, and maintained in incubators at 37°C in 5% CO2. The culture medium was replaced by serum-free culture medium. Subsequently, one-third of the culture medium was replaced with fresh culture medium twice a week. For correlative microscopy, cultures were coinfected with lentiviruses encoding PSD-95-EGFP and mCherry-gephyrin constructs (see below) for 5–7 d in vitro (DIV) before vitrification of the grid. Twelve hours after the infection, half of the culture medium was replaced by fresh medium.

To prevent overgrowth of glial cells, the cultures were treated with cytosine arabinoside (Sigma-Aldrich) at various stages. Cultures were used for cryo-EM imaging at 14–18 DIV, when healthy, low-density cultures formed patches of monolayer neuronal processes (Fig. 1B1). We judge whether the culture is healthy based on morphological criteria, e.g., smooth soma and dendrites with multiple branches viewed under phase-contrast LM, and ≥1 probable synapse each few micrometers along the dendrites of transfected neurons, as viewed under fluorescence microscopy. According to our experience, such criteria predict retention of functional properties of synaptic transmission (Vincze and Benjamins) and plasticity evaluated with patch-clamp recording and calcium imaging.
DNA constructs and lentivirus preparation. The PSD-95 cDNA was amplified from GW-PSD-95-EGFP plasmid (a generous gift from Dr. Weidong Yao) and subcloned into pLenti-CaMKII-mKate2 vector to produce pLenti-CaMKII-PSD-95-mKate2. The EGFP cDNA was amplified from the pEGFPN1 plasmid, and then subcloned into pLenti-CaMKII-PSD-95-mKate2 to produce the pLenti-CaMKII-PSD-95-EGFP plasmid. The mCherry-gephyrin lentiviral construct (Dobie and Craig, 2011) was a generous gift from Dr. Ann Marie Craig. Both PSD-95-EGFP and mCherry-gephyrin lentiviral constructs were packaged into lentivirus following a protocol from Dr. Karl Deisseroth’s laboratory (Zhang F et al., 2010).

Frozen-hydrated sample preparation. After being removed from the CO2 incubator, low-density neuronal cultures (14–18 DIV) on EM grids were first placed in extracellular solution (ECS; containing 150 mM NaCl, 3 mM KCl, 3 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, and 5 mM glucose, pH 7.3), then mounted on a Vitrobot IV (FEI). Protein A-coated colloidal gold beads (15 nm; CMC) were added to the grid (4 μl each, stock solution washed in ECS and diluted 10 times after centrifugation) as fiducial markers.
The grids were then plunged into liquid ethane for rapid vitrification of the samples, which were then stored in liquid nitrogen until use.

**Cryo-ET imaging.** Cryo-ET data were collected with single-axis tilt using either a Tecnai F20 transmission electron microscope (FEI) equipped with an Eagle 4K × 4K multiport CCD camera (FEI), or a Titan Krios (FEI) with a K2 Summit direct electron detector (K2 camera, Gatan). The Tecnai F20 was operated at an acceleration voltage of 200 kV. Tilt series were collected from −60 to +60° at 2° intervals using FEI Xplore 3D software, with the defocus value set at −12 to −18 μm, and the total electron dosage of ~100 e− Å². The final pixel size was 0.755 nm. The Titan Krios was operated at an acceleration voltage of 300 kV, with or without VPP and Gatan image filter (GIF). In either configura-

**Figure 2.** Synapses of various sizes, shapes, and ultrastructural details imaged with cryo-ET. A–C, Three tomographic slices showing structures of different synapses. In the synapses, structures, such as SVs and dense core vesicle in presynaptic boutons (Bouton), microtubules (MT) in boutons and dendritic shaft (Shaft), mitochondria (Mit) in presynaptic bouton and postsynaptic spine (Spine), are clearly visible. A1–C1, Zoomed-in views of corresponding boxed areas from A–C showing thick (A1, B1, dashed parallel lines) and thin (C1, dashed parallel lines) PSDs, as well as SVs attached (A1, cyan arrowhead) or fused (B1, pink arrowheads) to the presynaptic membrane. D, E, Two synapses sharing the same presynaptic axon (determined by following through their tomograms in 3D), both with thick PSDs (D1 and D2) or both with thin PSDs (E1 and E2), respectively. F, G, Two synapses sharing the same postsynaptic spine, both with thick PSDs (F1 and F2), or one with thin PSD (G1) and the other with thick PSD (G2).
tion, images were collected by the K2 camera in counting mode. In the absence of VPP and GIF, tilt series were acquired from $-64$ to $+64^\circ$ at 2$^\circ$ intervals using Leginon (Suloway et al., 2005), with the defocus value maintained at $-10 \mu m$, and the total accumulated dose of $\sim 120 e^-/\AA^2$. The final pixel size was 0.765 nm. When VPP and GIF were used, the energy filter slit was set at 20 eV, and VPP was conditioned by preirradiation for 60 s to achieve an initial phase shift of $-0.3 \pi$ (Fukuda et al., 2015). Tilt series were acquired from $-66$ to $+60^\circ$ at an interval of 2 or 3$^\circ$ using SerialEM (Mastronarde, 2005) with the defocus value maintained at $-1 \mu m$ and the total accumulated dose of $\sim 150 e^-/\AA^2$. The final pixel size was 0.435 nm.

For this study, we examined 78 grids, of which 12 were used for data collection. The rest were discarded because the grids were damaged during transfer or freezing, cultures were too dense and/or too thick, or cultures appeared not healthy with few or no synapses found. Usually 3–5 grid squares (each $\sim 100 \times 100 \mu m^2$) per grid were selected for imaging. To obtain high-quality cryo-ET images, it is critical to choose thin culture areas with healthy yet relatively low-density dendrites (Fig. 1B2). Generally, areas $>500 \mu m$ thick were ignored. At this thickness, subcellular structures, such as mitochondria, microtubules, and SVs, could not be distinguished in single-projection images.

Cryo-correlative light and electron microscopy imaging. The hardware of our cryo-light microscope system includes a custom-built cryo-chamber with liquid nitrogen supply, a Gatan 626 EM cryo-holder, and an Olympus IX71 inverted fluorescence microscope (Fig. 1C). The inside channel of the cryo-chamber was precooled to $-190^\circ C$ by liquid nitrogen, and maintained below $-180^\circ C$, as monitored by a thermoelectric sensor. Nitrogen gas flowed through the objectives and light-source windows during the experiment to prevent frost accumulation. Then, an EM grid with frozen-hydrated sample was loaded onto an EM cryo-holder, which was subsequently inserted into the cryo-chamber.

