

## **SUPPLEMENTARY MATERIALS**

### **The molecular infrastructure of glutamatergic synapses in the mammalian forebrain**

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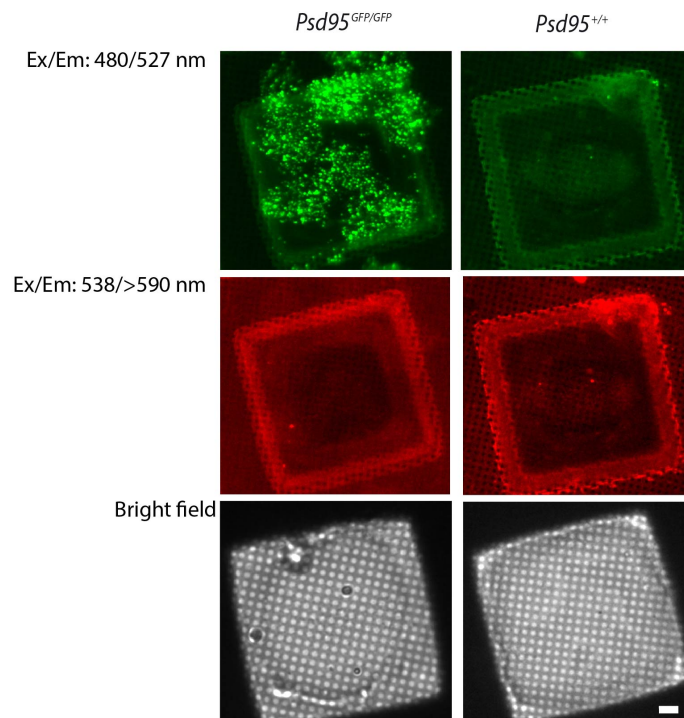
#### **Table S1**

A spreadsheet detailing the organelle and macromolecular constituents within each tomogram of the ultra-fresh synapse dataset, including within the PreSM and PoSM of PSD95-GFP containing synapses and additional subcellular compartments in the vicinity.

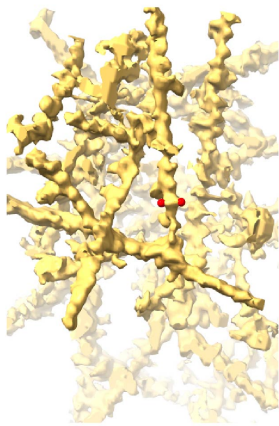
#### **Supplementary Figures 1 to 5**

**Figure S1A**

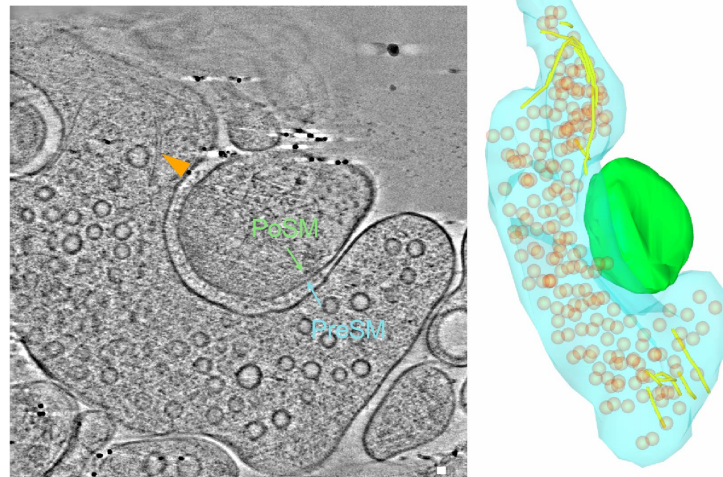
**A**



**B**



**C**



**Figure S1.** (A) Cryogenic fluorescence microscopy of forebrain (cortex and hippocampus) fast-fresh synapse preparation from *Psd95<sup>GFP/GFP</sup>* (left panels) and WT (right panels). Top, detection of GFP with excitation and emission filters of 480 nm and 527 nm, respectively. Middle, detection of red fluorescence with excitation and emission filters of 538 nm and >590 nm, respectively. Bottom, bright