For cryo-CLEM imaging, fluorescence images were taken using a 40X air-objective lens (Olympus LUCPLFN 40X; numerical aperture, 0.6) and an ANDOR NEO sCMOS camera (Andor) attached to the fluorescence microscope. For each field of view, three images were collected, one in bright field, another in the EGFP channel [exciter (Ex): 470/40; dichroic mirror (DM): 495; emitter (Em): 525/50; Chroma, 49002], and the third in the mCherry channel (Ex: 562/40; DM: 593; Em: 641/75; Semrock, mCherry-B-000). Typically, $\sim 10$ sets of images were sufficient to cover all good areas ($\sim 40$ squares) on each grid, which took $\sim 20$ min to complete; a “good area” was defined as a grid square ($\sim 100 \times 100 \mu m^2$) of appropriate sample thickness that displayed multiple dendritic branches, usually containing dozens of PSD-95-EGFP puncta or multiple mCherry-gephyrin puncta under fluorescence microscopy (Fig. 1D1).

Immediately after the LM imaging, the EM cryo-holder with grid was directly transferred into a Tecnai F20 scope. Areas of the sample imaged in cryo-light microscopy were identified in the EM using the indexes of areas containing corresponding structures in UCSF Chimera (RRID: SCR_003297; Kremer et al., 1996). After rough alignment, a set of holes on the carbon layer of the grid were picked from both the low-magnification EM images and their corresponding fluorescence images using 3dmod in the IMOD package. Transformation functions between the EM and LM images were calculated based on the selected positions by minimizing the mean squared error.

When the low-magnification EM image and LM image were optimally aligned (Fig. 1D3), $\sim 15$ holes on carbon (in one square) in the low-magnification EM image were selected, with their pixel positions recorded. The same holes were identified at 5000X magnification and the mechanical coordinates were recorded. Afterward, the transformation function from the pixel positions to EM mechanical coordinates was determined using similar linear regression methods. With the transformation functions, positions of selected fluorescent puncta (putative excitatory or inhibitory synapses) were converted into corresponding EM mechanical coordinates, which were used to guide EM-image acquisition. Tilt series were collected on the area with selected fluorescent signals. Finally, tomographic slices were fine-aligned and merged with the fluorescence images to identify each synapse (Fig. 1D4) using Midas and Python scripts (RRID:SCR_008394) to integrate the correlation procedures are available to interested readers upon request.

Three-dimensional reconstruction and rendering. Tilt series were aligned and reconstructed using IMOD. Gold beads added to the sample before plunge freezing were used as fiducial markers to align the tilt series. Reconstruction was performed using a simultaneous iterative reconstruction technique with 5 or 15 iterations. A fraction of the data collected was discarded during reconstruction for technical reasons: for Tecnai F20, $\sim 50\%$ of the data were discarded because of such issues as stage drift, beam blockage at high tilt angles, and occasional autofocus failure; for Titan Krios, $<25\%$ of the data were discarded, usually because of issues with beam blockade or autofocus failure at high tilt angle, GIF mistuning, or errors in VPP charging.

Cellular structures, including membranes, actin filaments, microtubules, mitochondria, endoplasmic reticulum, and putative membrane proteins in the tomograms were segmented by manually selecting areas containing corresponding structures in UCSF Chimera (RRID: SCR_004097; Pettersen et al., 2004) and filtered to make the densities of the grid were picked from both the low-magnification EM images and their corresponding fluorescence images using 3dmod in the IMOD package. Transformation functions between the EM and LM images were calculated based on the selected positions by minimizing the mean squared error.

When the low-magnification EM image and LM image were optimally aligned (Fig. 1D3), $\sim 15$ holes on carbon (in one square) in the low-magnification EM image were selected, with their pixel positions recorded. The same holes were identified at 5000X magnification and the mechanical coordinates were recorded. Afterward, the transformation function from the pixel positions to EM mechanical coordinates was determined using similar linear regression methods. With the transformation functions, positions of selected fluorescent puncta (putative excitatory or inhibitory synapses) were converted into corresponding EM mechanical coordinates, which were used to guide EM-image acquisition. Tilt series were collected on the area with selected fluorescent signals. Finally, tomographic slices were fine-aligned and merged with the fluorescence images to identify each synapse (Fig. 1D4) using Midas and Imagem (RRID:SCR_003070). Python scripts (RRID:SCR_008394) to integrate the correlation procedures are available to interested readers upon request.

Quantitative analyses of PSD and synaptic cleft. To analyze PSD profiles, we extracted a 10-nm-thick ($z$) subvolume containing a PSD from each synapse (the $z$-axis is parallel with the direction of the electron beam; $x$-axis is along the tilt axis; and $y$-axis is the direction perpendicular to the $z$ plane). Virtual slices within the slab were averaged along the $z$-axis using the Slicer tool in 3dmod to create a 2D projection (x, y). Then, a contour line was manually drawn to trace the postsynaptic membrane in this projection using 3dmod, and a set of virtual lines parallel to

![Figure 3. Identification of excitatory and inhibitory synapses with cryo-CLEM. A, B, Tomographic slices of an excitatory (A) and inhibitory (B) synapse colocalized with PSD-95-EGFP and mCherry-gephyrin puncta, respectively. A1, B1, Zoomed-in views of the boxed area in A and B showing the synapse with thick and thin PSD, respectively. Red dashed lines indicate the range of the PSD.](Image 313x491 to 429x608)
this contour line were defined at different distances from the contour line. Averaging along each virtual line yields a cross-sectional mean density value; the mean density values corresponding to different distances from the postsynaptic membrane constitute the density profile for the synapse. To compensate for variability in imaging conditions, the density profile was normalized by subtracting the mean value corresponding to a “flat” region 100 to 200 nm from the postsynaptic membrane, and then dividing by the SD of this “flat” region.

The density profile typically consists of distinct peaks corresponding to the presynaptic and postsynaptic membranes as well as the part of PSD with highest density (hereafter referred to as the PSD peak); the positions of these components were measured by Gaussian fitting around the corresponding peaks in the profile (see Fig. 4A2, B2). Synaptic cleft width was defined as the distance from the center of the postsynaptic membrane to the center of the presynaptic membrane. The position of the PSD peak (i.e., its distance from the postsynaptic membrane) was defined as \( d_1 \). From the PSD peak to the flat background into the postsynaptic side, the density profiles varied widely.

A simple, objective approach to quantify this was to fit it with an exponential decay function, as follows: \( v = Ae^{-d/\lambda} + B \), where \( d \) is the distance to PSD peak, \( v \) is density value, and \( \lambda \) is the length constant of the fitted curve. Combined with the distance of the PSD peak from the postsynaptic membrane, we define \( d_2 = d_1 + \lambda \), and consider \( d_2 \) as a measure of the thickness of the PSD.

**Quantitative analysis of SVs.** Size range and shape variation of all SVs were initially analyzed in two steps. First, we used template matching to identify vesicles. To do this, a set of featureless spherical shells 5 nm thick with diameter ranging from 25 to 70 nm at 1 nm intervals were designed as templates. These templates were Gaussian low-pass filtered to 10 nm resolution with 1498 resolution (the resolution along the \( z \) direction of the reconstructed 3D tomogram is much lower than the \( x/y \) resolution) because of missing-wedge effect due to the limited range of tilt angles. This causes the EM density along \( z \) direction to be blurred, but does not bias the shape of fitted vesicles.