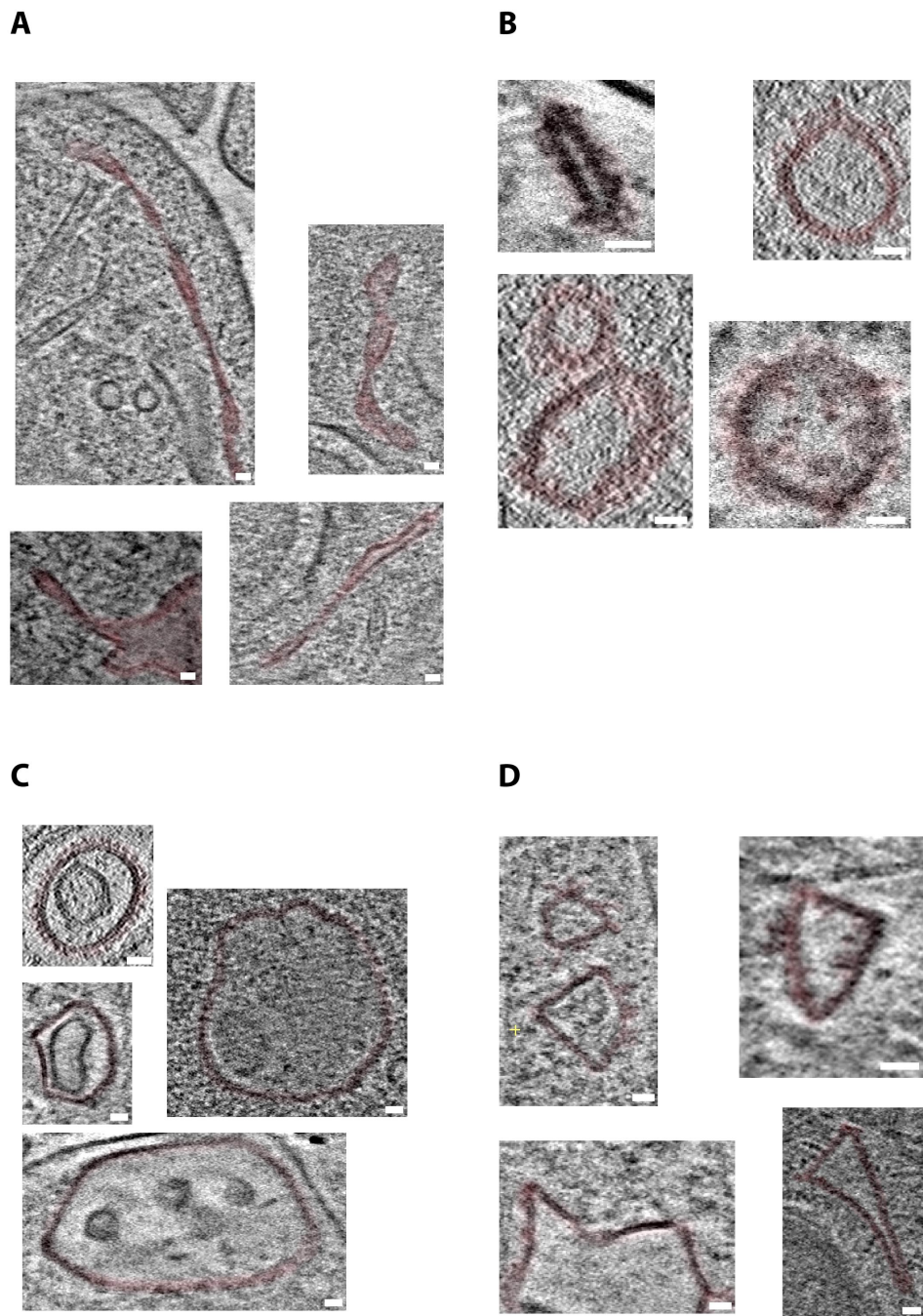
field image. Submicron-sized puncta in the GFP channel only detected exclusively in *Psd95<sup>GFP/GFP</sup>* samples.