**Analysis of receptor-like structures.** To analyze receptor-like structures, two high-quality synaptic tomograms, which were collected using a Titan Krios equipped with K2 camera, VPP, and GIF, were selected. One of them contained an excitatory spine synapse and the other an inhibitory shaft synapse. We extracted subvolumes \((x, y, z)\) containing particles on the cleft side of the postsynaptic membrane of the excitatory and the inhibitory synapse and performed the following analyses. Each subvolume was averaged (9.6 and 6.5 nm for the excitatory and inhibitory synapse, respectively) along the \( z \)-axis (parallel to the direction of the electron beam) to create a 2D projection \((x, y)\) of multiple virtual slices averaging using the Slicer tool in 3dmod). Particles with shape and size similar to those of known receptor structures were visually classified as putative receptors, and the rest were classified as putative nonreceptor structures. The length and width of each particle were also measured manually in a blind manner (independent of the visual classification). After that, projections of all particles classified as glutamate receptors or \( \text{GABA}_A \) receptors were aligned by matching the postsynaptic membrane-end of all these particles, and rotating the long axis of the particles to the vertical direction. Aligned projections were averaged subsequently using the Slicer tool in 3dmod.

To obtain the sizes (length and width) of specific transmitter receptors based on the known crystal structures, density maps of AMPA receptors (AMPARs), NMDA receptors (NMDARs), and \( \text{GABA}_A \) receptors were simulated and low-pass filtered to 27 Å resolution using e2pdb2mrc.py program in EMAN2.1 from their atomic models [NMDAR, PDB: 4TLL (Ie et al., 2014); AMPAR, PDB: 4U2P (Durr et al., 2014); \( \text{GABA}_A \), PDB: SCR_001622].

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Results

Cryo-ET of synapses in intact primary neurons grown on EM grids

To observe the in situ ultrastructure of intact hippocampal synapses, we directly grew hippocampal neurons on gold EM grids, which were plunge-frozen in liquid ethane at 14–18 DIV. This method preserved the structure of vitrified neuronal synapses near their native form, as evidenced by the smooth membranous and cytoskeletal structures (Fig. 1B2–B4). Structural deformations commonly seen in conventional EM (Korogod et al., 2015) were not detected in these frozen-hydrated samples.

To find synapses in these samples at low electron dosage (to minimize radiation damage), we usually started from selected grid squares covered by thin ice that contained many neurites (Fig. 1B2). We then took a series of single-projection images at high magnification along dendrites to look for synapse-like structures with characteristic features, including closely apposed membranes, with one of which containing a dense population of vesicles of similar sizes, and a relatively uniform cleft in between (Fig. 1B3). These membranous structures are easily identifiable under cryo-EM, likely due to higher phase contrast of phospholipids than amorphous background water. In our experiments, only synaptic contacts with approximately normal orientation (i.e., the presynaptic and postsynaptic compartment do not overlap in the single-projection image) were selected for further study, as other contacts could not be easily identified as synapses. These features would become more distinct, along with other fine structural details, in the 3D tomogram reconstructed based on the tilt series collected for each synapse (Fig. 1B4). Besides the above characteristic features, the synaptic cleft also contains translocute filaments and an electron-dense intercellular band similar to the “intermediate band” described previously (Gray, 1959; Fig. 1B4, inset).

Based on the above criteria, we identified 101 synapses of various sizes, shapes, and ultrastructural details in 90 tomograms (Fig. 2). Some of the synapses were formed directly on dendritic shafts, with microtubules readily visible in the postsynaptic compartment (Fig. 2A). More synapses were formed onto probable spines (Fig. 2B, C), with a mushroom-like postsynaptic compartment containing no microtubules and a thin neck linking it to the dendrite, as more clearly viewed in 3D tomograms (Fig. 1B4). In most synapses, a thick electron density was observed near the postsynaptic membrane (Fig. 2A, B), analogous to the PSDs of excitatory synapses described in previous studies using conventional EM (Colonnier, 1968; Peters and Palay, 1996). Intriguingly, we also found that ~18% (18 of 101) of synapses had no “typical” thick PSD structure, but a distinct thin sheet-like structure near the postsynaptic membrane (Fig. 2C). This thin sheet-like structure, which has not been reported previously, is reminiscent of the thickened postsynaptic membrane observed in some “symmetric” inhibitory synapses (Colonnier, 1968; Peters and Palay, 1996), as well as the postsynaptic specialization in the glycineric synapses in the anteroverentral cochlear nucleus (Tat-
suoka and Reese, 1989). We suspected that such thin sheets were PSDs of inhibitory hippocampal synapses. Indeed, in the few tomograms that captured multiple spines forming synapses onto the same presynaptic bouton or different boutons of the same axon, the seven pairs of spines we observed sharing the same presynaptic cell were always the same type, with either “thick” PSDs (four pairs; Fig. 2D) or “thin sheet-like” PSDs (three pairs; Fig. 2E). In contrast, when separate boutons formed synapses on the same postsynaptic spine, the corresponding PSDs could be the same or different types (Fig. 2F, G). This suggests that cryo-ET reveals distinct PSD features of intact excitatory and inhibitory synapses in their native state.

Figure 6. Heterogeneity of synaptic vesicles in excitatory and inhibitory synapses. A, B, Tomographic slices of an excitatory and an inhibitory synapse respectively. Insets are zoomed-in views showing thick and thin PSDs from A and B respectively. C, Scatter plot showing the major and minor axes of SVs in the two synapses in A and B measured by 2D fitting. D, Distribution of vesicle sizes in excitatory and inhibitory synapses (16,476 vesicles in 35 excitatory synapses and 4766 vesicles in 15 inhibitory synapses). E, Distribution of ellipticity of SVs (major to minor axis ratio) in excitatory and inhibitory synapses. A threshold (dashed line) was set at major/minor ∼1.14, which is approximately twice the peak position (major/minor ∼1.07) from perfect circle (major/minor ∼1) to separate ellipsoidal from spherical vesicles. Coincidentally, this threshold is also close to the cross point of the two distribution curves. F, Cumulative frequency of the fraction of ellipsoidal vesicles in excitatory and inhibitory synapses. G, Tomographic slices of spherical (left column), discus-shaped (i.e., oblate spheroid, middle column), and olive-shaped (i.e., prolate spheroid, right column) SVs viewed in three orthogonal planes rotated so that each of the three principle axes (a–c) of the vesicles can be measured horizontally in the corresponding plane. For the spherical vesicles, a=b=c; for discus-shaped ones, a>b≈c; for olive-shaped ones, a≈b<c. H, Long to middle axis ratio versus middle to short axis ratio of 70 ellipsoidal SVs and 70 adjacent spherical SVs in five excitatory and five inhibitory synapses. Schemes depict the shapes at the given positions in the plot.
Identification of excitatory and inhibitory synapses by cryo-CLEM