(B) Masked tomographic map of branched filamentous actin network in PSD95-containing PoSM compartment. Red spherical markers, spaced 7.1 nm apart on the outside edges of a filament.

(C) Molecular architecture of f-actin in the presynaptic compartment.

*Left*, virtual slice through tomogram of synapse located by PSD95-GFP cryoCLEM. F-actin parallel bundle indicated by yellow arrowhead within PreSM (cyan) compartment that was adhered to PoSM (green). *Right*, 3D segmented model of f-actin (yellow tubes) in PresM (cyan) opposing PoSM (green). Synaptic vesicles (orange). Short f-actin filaments connect a fraction of synaptic vesicles to the plasma membrane. Scale bar, 20 nm.

**Figure S2**



**Figure S2.** Classification of membrane-bound organelles in PSD95-GFP-containing synapses. Gallery of virtual slices showing intracellular organelle subtype (pseudo-coloured magenta) of four different PSD95-GFP containing synapses of

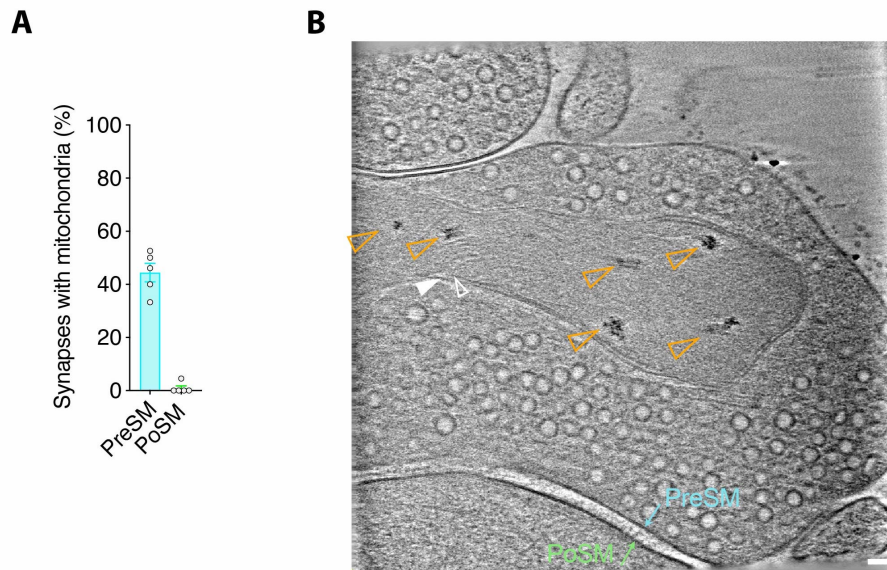
- (A) Flat tubular twisted membranes.
- (B) Clustered membrane proteins in vesicles.

(C) Multivesicular bodies.

(D) Polyhedral membranes.

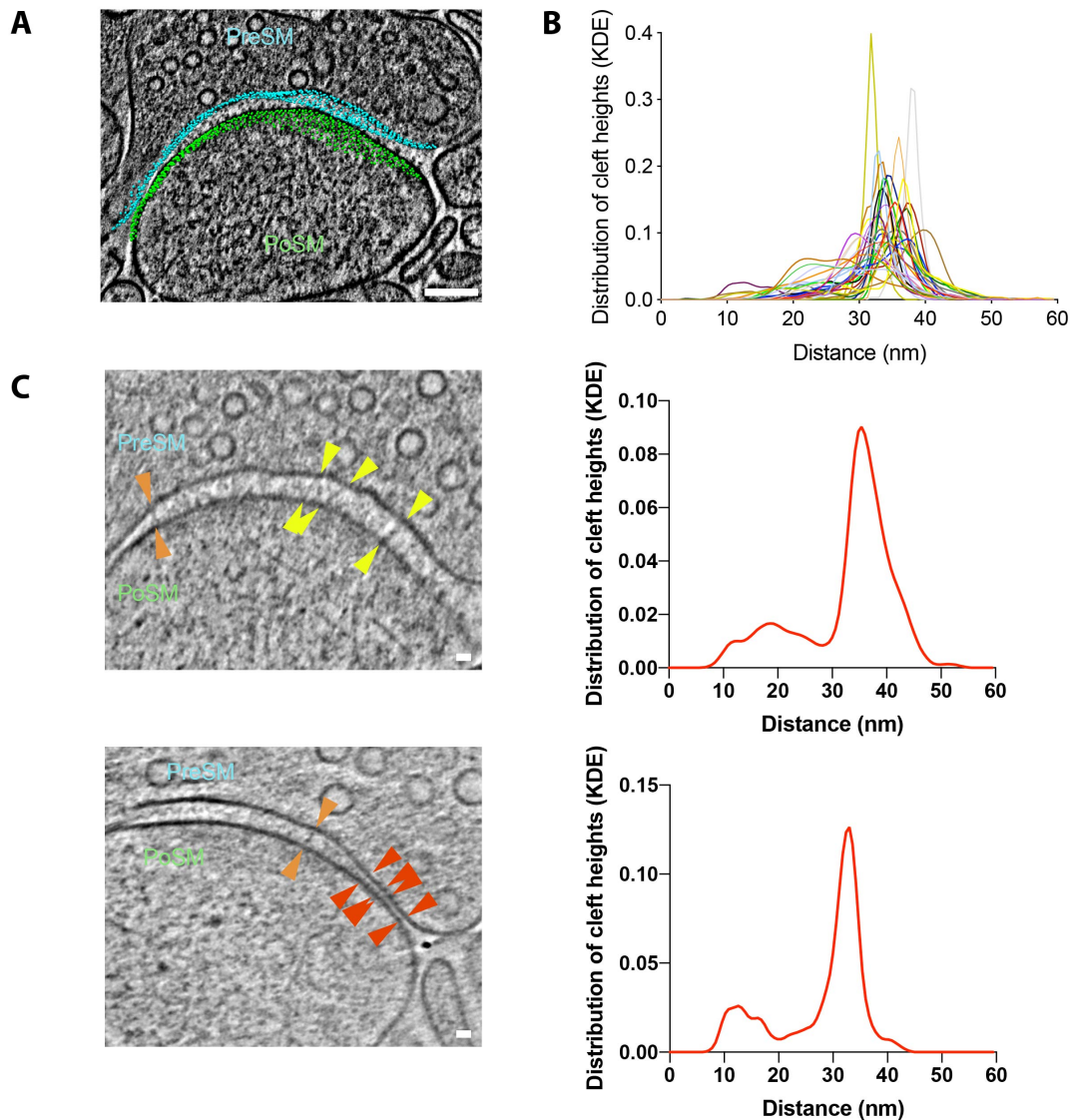
Scale bar, 20 nm

**Figure S3.**



**Figure S3.** Synaptic mitochondria. (A) bar chart showing the prevalence of mitochondria in the pre-synaptic membrane (PreSM) and post-synaptic membrane (PoSM) compartments of glutamatergic synapses. (B) Virtual slice through tomogram of synapse located by PSD95 cryoCLEM showing PreSM containing mitochondria identifiable by surrounding outer membrane (solid white arrowhead) and inner cristae membrane (open white arrowhead). Electron-dense deposits in the mitochondrial matrix are indicated with open orange arrowheads. Scale bar, 50 nm

**Figure S4.**



**Figure S4.** Quantification and variability of cleft height in glutamatergic synapses.

(A) Virtual slice of tomographic map through PSD95-GFP containing synapse. 3D model of computationally determined coordinates of synaptic cleft are shown with presynaptic and postsynaptic cleft membrane coordinates depicted in cyan and green, respectively. Scale bar, 100 nm.