To unambiguously identify the types of individual synapses visualized by cryo-ET, we developed a cryo-CLEM system (Fig. 1A) that took advantage of the specificity of fluorescent protein tagging. In this system, a cryo-chamber built to fit on a light microscope (Fig. 1C) can accept an EM cryo-holder through a side port to position the EM grid above the objective lens of the light microscope. This design makes it possible to shuttle the EM cryo-holder between light and electron microscopes without repeated sample transfer, thus minimizing ice contamination and grid damage. For fiducial markers, we used the patterned carbon holes on Quantifoil EM grids that can be visualized by both bright-field LM and EM. Based on these patterns, accurate correlation between LM and EM was obtained using a custom-developed program (see Materials and Methods). This approach differs from existing cryo-CLEM methods that rely on the use of large (100–200 nm) fluorescent beads (Schorb and Briggs, 2014; Liu et al., 2015), which may interfere with sample imaging.

We used lentivirus-mediated overexpression of PSD-95-EGFP and mCherry-gephyrin to specifically label glutamatergic and GABAergic synapses, respectively. LM and EM images obtained from different stages of cryo-CLEM are shown in Figure 1D. The contrast of the fluorescence images was adjusted for easier visualization of putative synapses seen as fluorescent puncta. Note that the size of a fluorescent punctum does not reflect the true size of a synapse because of limited optical resolution. Using this system, we collected 14 excitatory and eight inhibitory synapses that were identified based on their colocalization with PSD-95-EGFP fluorescence (Fig. 3A) and mCherry-gephyrin fluorescence (Fig. 3B), respectively. The EM images of these synapses were virtually indistinguishable from those without fluorescent protein labeling, indicating that the overexpression of these tagged scaffolding molecules did not significantly alter synaptic ultrastructure.

Among the 22 synapses identified by fluorescence, and the 101 synapses obtained by cryo-ET only, we observed docked and sometimes partially fused vesicles at the presynaptic membrane (Fig. 2A,B), but no distinctive high-density “active zone” structure in the presynaptic area (Fig. 2), as described in previous studies using conventional EM (Phillips et al., 2001; Südhof, 2012). By contrast, the postsynaptic sides contained distinctive densities (Figs. 2, 3). Thick (>20 nm) PSD structures next to the plasma membrane were easily identifiable in 13 of the 14 excitatory synapses (Fig. 3A) and spanned nearly the entire area of the uniform synaptic cleft (Fig. 3A1). The existence of such thick PSDs is consistent with the common belief that excitatory synapses are “asymmetric,” with dense molecular scaffolds on the postsynaptic side (Colonner, 1968; Peters and Palay, 1996). In contrast, nearly all (seven of eight) inhibitory synapses identified by cryo-CLEM (Fig. 3B) had distinct thin sheet-like PSD (thin PSD for short) positioned in parallel and close to the postsynaptic membrane (Fig. 3B1). Thus, the previously termed “symmetric” inhibitory synapses are in fact asymmetric under cryo-ET.

Quantitative analyses of PSD structures in excitatory and inhibitory synapses

By plotting the mean pixel density as a function of its distance to the postsynaptic membrane, we quantified the PSD profiles of the above 20 identified synapses together with 90 additional synapses with visible PSDs from the 101 synapses obtained with cryo-ET but not CLEM (Fig. 4A,B). The curve contains two major peaks...
representing the presynaptic and postsynaptic membranes. There are also two smaller peaks on the curve, one between the presynaptic and postsynaptic membrane, representing an electron-dense band within the synaptic cleft reported previously as an intermediate band (Gray, 1959), and the other to the right of the postsynaptic membrane defined as “PSD peak,” presumably indicating a postsynaptic proteinaceous layer. This quantitative approach allowed us to identify $d_1$, the peak position of the PSD, and $d_2$, which provides a measure of the thickness of the PSD (Fig. 4A2,B2). Scatter plot of PSD thickness and PSD peak positions of all synapses that contain visible PSDs shows two well-defined clusters, thick and thin, which overlap with the two distinct populations formed by the CLEM-identified excitatory and inhibitory synapses, respectively (Fig. 4C). Thus, the distinct PSD patterns detected by cryo-ET can be used as hallmarks to distinguish between the two types of synapses.

With the PSD pattern as an unequivocal criterion, we systematically characterized presynaptic and postsynaptic features in all 110 synapses with visible PSDs analyzed above, including 85 excitatory synapses and 25 inhibitory synapses. Their postsynaptic densities exhibit distinct PSD peak positions ($14.7 \pm 3.0\,\text{nm}$, $n = 85$ for excitatory synapse; $9.1 \pm 1.1\,\text{nm}$, $n = 25$ for inhibitory synapse) and thickness ($32.7 \pm 7.7\,\text{nm}$, $n = 85$ for excitatory synapse; $12.3 \pm 1.8\,\text{nm}$, $n = 25$ for inhibitory synapse). Compared with the uniformly thin PSDs of inhibitory synapses, the thick PSDs of excitatory synapses exhibit substantial variability (Fig. 4C,D). By averaging all density profiles for excitatory and inhibitory synapses, respectively, we found that the two types of synapses have similar cleft density profiles for excitatory and inhibitory synapses, respectively ($8.3 \pm 2.7\,\text{nm}$, $n = 85$ for excitatory synapse; $1.8 \pm 0.5\,\text{nm}$, $n = 25$ for inhibitory synapse). On the presynaptic side, we found that in both excitatory and inhibitory synapses most SVs are spherical (Tatsuoka and Reese, 1989), but is consistent with findings using HPF-FS (Korogod et al., 2015). They differ from the classical EM observation that inhibitory vesicles tend to be smaller than excitatory vesicles (Peters and Palay, 1996). Inside the cleft, distinctive band-like structures are visible in all synapses (Fig. 4A,B), as reported previously (Gray, 1959; Zuber et al., 2005). We speculate that these structures are protein complexes involved in cell adhesion (Missler et al., 2012). Intriguingly, the density profile around the presynaptic membrane peak is asymmetric; the density values on the cytoplasmic side are slightly higher than that on the cleft side, especially in excitatory synapses. This presumably reflects extra proteins on the cytoplasmic side of the presynaptic membrane, forming a weak version of the active zone commonly observed in conventional EM (Phillips et al., 2001; Südhof, 2012).