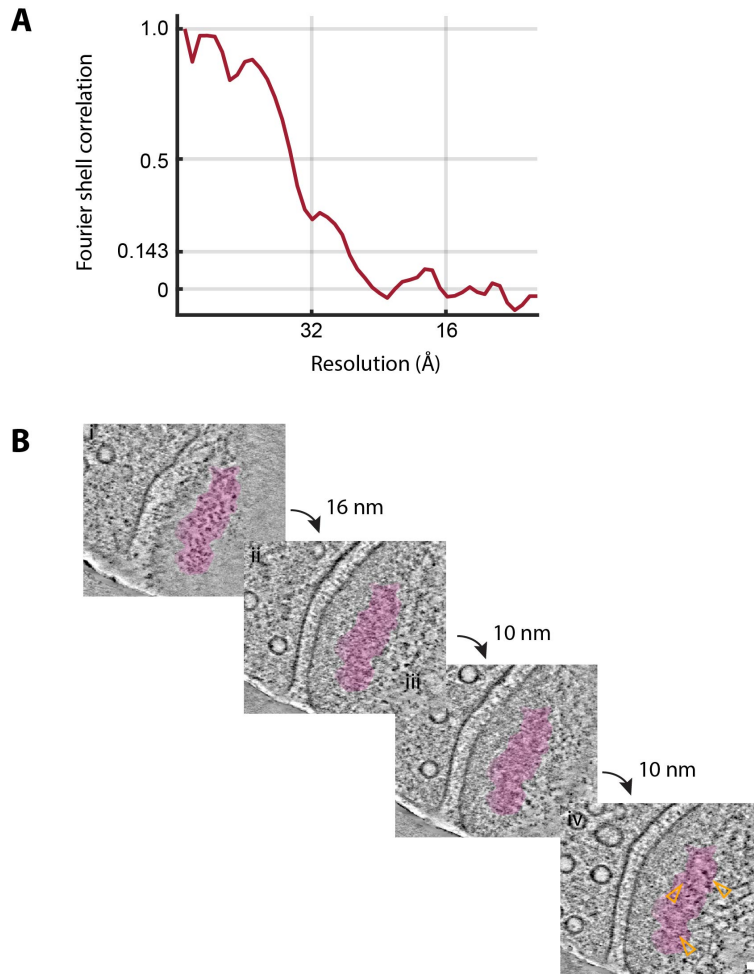
(B) Measuring the nearest neighbour distance between presynaptic and postsynaptic coordinates of the cleft were used to quantify the cleft heights for each synapse. The distribution of cleft height distances is shown using kernel density estimation (KDE) plotted with a different colour for each synapse.

(C) Synapses with a bimodal distribution of cleft heights. *Left*, Virtual slice through tomographic map of PSD95-GFP containing synapses showing subsynaptic regions with varying cleft height and corresponding sizes of transsynaptic adhesion proteins bridging the PreSM (cyan) and PoSM (green). Red arrowheads, 5-15 nm transsynaptic adhesion complexes. Orange arrowheads, 22-28 nm transsynaptic adhesion complexes. Yellow arrowheads, 32-36 nm transsynaptic adhesion complexes.

Scale bar, 20 nm. The corresponding distribution of cleft height distance for each synapse is plotted using kernel density estimation (KDE) on the *right*.



**Figure S5.**



**Figure S5.** Subtomogram averaging and anchoring of ionotropic glutamate receptors.

(A) Global Fourier shell correlation curve estimating the resolution of subtomogram averaged ionotropic glutamate receptors.

(B) Cytoplasmic anchoring of perisynaptic ionotropic glutamate receptor clusters (pseudo-coloured in magenta). Cluster was defined using DBSCAN. Virtual slice series through tomogram of PSD95-GFP containing postsynaptic compartment. Virtual slices separated by 16, 10, 10 and 10 nm, showing i) extracellular membrane protein domains, ii) proximal and iii) distal cytoplasmic macromolecular complexes, iv) associated branched f-actin network (orange open arrowhead). Scale bar, 20 nm.