In addition to synapses with visible PSD, we also observed 13 structures that met our criteria for synapses but exhibited no visible PSD (Fig. 5A). They differ from the more frequently observed nonsynaptic boutons similar to those reported previously (Bourne et al., 2013), because they had distinct uniform synaptic cleft structures that were absent in the latter. These “PSD-free synapses” could be either excitatory or inhibitory as evidenced from the cryo-CLEM data (Fig. 5B,C). They might reflect a special transient stage, e.g., at an early phase of synaptogenesis or on the way toward elimination (Klemann and Roubos, 2011).
circular, its 3D shape can be either spherical, “discus-shaped” (i.e., oblate spheroid), or “olive-shaped” (i.e., prolate spheroid; Fig. 6G). Therefore, we measured the three principal axes of each vesicle using a 3D fitting program (see Materials and Methods) similar to a method described previously (Kukulski et al., 2012). Measurements of axes with this program on 70 ellipsoidal vesicles and 70 of their neighboring spherical vesicles in five excitatory and five inhibitory synapses (in high-resolution tomograms obtained with VPP, electron filtering, and counting) revealed that most ellipsoidal vesicles were discus-shaped rather than olive-shaped for both excitatory and inhibitory synapses (Fig. 6H). The ellipticity of the ellipsoidal vesicles varied widely (Fig. 6H), which may reflect their different compositions and functional roles in synaptic transmission.

**Visualization of putative receptors and scaffolding proteins in individual synapses**

New tools that enable cryo-ET to achieve higher resolution, including direct electron detection, VPP, and electron energy filter, have facilitated characterization of molecular complexes, such as proteasome in intact cultured neurons (Asano et al., 2015). Using these tools, we obtained high-quality tilt series (Fig. 7A, B; Movie 1) and tomograms with high contrast and high resolution, permitting visualization of features, such as the two leaflets of the membrane bilayer, microtubule protofilaments, and putative proteasomes (Fig. 7C–E). Two high-quality synaptic tomograms obtained using VPP and electron filtering and counting, were selected for further study. One is a spine excitatory synapse with thick PSD structure. The other is a shaft inhibitory synapse with thin sheet-like PSD.

**Figure 8.** Putative receptors and scaffolding proteins in an excitatory synapse. A. An 8.2-nm-thick tomographic slice of an excitatory synapse. Circles: SVs (green), DCVs (purple), ribosome-like structures (cyan); arrows: ellipsoidal vesicle (green), putative actin filaments (red), ER, Endoplasmic reticulum; Mit, mitochondria; MT, microtubule. B. 3D segmented structures of the whole tomogram (~300 nm thickness) of the same synapse shown in A rendered as surfaces, colored as follows: outer-Mit, gold; inner-Mit, light pink; MT, yellow; ER, orange; ribosomes, cyan; actin filaments, red; presynaptic membrane, light yellow; postsynaptic membrane, cyan; presynaptic putative adhesion molecules, magenta; postsynaptic putative adhesion molecules, yellow; putative glutamate receptors, red; PSD filaments attached to the postsynaptic membrane, blue; PSD filaments away from the postsynaptic membrane, purple. Except for DCVs (purple), the size of SVs was color-coded (top). The same code also applies to Figure 9 and Movies 2–5. C. Zoomed-in view of the dashed-box area in A with arrows pointing to putative proteins on the postsynaptic membrane: glutamate receptors, red; other cleft structures, yellow; PSD filaments, blue. D. Scatter plot of length and width dimensions of the particles on the postsynaptic membrane at the synaptic cleft side. Red dots are putative glutamate receptors, and yellow dots are putative nonreceptor structures identified by visual inspection. The sizes of putative receptors (length: 12.1 ± 1.4 nm; width: 8.6 ± 1.4 nm, n = 81) are similar to that of extracellular domains of the crystal structures of AMPAR (green; length: 12.0 ± 0.2 nm; width: 10.5 ± 2.4 nm) and NMDAR (magenta; length: 10.5 ± 0.2 nm; width: 10.3 ± 1.4 nm; see detailed calculation of averaged dimensions in Materials and Methods). D1. Averaged 2D image of all particles in the red cluster in D. D2 with AMPAR (green) and NMDAR (magenta) superposed. E. F. Segmented structures on the postsynaptic membrane either superposed on a 1.54-nm-thick (gray) tomographic slice (E) or 90°-rotated (F) to reveal their deposition on the postsynaptic membrane (cyan). Structures were colored as follows: putative glutamate receptors, red; putative nonreceptor structures on the cleft side, yellow; putative scaffolding proteins on the cytoplasmic side, blue. G. Four types of glutamate receptor-like particles with their interactions on the cytoplasmic side. G1, NMDAR-like structure (extracellular domain: red) had a ~10 nm globular cytoplasmic domain (pink), which linked to one filamentous structure (blue). G2, AMPAR-like structure (extracellular domain; red) linked to one and two filamentous structures (blue). G3, AMPAR-like structure with no associated filamentous structure. The postsynaptic membrane in all four panels is shown in cyan.
In the excitatory synapse (Fig. 8A), large features, such as membranous organelles and ribosomes, as well as actin and microtubule filaments with ultrastructural details, were readily identified and segmented (Fig. 8B; Movie 2). Furthermore, numerous particles and filamentous structures of various sizes and shapes were visualized within and across presynaptic and postsynaptic compartments (Fig. 8C). These structures were presumably individual protein molecules and complexes. Of special interest were particles near the postsynaptic membrane, some of which have shapes similar to that known for glutamate receptors, including NMDARs and AMPARs, which constitute a major fraction of the postsynaptic membrane proteins (Valtschanoff and Weinberg, 2001; Chen et al., 2008; Dani et al., 2010; Jacob and Weinberg, 2015). We thus visually classified these particles as putative “glutamate receptors,” and defined the remaining particles visible on the cleft side of the postsynaptic membrane as “nonreceptor” particles (Fig. 8C). Plotting the length and width of all these particles reveals that the visually identified putative glutamate receptors form a cluster, although not well separated from the nonreceptor particles (Fig. 8D). The average length (12.1 ± 1.4 nm, n = 81) and width (8.6 ± 1.4 nm, n = 81) of particles in this cluster are similar to those of the extracellular domain of AMPARs (length: 12.0 ± 0.2 nm; width: 10.5 ± 2.4 nm) and NMDARs (length: 10.5 ± 0.2 nm; width: 10.3 ± 1.4 nm) based on their crystal structures (see detailed calculation of averaged dimensions in Materials and Methods; Fig. 8D–D2). In total, this synapse contained 81 putative glutamate receptors, intermingled with other membrane proteins to occupy the surface of the postsynaptic membrane area (Fig. 8E,F; Movie 3). This number agrees with estimates of the total number of AMPARs and NMDARs in a glutamatergic synapse based on quantitative immuno-EM (Nusser et al., 1998b; Takumi et al., 1999), quantitative mass spectrometry (Sheng and Hoogenraad, 2007; Loventhal et al., 2015), and visual identification with ET after HPF-FS (Chen et al., 2008, 2015).

Among the 81 receptor-like structures in the excitatory synapse, 16 displayed a clear globular density (~10 nm in diameter) on the cytoplasmic side (Fig. 8G). Such globular densities are unlikely to belong to the MAGUK (membrane-associated guanylate kinase)-family proteins, which have filamentous shapes (Nakagawa et al., 2004; Chen et al., 2008, 2011). We thus suspect that these 16 structures are likely NMDARs, known to have much larger cytoplasmic domains than AMPARs (Chen et al., 2008). Of the remaining 65 receptor-like structures, 11 had relatively low image quality and thus prevented classification based on their cytoplasmic structures, whereas 54 structures could be visually identified as AMPAR-like structures based on the lack of high globular density. Among them, 44 were found to each link to one or two filamentous structure that might represent PSD-95 or similar MAGUK-family proteins (Fig. 8G2,G3). These putative scaffolding structures appeared to contact the cytoplasmic side of AMPAR-like structures (Fig. 8G2,G3), reminiscent of PSD-95 anchoring AMPAR through its interaction with stargazin, which binds to the side of the AMPAR (Meyer et al., 2004; Nakagawa et al., 2006). We also observed 10 AMPAR-like structures not associated with any PSD-95-like structures (Fig. 8G4). Most of these PSD-95-like structures linking to AMPAR-like structures were in near-perpendicular orientation with respect to the postsynaptic membrane (Fig. 8G). Together with ~200 similar filaments connecting directly to the membrane, they form a set of “vertical pillars” to shape an overall core structure of the PSD (Movie 3, as also seen by ET of samples prepared using HPF-FS (Chen et al., 2008).

A high-resolution tomogram of an inhibitory synapse also revealed rich ultrastructural details (Fig. 9A–C; Movie 4). On the cleft side of the postsynaptic membrane, many particles (Fig. 9C) were found with shapes similar to that of the type-A GABA<sub>AR</sub>, the primary inhibitory transmitter receptor in these hippocampal neurons (Bi and Poo, 1998; Nusser et al., 1998a). With visual inspection, we provisionally identified ~143 particles as GABA<sub>AR</sub>, and ~109 other particles visible on the extracellular side of the postsynaptic membrane as “nonreceptor” particles that likely represent other synaptic proteins, such as adhesion molecules. Plotting the length and width of all these particles revealed that the visually identified putative GABA<sub>AR</sub> formed a cluster, and that the sizes of these putative GABA<sub>AR</sub> (length: 7.1 ± 0.9 nm; width: 5.9 ± 0.9 nm, n = 143) were similar to those of the extracellular domain of GABA<sub>AR</sub> based on its...
crystal structure (length: 6.2 ± 0.1 nm; width: 6.4 ± 0.1 nm; Fig. 9D–D2). Note that the averaged putative GABA<sub>R</sub> resembles the averaged putative glutamate receptor, is surrounded by a “halo” (Figs. 8D1, 9D1), which could be partially due to fringes arising from uncorrected contrast transfer function. However, for individual particles, such effects appeared to be minimal and did not affect the measurements of particle sizes. Most putative nonreceptor particles were uniformly skinny but with variable lengths (Fig. 9D). Within this inhibitory synapse, the 143 putative GABA<sub>R</sub>Rs lying amid 109 nonreceptor membrane protein molecules covered the entire ~0.1 μm<sup>2</sup> postsynaptic membrane (Fig. 9E, F; Movie 5). The number and density of GABA<sub>R</sub>-like particles are consistent with a previous estimate of 30–200 GABA<sub>R</sub>Rs per GABAergic synapse (1250 receptors/μm<sup>2</sup>; Nusser et al., 1997).

Most GABA<sub>R</sub>-like particles were associated with one or two “hammer-shaped” structures on the cytoplasmic side, each with a dense “head” and a thin “neck” (Fig. 9G). The heads of these hammer-shaped structures were consistently located ~12 nm from the postsynaptic membrane, and the necks bridged the transmembrane domain between the GABA<sub>R</sub>-like structure and the dense head (Fig. 9G1–G3). We speculate that these hammer-shaped structures are protein complexes containing gephyrin molecules, the major postsynaptic scaffolding component of the inhibitory synapse (Tretter et al., 2012). Some of the GABA<sub>R</sub>-like structures had only thin necks on their cytoplasmic side and thus lacking the head density (Fig. 9G4), suggesting that the neck might be the cytosolic domain of GABA<sub>R</sub>Rs. These putative receptor-linked gephyrin-like structures, together with similar particles not linked to receptor-like particles but also lying ~12 nm from the postsynaptic membrane, appeared to form a cross-linked matrix, which could provide anchoring sites and structural support for GABA<sub>R</sub>Rs, as previously proposed (Tyagarajan and Fritschy, 2014). Interestingly, we also found many GABA<sub>R</sub>-like structures also linked to densities on the cleft side (Fig. 9G3, G4). These densities might represent the cell adhesion molecule neurexin, previously reported to bind directly to GABA<sub>R</sub>Rs (Zhang C et al., 2010).

Discussion
The complex and highly organized molecular machinery inside neuronal synapses provides the structural basis for synaptic transmission and plasticity. In this study, we have developed an approach of cryo-correlative microscopy to distinguish between excitatory and inhibitory synapses in intact neurons in culture and to visualize their 3D structures in their native state. By quantifying ultrastructural features of >100 hippocampal syn-
apses, we have characterized the ultrastructural features across excitatory and inhibitory central synapses. Because the neurons we used were from the embryonic brain and cultured for only a couple weeks, their synapses may not be as mature as those in the adult brain. Nonetheless, such cultured neurons have been shown to exhibit basic physiological properties of synaptic transmission and plasticity similar to those in more intact preparations, such as brain slices. Thus, it is likely that the basic ultrastructural features we observed also reflect synaptic architecture in the brain, at least during its early development.

Our approach allows for unequivocal differentiation of the ultrastructure of excitatory and inhibitory synapses in hippocampal neurons. Our results show that excitatory synapses have distinct thick PSDs, and inhibitory synapses have more uniformly thin PSDs. These corroborate classic findings regarding the ultrastructure of excitatory and inhibitory synapses (Colonnier, 1968; Gray, 1969), while providing an updated description of different PSD types in their native state. Quantitative analysis reveals that excitatory PSDs have a broad distribution of thickness. This is consistent with the idea that the dynamically interacting PSD molecules may be in a mixed gel/liquid phase (Zeng et al., 2016), and suggests the existence of multiple structural configurations, perhaps reflecting different states of their activation (Dosemeci et al., 2001) and plasticity (Bi and Poo, 1998; Montgomery and Madison, 2004). The existence of multiple functional and plasticity states in excitatory synapses could be critical for optimal learning and memory storage in neuronal circuits, as suggested by theoretical studies (Fusi et al., 2005; Fusi and Abbott, 2007). In contrast, inhibitory PSDs are much more uniformly and regularly organized, consistent with the meager evidence for structural plasticity in such synapses. Intriguingly, there is a “gap” area with relatively lower electron density between the postsynaptic membrane and the PSD peak in both excitatory and inhibitory synapses (see the density profiles of Fig. 4A2,B2). We suspect that much of this gap reflects the relatively lower protein content here, compared with the PSD peak where more extensive interactions between protein molecules may occur.

High-resolution cryo-ET has allowed visualization of the distinct molecular organization underlying the different functional properties of excitatory and inhibitory synapses. In the excitatory synapse, a set of PSD-95-like filamentous structures formed “vertical pillars” immediately underneath the postsynaptic membrane to organize the thick PSD meshwork. These filaments have not been observed in classic studies with conventional EM (Gray,
presence of discus-shaped ellipsoidal vesicles in both excitatory and inhibitory synapses. “Pleomorphic” vesicles have been observed since early EM studies of the synapse, and were generally considered an indicator of inhibitory synapses (Uchizono, 1965). The nature of such vesicles has been debated as more recent studies indicated that their occurrence was associated with specific conditions of sample processing (Tatsukawa and Reese, 1989; Peters and Palay, 1996; Korogod et al., 2015). Although synapses of cultured hippocampal neurons may have different characteristics compared with mature synapses in brain slices, our results suggest that ellipsoidal vesicles could exist in both excitatory and inhibitory native synapses, but their existence may not be used as a definitive criterion to classify synapse types.

Another advantage of our approach is that direct plunge-freezing of neurons cultured on EM grids at low density prevents unwanted disturbance to the synapses, which was unavoidable in the synaptosome preparations previously used for cryo-ET studies (Fernández-Busnadiego et al., 2010; Shi et al., 2014; Perez de Arce et al., 2015). Therefore, the ultrastructure of intact synapses can be preserved near their physiological state. However, plunge-freezing is limited to monolayer cultured neurons and synapses no more than a few hundred nanometers thick. High-pressure freezing and cryo-sectioning could provide an appropriate tool to extend cryo-ET to native circuits in brain tissue (Zuber et al., 2005). By implementing the latest cryo-ET technologies, including VPP, electron energy filter, and direct electron detection, which greatly improve resolution and signal-to-noise ratio (Daney et al., 2014; Fukuda et al., 2015), we were able to visualize synaptic ultrastructural features. Individual molecules in the synapses, such as GABA_ARs previously not accessible, could be identified, localized, and counted, thus providing a straightforward method for unambiguous identification of excitatory and inhibitory synapse types. This is potentially extendable to broader applications, especially in light of functional heterogeneity of synapses in neuronal circuits (Dobrunz and Stevens, 1997; Bi and Poo, 2001; Craig and Boudin, 2001; Vogels and Abbott, 2009; Letellier et al., 2016). Compared with immuno-EM labeling, the use of fluorescent protein tagging ensured high label density, while avoiding additional staining steps that can cause significant structural distortions and artifacts. The platform we developed here uses the same EM cryo-holder for shutting the sample between the light and electron microscopes. Besides being more convenient for reliable correlation between LM and EM, this method also avoids repeated grid transfers, thus protecting the sample from potential damage and contamination. This, together with our method for accurate correlation between LM and EM, greatly improved the efficiency of our approach, and was key to the success of our cryo-CLEM experiments.

Figure 8B. Structures of an inhibitory synapse. This movie shows the tomogram of an inhibitory synapse (same data as in Fig. 8A,B) displayed as z-stack and 3D surface rendering of the segmented structures, including presynaptic membrane (light yellow), postsynaptic membrane (cyan), mitochondrial membrane (outer membrane, gold; inner membrane, light pink), endoplasmic reticulum or endosomes (orange), microtubules (yellow), ribosome-like structures (cyan), putative actin filaments (red), presynaptic putative adhesion molecules (magenta), postsynaptic putative adhesion molecules (yellow), GABA_A-like particles (red), PSD particles on the postsynaptic membrane (blue), and postsynaptic vesicles (beige). Additionally, except for dense core vesicles (purple), all other spherical and ellipsoidal shapes are SVs and their varied colors reflect their varying sizes as shown in Figure 8B.
way to study key synaptic proteins in individual synapses. With further technical improvements along the lines outlined here, future studies with 3D classification and subtomogram averaging could identify additional synaptic proteins more confidently, and reveal finer structural details of protein complexes in situ.

References

Asano S, Fukuda Y, Beck F, Außerheide A, Förster F, Danev R, Baumeister W (2015) Proteasomes. A molecular census of 26S proteasomes in intact neurons. Science 347:439–442. CrossRef Medline

Bi GQ, Poo MM (1998) Synaptic modifications in cultured hippocampal neurons: dependence on spike timing, synaptic strength, and postsynaptic cell type. J Neurosci 18:10464–10472. Medline

Bi G, Poo M (2001) Synaptic modification by correlated activity: Hebb’s postulate revisited. Annu Rev Neurosci 24:139–166. CrossRef Medline

Bourne JN, Chirillo MA, Harris KM (2013) Presynaptic ultrastructural plasticity along CA3→CA1 axons during long-term potentiation in mature hippocampus. J Comp Neurol 521:3898–3912. CrossRef Medline

Brandt F, Carlson LA, Hartl FU, Baumeister W, Grünwald K (2010) The three-dimensional organization of polyribosomes in intact human cells. Mol Cell 39:560–569. CrossRef Medline

Buret AC, Lesperance T, Crum J, Martone M, Vollmann N, Ellisman MH, Weinberg RJ (2012) Electron tomographic analysis of synaptic ultrastructure. J Comp Neurol 520:2697–2711. CrossRef Medline

Chen X, Winters C, Azzam R, Li X, Galbraith JA, Leapman RD, Reese TS (2008) Organization of the core structure of the postsynaptic density. Proc Natl Acad Sci U S A 105:4453–4458. CrossRef Medline

Chen X, Nelson CD, Li X, Winters CA, Azzam R, Sousa AA, Leapman RD, Gainer H, Sheng M, Reese TS (2011) PSD-95 is required to sustain the molecular organization of the postsynaptic density. J Neurosci 31:6329–6338. CrossRef Medline

Chen X, Levy JM, Hou A, Winters C, Azzam R, Sousa AA, Leapman RD, Nicoll RA, Reese TS (2015) PSD-95 family MAGUKs are essential for anchoring AMPA and NMDA receptor complexes at the postsynaptic density. Proc Natl Acad Sci U S A 112:E6983-E6992. CrossRef Medline

Colonnier M (1968) Synaptic patterns on different cell types in the different laminae of the cat visual cortex. An electron microscope study. Brain Res 9:268–287. CrossRef Medline

Craig AM, Boudin H (2001) Molecular heterogeneity of central synapses: afferent and target regulation. Nat Neurosci 4:569–578. CrossRef Medline

Danev R, Buijse B, Khoshouei M, Plitzko JM, Baumeister W (2014) Volta potential phase plate for in-focus phase contrast transmission electron microscopy. Proc Natl Acad Sci U S A 111:15635–15640. CrossRef Medline

Dani A, Huang B, Bergan J, Dulac C, Zhuang X (2010) Superresolution imaging of chemical synapses in the brain. Neuron 68:843–856. CrossRef Medline

Dobie FA, Craig AM (2011) Inhibitory synapse dynamics: coordinated presynaptic and postsynaptic mobility and the major contribution of recycled vesicles to new synapse formation. J Neurosci 31:10481–10493. CrossRef Medline

Dobrunz LE, Stevens CF (1997) Heterogeneity of release probability, facilitation, and depletion at central synapses. Neuron 18:1095–1108. CrossRef Medline

Dosemeci A, Tao-Cheng JH, Vinade L, Winters CA, Pozzo-Miller L, Reese TS (2001) Glutamate-induced transient modification of the postsynaptic density. Proc Natl Acad Sci U S A 98:10428–10432. CrossRef Medline

Dürr KL, Chen L, Stein RA, De Zorzi R, Folea IM, Walz T, Mchaourab HS, Gouaux E (2014) Structure and dynamics of AMPA receptor GluA2 in resting, pre-open, and desensitized states. Cell 158:778–792. CrossRef Medline

Eccles JC (1964) The physiology of synapses. Berlin, Germany: Springer.

Fernández-Busnadiego R, Zuber B, Maurer UE, Cyrklaff M, Baumeister W, Lucic V (2010) Quantitative analysis of the native presynaptic cytomatrix by cryo-electron tomography. J Cell Biol 188:145–156. CrossRef Medline

Fernández-Busnadiego R, Asano S, Oprisoreanu AM, Sakata E, Dongmi G, Kochovski Z, Zürner M, Stein V, Schoch S, Baumeister W, Lucic V (2013) Cryo-electron tomography reveals a critical role of RIM1alpha in synaptic vesicle tethering. The Journal of cell biology 201:725–740. CrossRef Medline

Fitzgibbon A, Pilu M, Fisher RB (1999) Direct least square fitting of ellipses. IEEE Trans Pattern Anal Mach Intell 21:476–480. CrossRef Medline

Fukuda Y, Laugks U, Lucic V (2010) Quantitative analysis of the native presynaptic cytomatrix by cryo-electron tomography. J Cell Biol 188:145–156. CrossRef Medline

Fukuda Y, Langk U, Lucic V, Baumeister W, Danev R (2015) Electron cryotomography of vitrified cells with a Volta phase plate. J Struct Biol 190:439–442. CrossRef Medline

Fusi S, Abbott LF (2007) Limits on the memory storage capacity of bounded synapses. Nat Neurosci 10:485–493. CrossRef Medline

Fusi S, Drew PJ, Abbott LF (2005) Cascade models of synaptically stored memories. Neuron 45:599–611. CrossRef Medline

Gray EG (1939) Axo-somatic and axo-dendritic synapses of the cerebral cortex: an electron microscope study. J Anat 93:420–433. Medline
Suèdhof TC (2012) The presynaptic active zone. Neuron 75:11–25. CrossRef Medline
Suèdhof TC, Malenka RC (2008) Understanding synapses: past, Present, and Future. Neuron 60:469–476. CrossRef Medline
Suloway C, Pulokas J, Fellmann D, Cheng A, Guerra F, Quipe J, Stagg S, Potter CS, Carragher B (2005) Automated molecular microscopy: the new legion system. J Struct Biol 151:41–60. CrossRef Medline
Takumi Y, Ramírez-León V, Laake P, Rinvik E, Ottersen OP (1999) Different modes of expression of AMPA and NMDA receptors in hippocampal synapses. Nat Neurosci 2:618–624. CrossRef Medline
Tao C, Xia C, Chen X, Zhou ZH, Bi G (2012) Ultrastructural analysis of neuronal synapses using state-of-the-art nano-imaging techniques. Neurosci Bull 28:321–332. CrossRef Medline
Tatsuoka H, Reese TS (1989) New structural features of synapses in the anteroventral cochlear nucleus prepared by direct freezing and freeze-substitution. J Comp Neurol 290:343–357. CrossRef Medline
Tretter V, Mukherjee J, Maric HM, Schindelin H, Sieghart W, Moss SJ (2012) Gephyrin, the enigmatic organizer at GABAergic synapses. Front Cell Neurosci 6:23. CrossRef Medline
Tyagarajan SK, Fritschy JM (2014) Gephyrin: a master regulator of neuronal function? Nat Rev Neurosci 15:141–156. CrossRef Medline
Uchizono K (1965) Characteristics of excitatory and inhibitory synapses in the central nervous system of the cat. Nature 207:642–643. CrossRef Medline
Valtchanoff JG, Weinberg RJ (2001) Laminar organization of the NMDA receptor complex within the postsynaptic density. J Neurosci 21:1211–1217. Medline
Verbeek J, Van Dyck D, Van Tendeloo G (2004) Energy-filtered transmission electron microscopy: an overview. Spectrochim Acta B 59:1529–1534. CrossRef
Vogels TP, Abbott LF (2009) Gating multiple signals through detailed balance of excitation and inhibition in spiking networks. Nat Neurosci 12:483–491. CrossRef Medline
Watanabe S, Rost BR, Camacho-Pérez M, Davis MW, Sölh-Kielczynski B, Rosenmund C, Jorgensen EM (2013) Ultrafast endocytosis at mouse hippocampal synapses. Nature 504:242–247. CrossRef Medline
Wilhelm BG, Mandad S, Truckenbrodt S, Krohnert K, Schafer C, Rammner B, Koo SJ, Claßen GA, Krauss M, Haucke V, Urlaub H, Rizzoli SO (2014) Composition of isolated synaptic boutons reveals the amounts of vesicle trafficking proteins. Science 344:1023–1028. CrossRef Medline
Zeng M, Shang Y, Araki Y, Guo T, Huganir RL, Zhang M (2016) Phase transition in postsynaptic densities underlies formation of synaptic complexes and synaptic plasticity. Cell 166:1163–1175.e12. CrossRef Medline
Zhang C, Atasoy D, Arac D, Yang X, Fucillo MV, Robison AJ, Ko J, Brunger AT, Südhof TC (2010) Neurexins physically and functionally interact with GABA(A) receptors. Neuron 66:403–416. CrossRef Medline
Zhang F, Gradinaru V, Adamantidis AR, Durand R, Airan RD, de Lecea L, Deisseroth K (2010) Optogenetic interrogation of neural circuits: technology for probing mammalian brain structures. Nat Protoc 5:439–456. CrossRef Medline
Zuber B, Nikonenko I, Klauer P, Muller D, Dubocchet J (2005) The mammalian central nervous synaptic cleft contains a high density of periodically organized complexes. Proc Natl Acad Sci U S A 102:19192–19197. CrossRef Medline